The Journal of Experimental Biology 213, 1069-1078

doi:10.1242/jeb.038885

Fibre architecture and song activation rates of syringeal muscles are not lateralized in the European starling

A. M. Uchida[1], R. A. Meyers[1], B. G. Cooper[2,*] and F. Goller[2,†]

[1]Department of Zoology, Weber State University, Ogden, UT 84408-2505, USA and [2]Department of Biology, University of Utah, Salt Lake City, UT 84112, USA

*Present address: Department of Psychology, Texas Christian University, Fort Worth, TX 76129, USA
†Author for correspondence (goller@biology.utah.edu)

Accepted 7 December 2009

SUMMARY

The songbird vocal organ, the syrinx, is composed of two sound generators, which are independently controlled by sets of two extrinsic and four intrinsic muscles. These muscles rank among the fastest vertebrate muscles, but the molecular and morphological foundations of this rapid physiological performance are unknown. Here we show that the four intrinsic muscles in the syrinx of male European starlings (*Sturnus vulgaris*) are composed of fast oxidative and superfast fibres. Dorsal and ventral tracheobronchialis muscles contain slightly more superfast fibres relative to the number of fast oxidative fibres than dorsal and ventral syringealis muscles. This morphological difference is not reflected in the highest, burst-like activation rate of the two muscle groups during song as assessed with electromyographic recordings. No difference in fibre type ratio was found between the corresponding muscles of the left and right sound generators. Airflow and electromyographic measurements during song indicate that maximal activation rate and speed of airflow regulation do not differ between the two sound sources. Whereas the potential for high-speed muscular control exists on both sides, the two sound generators are used differentially for modulation of acoustic parameters. These results show that large numbers of superfast fibre types are present in intrinsic syringeal muscles of a songbird, providing further confirmation of rapid contraction kinetics. However, syringeal muscles are composed of two fibre types which raises questions about the neuromuscular control of this heterogeneous muscle architecture.

Key words: birdsong, vocal muscle, vocal control.

INTRODUCTION

In non-avian tetrapods, the larynx is the main sound-producing organ. Birds, however, have evolved a unique phonatory organ, the syrinx, located at the basal end of the trachea. Like the mammalian larynx, the syrinx is controlled by muscles whose action influences acoustic parameters and regulates airflow by closing and opening of the airways. Syrinx morphology and the number of muscles controlling it vary between different bird groups. Despite substantial variation in vocal control, vocal behaviour of birds ranks among the most elaborate use of sound as a communication signal. Because song is an important behaviour for mate attraction and territorial defence, vocal quality is assumed to be under strong sexual selection (e.g. Andersson, 1994). In oscines, song is a learned vocal behaviour, which often displays highly complex temporal and acoustic features. Production of rapid temporal sequences and high rates of amplitude and frequency modulation require precise control of sound onset and offset. Many rapid acoustic phenomena arise from direct muscular control, although passive transitions intrinsic to the dynamics of the vibrating labia have also been proposed as a potential source (e.g. Fee et al., 1998).

The oscine syrinx is a bipartite structure, in which six pairs of syringeal muscles control two independent sound generators (reviewed by Suthers et al., 1999). Different muscles contribute in different ways to the control of sound production. For example, in brown thrashers (*Toxostoma rufum*) electromyographic (EMG) recordings from syringeal muscles during spontaneous singing suggest that the tracheobronchial muscles regulate airflow by adducting (m. tracheobronchialis dorsalis) and abducting (m. tracheobronchialis ventralis) the lateral labium (Goller and Suthers, 1996a). The main function of the ventral syringeal muscle, m. syringealis ventralis, is control of sound frequency (Goller and Suthers, 1996b).

Syringeal muscles involved in gating airflow through the labial valve have very fast contractile properties in the European starling (*Sturnus vulgaris*) and the zebra finch (*Taeniopygia guttata*) (Elemans et al., 2008). In a work loop assay, dorsal tracheobronchial muscles in the starling produced positive work up to 250 Hz. Oscillatory stimulation of both tracheobronchial muscles *in situ* produced modulation of airflow up to 250 Hz, indicating that these muscles can exert biomechanical effects in the intact syrinx at these high rates. This direct evidence is accompanied by indirect evidence from EMG recordings of the muscles controlling airflow in brown thrashers and starlings to show activation at high rates during spontaneous song. Burst-like activation patterns of syringeal muscles accompany changes in airflow and amplitude modulation of sound at rates up to 125 Hz in thrashers (Goller and Suthers, 1996a) and above 200 Hz in starlings (Elemans et al., 2008).

These data strongly suggest that the syringeal muscles are highly specialized for providing rapid control, a feature they share with a number of other, independently evolved muscles in sound-generating systems. With regard to their adaptations for rapid physiological performance, the best-studied examples for very fast sonic muscles are the swimbladder muscle of some fishes and the tail shaker muscle of rattlesnakes (Rome et al., 1996; Rome et al., 1999). In these muscles, individual contractions give rise to sound waves; thus contraction rate corresponds to sound frequency. Muscle fibre type composition is homogeneous (Fine and Pennypacker, 1988; Schultz

et al., 1980) and contraction kinetics are very rapid but force production is low (Rome and Lindstedt, 1998; Rome, 2006).

In contrast, birds and mammals produce sound by vibrating tissue masses, and vocal muscles control acoustic parameters such as frequency and amplitude. Laryngeal muscles in mammals are fast, but do not reach such high contraction rates as starling muscles (Martensson and Skoglund, 1964; Alipour-Haghighi et al., 1987; Alipour and Titze, 1999; Sciote et al., 2002; Hoh, 2005). Unlike toadfish sonic and rattlesnake tailshaker muscles, laryngeal muscles show heterogeneous fibre type composition (Hoh, 2005). For example, fibre composition of all five laryngeal muscles has been studied in a number of mammalian species (Hoh, 2005) and shows species-specific patterns as well as muscle-specific differences in composition. The percentage of slow fibres ranges from 0 in the thyroarytenoid muscle of small species to near 50% in the vocalis part in large mammals.

Whereas many aspects of mammalian muscles have been studied in detail, very little is known about muscle morphology, fibre type composition and molecular make-up of syringeal muscles of birds. Histochemical and ultrastructural analyses of extrinsic syringeal muscles (m. sternotrachealis) in ducks and geese show heterogeneous composition of slow and fast fibres (Gopalakrishnakone, 1985; Lalatta-Costerbosa et al., 1990). Percentages of respective fibre types vary between species and clear sex differences exist. In general, however, the muscles predominantly contain fast fibres, although slow fibres are also present (Lalatta-Costerbosa et al., 1990). In addition, ultrastructural evidence indicates that extrinsic syringeal muscles in oilbirds (*Steatornis caripensis*) are composed of slow and fast muscle fibres (Suthers and Hector, 1985), and that the extrinsic muscles of the dove most likely contain several different fibre types (Elemans, 2004). The functional morphology of intrinsic muscles in oscine songbirds has not been studied in the same detail. In the zebra finch, intrinsic syringeal muscles show a difference in fibre cross-sectional area between right and left sides, and there is an apparent difference in mean cross-sectional area between ventral and dorsal muscles, although this difference was not the focus of the study and statistical treatment of this comparison is not available (Wade and Buhlman, 2000). These data are based on measurements using traditional histology of muscle cross-sections, which does not permit differentiation of fibre types.

This morphological difference between the left and right sides of the syrinx suggests that a lateralization of use occurs. In a number of species, production of song or specific song elements is lateralized to one syringeal sound generator (reviewed by Suthers and Goller, 1997; Suthers and Zollinger, 2008). Such a functional difference was documented in the brown thrasher. The right side of the syrinx contributed more to amplitude modulation (Goller and Suthers, 1996a), but it is not known whether the sides differ in the maximal modulation rate that can be generated. It is possible that specializations for production of rapid acoustic phenomena are lateralized and therefore correspond to a potential asymmetry in muscle morphology.

The European starling is an ideal species in which to study these questions. Song is characterized by a large number of different syllable types that are rich in amplitude and frequency modulation. *In situ* observations of their syringeal muscles show that these muscles can effect acoustic modulation at very high rates (Elemans et al., 2008). We therefore used this species to investigate the activity of the intrinsic syringeal muscles during song and the fibre type composition and architecture of these muscles to ask the following questions. (1) What is the fibre type composition of intrinsic syringeal muscles? (2) Do fibre composition and activation, as indirect measurements of temporal performance, vary between syringeal muscles? (3) Is there lateralization of rapid acoustic phenomena, and is this reflected in the performance of left and right syringeal muscles?

MATERIALS AND METHODS

Tissue

Fresh intrinsic syringeal muscles were collected from eight male European starlings (*Sturnus vulgaris* L.) during the summer months between the years 2004 and 2007. In seven individuals, we collected all four pairs of intrinsic muscles (ventral and dorsal tracheobronchialis muscles, vTB and dTB, and ventral and dorsal syringealis muscles, vS and dS, from the left and right sides); in the eighth bird we collected only the ventral muscle mass (Fig. 1). Physiological data had previously been collected from some (but not all) of these individuals (see below).

Histology

Starlings were killed with an overdose of isoflurane, and immediately afterwards the syringeal muscles were removed and mounted on cork blocks using 5% gum tragacanth. Samples were flash-frozen in 2-methylbutane cooled in liquid nitrogen to approximately –150°C and stored in an ultracold freezer at –80°C. Frozen tissue sections of 10–12 μm were obtained using a cryostat (Tissue-Tek II, Microtome/Cryostat, models 4551 and 4553; Naperville, IL, USA) set to –20°C and placed onto glass microscope slides. Cross-sections were taken throughout the entire lengths of both tracheobronchialis and syringealis. Because borders between individual muscles could not always be clearly identified from sections through the main syringeal mass, we used the most rostral sections for quantification of data for tracheobronchialis, and the medial portion of the main syringeal mass for quantification of data for syringealis (Fig. 1). This avoided any misidentification of muscles more laterally where tracheobronchialis and syringealis overlap, and boundaries between them are not clearly identifiable.

To differentiate muscle fibre types, a series of histochemical reactions were performed on serial cross-sections of the tissue.

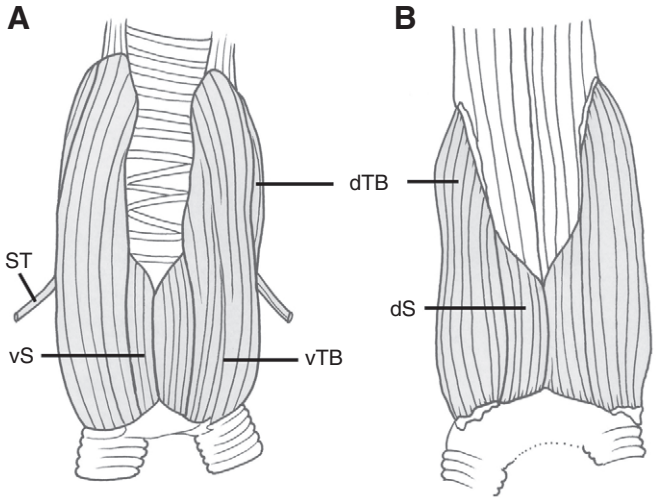

Fig. 1. Morphology of the syrinx of a male European starling. A, ventral view; B, dorsal view. Abbreviations: dS, dorsal m. syringealis; dTB, dorsal m. tracheobronchialis; vTB, ventral m. tracheobronchialis; ST, m. sternotrachealis; vS, ventral m. syringealis. M. sternotrachealis not illustrated on the dorsal view.

Following a previously used protocol (McFarland and Meyers, 2008), muscle sections were reacted for myosin ATPase using pH 4.2–4.3 (acidic) or pH 10.2–10.4 (alkaline) pre-incubations. Staining procedures with antifast antibody MY32 (Sigma Chemical Co., St Louis, MO, USA) and antislow antibody ALD58 (University of Iowa, Hybridoma Bank) were as previously described (Meyers and Stakebake, 2005). In short, tissue sections were reacted in a humidified chamber at 25°C for 2 h, then rinsed with phosphate-buffered saline, incubated in goat antimouse antibody, and stained with streptavidin peroxidase system (SPS kit; Zymed Labs, San Francisco, CA, USA). Alkaline myosin ATPase and antifast antibody MY32 reactions positively identify fast muscle fibres, while acidic myosin ATPase and antislow antibody ALD58 identify slow fibres (Fig. 2).

Following well-established protocols (Meijer, 1968; Novikoff et al., 1961), α-glycerophosphate dehydrogenase (α-GDP) and nicotinamide adenine dinucleotide diaphorase (NADH-D) reactions were used to identify glycolytic and oxidative properties, respectively (Fig. 2). However, these properties were not analysed statistically or quantified.

Reacted tissue sections and a corresponding scale bar were photographed using a Nikon Coolpix 995 or Olympus E-330 digital

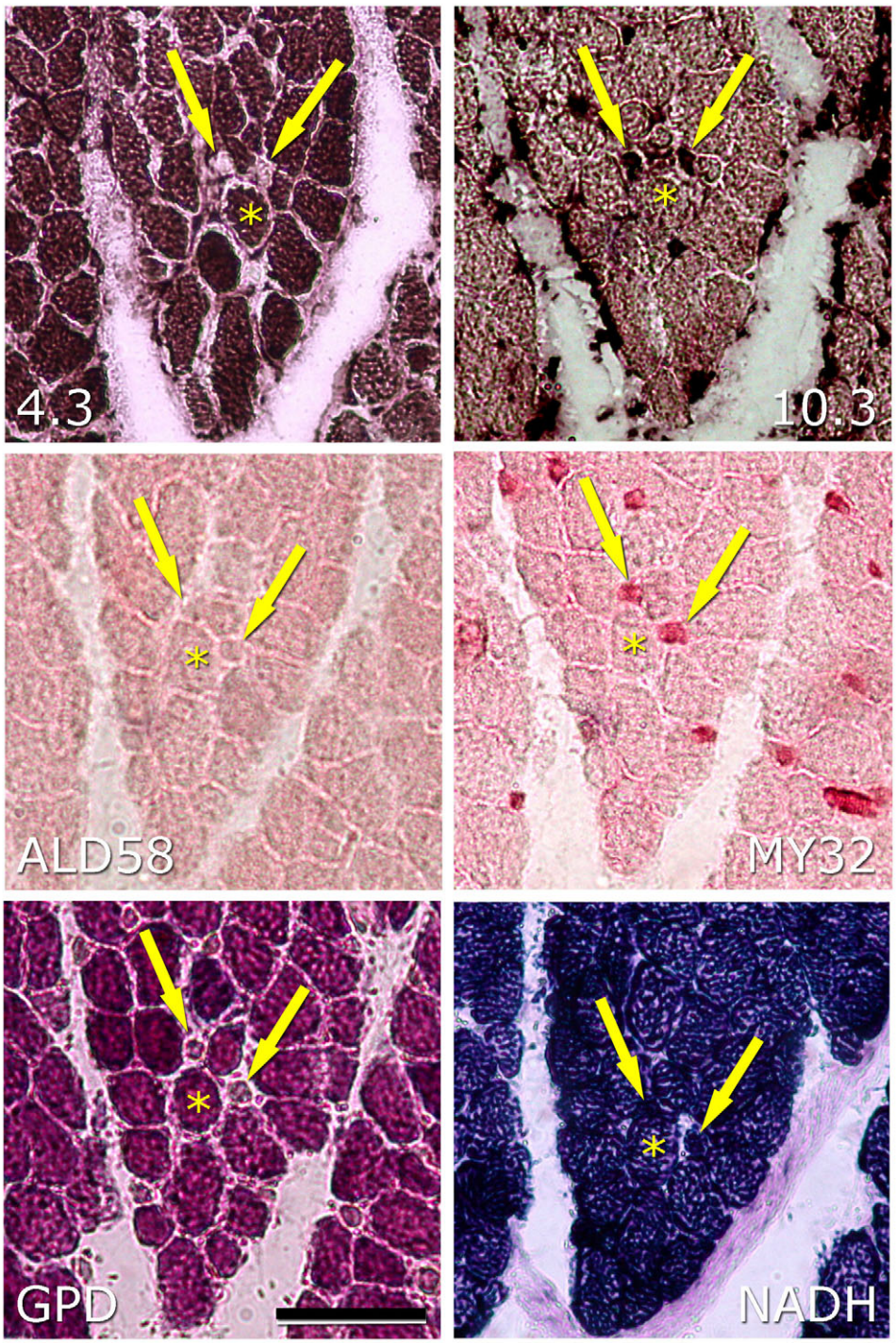

Fig. 2. Serial sections showing fibre staining profiles of the ventral tracheobronchialis (vTB) of a male European starling. Fibres indicated by arrows are smaller, fast fibres while the fibre indicated by an asterisk represents the larger, superfast muscle fibres. Acid preincubation (pH 4.3) reacts darkly for slow fibres; no positive reaction was found in any syringeal muscle. Alkaline (pH 10.3) preincubation reacts positively for fast muscle fibres as indicated by arrows. The antibodies ALD58 and MY32 react positively with slow and fast muscle fibres, respectively. Note the uniformly negative ALD58 reaction indicating no slow fibres, and the positive reaction with the smaller fibres in MY32. GPD demonstrates glycolytic properties, with more intense staining indicating greater glycolytic activity, seen in the superfast fibres. Oxidative capacity was determined through reaction with NADH, and both fibre types reacted positively, with the smaller, fast fibres slightly more intense. Scale bar, 100 μm.

camera mounted on a Zeiss Axioskop 40/40 FL microscope. Using Adobe Photoshop CS2 (v. 9.0), overlapping digital images of each muscle were merged to create a complete cross-sectional photomontage for each muscle. The images were used for analysis and quantification of fibres based on their type, location and size. All of the muscle fibres within a cross-section photomontage were utilized for fibre type counts (and percentages).

Muscle fibre comparisons

Initial comparisons were made for both fibre types between left and right sides of four muscles: dorsal tracheobronchialis (dTB), ventral tracheobronchialis (vTB), dorsal syringealis (dS) and ventral syringealis (vS). Both muscle fibre diameters and ratios of fibre types were quantified.

Fibre diameters were approximated by averaging the narrowest and widest points of individual fibres measured using Mitutoyo digital calipers. This was done for 100 randomly selected fibres of each fibre type, from each of the syringeal muscles. Mean values were then used to calculate a grand mean for each fibre type from each muscle from four male starlings (Tables 1 and 2). Rostral sections were used to measure tracheobronchialis fibre diameters, while medial portions of the syringeal mass (more caudally) were used to measure diameters from the syringealis muscles. Muscle fibres of both types were counted and compared statistically, although percentages are more biologically relevant and are also reported. Comparisons were made using an independent sample *t*-test with Bonferroni correction for multiple comparisons.

Muscle architecture

Two additional whole syringes were removed and placed in a solution of 15% nitric acid. The acid digested the connective tissue surrounding the muscle, enabling isolation of individual muscle

Table 1. Fibre type percentages and diameters for m. tracheobronchialis

		No. of fibres		% Superfast fibres	Mean % superfast fibres (±s.d.)	Fibre diameter (μm)		Mean diameter (±s.d.) (μm)	
Muscle	Bird	Fast	Superfast			Fast	Superfast	Fast	Superfast
Left vTB	53	394	1421	78.3	71.24±5.0	19.2	32.36	17.2±2.2	33.9±1.6
	94	433	915	67.9		19.03	33.54		
	12	819	2029	71.2		15.16	33.36		
	63	805	1674	67.5		15.55	36.2		
Right vTB	53	634	1728	73.2	66.89±4.4	17.09	35.74	15.9±2.5	36.0±3.2
	94	712	1216	63.1		18.46	40.53		
	12	1077	2114	66.2		12.6	34.0		
	63	976	1835	65.3		15.39	33.7		
Left dTB	53	361	1511	80.7	74.86±5.5	16.47	41.39	18.2±1.5	37.4±4.5
	94	293	833	47		18.97	32.57		
	12	–	–	–		–	–		
	63	531	1262	69.9		19.14	38.12		
Right dTB	53	227	997	81.5	76.22±4.8	20.39	40.26	20.8±1.6	38.7±0.9
	94	218	657	75.1		22.62	38.17		
	12	–	–	–		–	–		
	63	449	1161	72.1		19.43	36.55		

vTB, ventral tracheobronchialis; dTB, dorsal tracheobronchialis.

Table 2. Fibre type percentages and diameters for m. syringealis

		No. of fibres		% Superfast fibres	Mean % superfast fibres (±s.d.)	Fibre diameter (μm)		Mean diameter (±s.d.) (μm)	
Muscle	Bird	Fast	Superfast			Fast	Superfast	Fast	Superfast
Left vS	53	–	–	–	60.9±11.14	–	–	21.73±1.9	32.6±3.0
	94	489	1353	73.5		20.0	33.9		
	12	985	1070	52.1		22.7	29.6		
	63	417	560	57.3		22.5	34.3		
Right vS	53	–	–	–	41.0±28.1	–	–	27.47±10.0	35.3±1.9
	94	695	1357	66.1		21.8	37.1		
	12	1599	191	10.7		36.0	34.4		
	63	476	409	46.2		24.6	34.4		
Left dS	53	275	501	64.6	49.4±15.42	25.5	40.1	28.07±1.3	46.7±0.2
	94	201	100	49.9		27.3	39.8		
	12	–	–	–		–	–		
	63	336	171	33.7		31.4	60.2		
Right dS	53	262	226	46.3	42.0±4.023	33.0	49.5	30.37±3.1	43.67±7.1
	94	331	206	38.4		28.6	39.4		
	12	–	–	–		–	–		
	63	279	196	41.3		29.5	42.1		

vS, ventral syringealis; dS, dorsal syringealis.

fibres [following the protocol of Meyers and Hermanson (Meyers and Hermanson, 1994)]. Muscles remained in the acid for 4–10 days, depending on the connective tissue and ease of liberating individual fibres from each other. Once connective tissue was adequately digested, the muscle was placed in a 50% solution of glycerin for storage and analysis. A Zeiss Discovery V12 stereoscope was used to separate and measure individual muscle fibres. Fifty fibres from each muscle were randomly isolated – based primarily on accessibility – and measured for length. Left and right muscles were not differentiated in this case.

Physiological recordings

The procedures for recording air sac pressure and bilateral airflow or EMGs from syringeal muscles have been described in detail elsewhere (Suthers et al., 1994; Goller and Suthers, 1996a; Goller and Suthers, 1996b). In brief, vigorously singing birds were accustomed to wearing an elastic belt around the thorax with a Velcro tab for later attachment of a backpack. Once they resumed singing they were anaesthetized with chloropent injections (4 µl g^{-1} i.m.). A flexible cannula was implanted below the last rib into one anterior thoracic air sac and sutured to the rib cage. The free end was connected to a miniature piezoresistive pressure transducer (Fujikura model FPM-02PG; Tokyo, Japan) mounted on the backpack. Then the syrinx was accessed by opening the skin and interclavicular air sac membrane in the space surrounded by the furcula. For airflow experiments, a custom-built flow probe, manufactured from a microbead thermistor (Thermometrics BB05JA202; Edison, NJ, USA), was inserted into the bronchi below each syringeal sound generator. For EMG experiments, up to four bipolar electrode pairs were inserted into different syringeal muscles. Wires were routed out of the interclavicular air sac and then subcutaneously to the backpack, where connections to stronger wires, leading to signal conditioning instrumentation, were made. The air sac membrane was then closed with surgical suture and tissue adhesive (Nexaband) and the skin incision was sutured closed.

Birds were then allowed to recover for several days and recording started as soon as they resumed singing activity. Airflow was determined *via* a feedback circuit (Hector Engineering, Ellettsville, IN, USA), which heated the thermistor slightly and registered the required current for maintaining the temperature. EMG signals were differentially amplified (×1000) and band-pass filtered (100–3000 Hz) with either a Dagan amplifier (Minneapolis, MN, USA) or a Brownlee Precision 440 amplifier (San Jose, CA, USA). Sound was recorded with a microphone (Audiotechnica AT 8356; Stow, OH, USA) placed approximately 30 cm in front of the cage. Avisoft Recorder software (Avisoft Bioacoustics, Berlin, Germany) was used to record all channels simultaneously to a computer at 44.1 kHz *via* a DT 2821G A/D board.

RESULTS

All intrinsic syringeal muscles are composed of two fibre types (Figs 2 and 3). The smaller fibre type population reacted like typical IIA muscle fibres: positive reactions for the antifast antibody MY32 and alkaline pH preincubations; negative reactions for ALD58 and acidic pH preincubations. The larger fibre population did not react like type I, type IIA or type IIB, or tonic muscle fibres: negative reactions for MY32 and ALD58; intermediate reactions in both acidic and alkaline pH preincubations (Fig. 2). DelGaudio and colleagues (DelGaudio et al., 1995) likewise described a population of fibres within the muscles of the rat larynx that reacted atypically compared with the other fibres of the larynx. These fibres, designated type IIL, have been subsequently identified as superfast (Shiotani et al., 1999).

Comparing left and right syringeal sides

Muscle fibre types

There was no difference in fibre type counts for fast or superfast fibres between left and right dTB (fast fibres: mean 395 *vs* 298; t=0.937; d.f.=4; P=0.401; superfast fibres: mean 1202 *vs* 938; t=1.066; d.f.=4; P=0.347) or vTB (fast fibres: mean 612 *vs* 849; t=–1.517; d.f.=6; P=0.179; superfast fibres: mean=1509 *vs* 1723; t=–0.711; d.f.=6; P=0.503) (Table 1).

Comparison between left and right dS (fast fibres: mean 270 *vs* 290; t=–0.452; d.f.=4; P=0.674; superfast fibres: mean 257 *vs* 209; t=0.388; d.f.=4; P=0.718) (Table 2) indicated no significant difference between fibre type numbers. Similarly, fast fibre counts (mean 630 *vs* 923; t=–0.756; d.f.=4; P=0.491) as well as superfast counts (mean 994 *vs* 652; t=0.801; d.f.=4; P=0.467) between left and right vS were also statistically similar.

There were no differences detected in the diameters of fast or superfast fibres between left and right dTB (fast fibres: mean 18.2 µm *vs* 20.8 µm; t=0.937; d.f.=4; P=0.401; superfast fibres: mean 37.4 µm *vs* 38.7 µm; t=–0.347; d.f.=4; P=0.746) or vTB (fast fibres: mean 17.2 µm *vs* 15.9 µm; t=0.809; d.f.=6; P=0.449; superfast fibres: mean 33.9 µm *vs* 36.0 µm; t=–1.19; d.f.=6; P=0.277) (Table 1). Likewise,

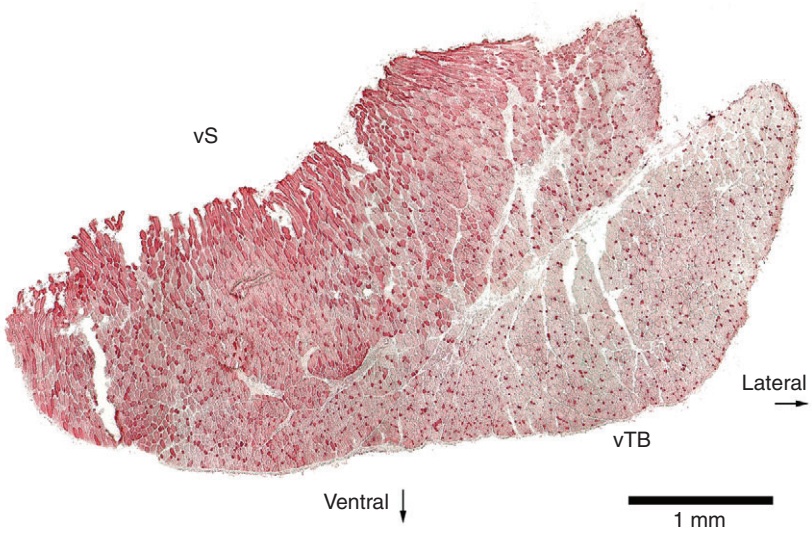

Fig. 3. Immunohistochemical reaction, using antifast antibody MY32, of the right ventral mass (tracheobronchialis and syringealis) of a male European starling. Dark red fibres indicate fast muscle fibres, while unreacted fibres are superfast. Ventral is to the bottom of the image, lateral is to the right. Tracheobronchialis ventralis (vTB) is more ventral and lateral than syringealis ventralis (vS). Note that the tracheobronchialis has smaller fast fibres and a higher percentage of superfast fibres compared with the syringealis muscle.

neither fast nor superfast fibres differed significantly in diameter for left and right dS (fast fibres: mean 28.1 µm *vs* 30.4 µm; t=–1.04; d.f.=4; P=0.355; superfast fibres: mean 46.7 µm *vs* 43.7 µm; t=0.410; d.f.=4; P=0.703) or left and right vS (fast fibres: mean 21.7 µm *vs* 27.5 µm; t=–1.29; d.f.=4; P=0.265; superfast fibres: mean 32.6 µm *vs* 35.3 µm; t=–1.54; d.f.=4; P=0.198) (Table 2). Thus, no left–right differences were observed for any of the fibre types in the four muscles examined.

Left–right activation during song

This lack of morphological differences between left and right syringeal sides is also reflected in our indirect physiological estimates of speed of contraction. We used airflow recordings and modulation of airflow to infer gating muscle activity. During song, rapid gating occurs during the switch from expiration to inspiration. Although substantial variation can be observed in the speed with which each labial valve is opened at the onset of mini-breaths, the two syringeal valves show similar maximal flow (Figs 4 and 5; Table 3). The maximal rate indicates that left and right abductor muscles can perform at similar speed. Airflow is also rapidly turned on and off during the course of a syllable as the bird switches sound production between sides. Again, no difference could be detected between the maximal rate for left and right opening events (Fig. 5; Table 3). Next, we selected segments where airflow was modulated in an oscillatory fashion. If air sac pressure did not parallel the changes in airflow, we assumed that the modulation was caused by the gating muscles of the syrinx (Goller and Suthers, 1996a) (Fig. 5). Data from all four starlings reveal an inverse correlation between the modulation depth and the period of the modulation. The data from the two individuals from which the highest sample sizes could be obtained are shown in Fig. 6. The time constant of the thermistors (90% of full scale in 6 ms) could theoretically contribute to the observed trend, as more rapid flow fluctuations would lead to a lower voltage response. However, it is unlikely that this possibility accounts for the observed relationship (Fig. 6), because the continuous trend spans a range of period durations which are clearly not affected by the temporal limitation of the thermistors. These data suggest therefore that the temporal limit for effective valve control is slightly above 200 Hz modulation rate, but no systematic difference between the left and right syringeal halves can be detected. In addition, EMG burst rates also do not indicate a left–right difference in the highest modulation rate (Table 4).

Comparing fibres within tracheobronchialis and syringealis muscles.

With no left–right differences, we averaged the fibre count and diameter data from left and right sides for comparison of the two fibre types within tracheobronchialis and syringealis muscles. Tracheobronchialis muscles contained significantly more superfast than fast fibres (mean 1468 *vs* 614; t=–3.14; d.f.=6; P=0.019; mean percentage 72.3% *vs* 27.7%). Fibre diameter was also significantly larger for superfast than fast fibres (mean diameter 35.8 µm *vs* 17.3 µm; t=–12.54; d.f.=6; P=0.00002) (Table 1; Fig. 3).

In syringealis muscles, there was no significant difference between the number of superfast and fast muscle fibres (mean 520 *vs* 591; t=0.276; d.f.=6; P=0.791; mean percentage 48% *vs* 52%). However, superfast fibres were again significantly larger in diameter (mean diameter 39.3±7.1 µm *vs* 27.5±3.6 µm; t=–3.80; d.f.=6; P=0.009) (Table 2; Fig. 3).

Architecturally, tracheobronchialis muscles are trapezoidally shaped (Fig. 1) with a shorter medial border and longer lateral edge.

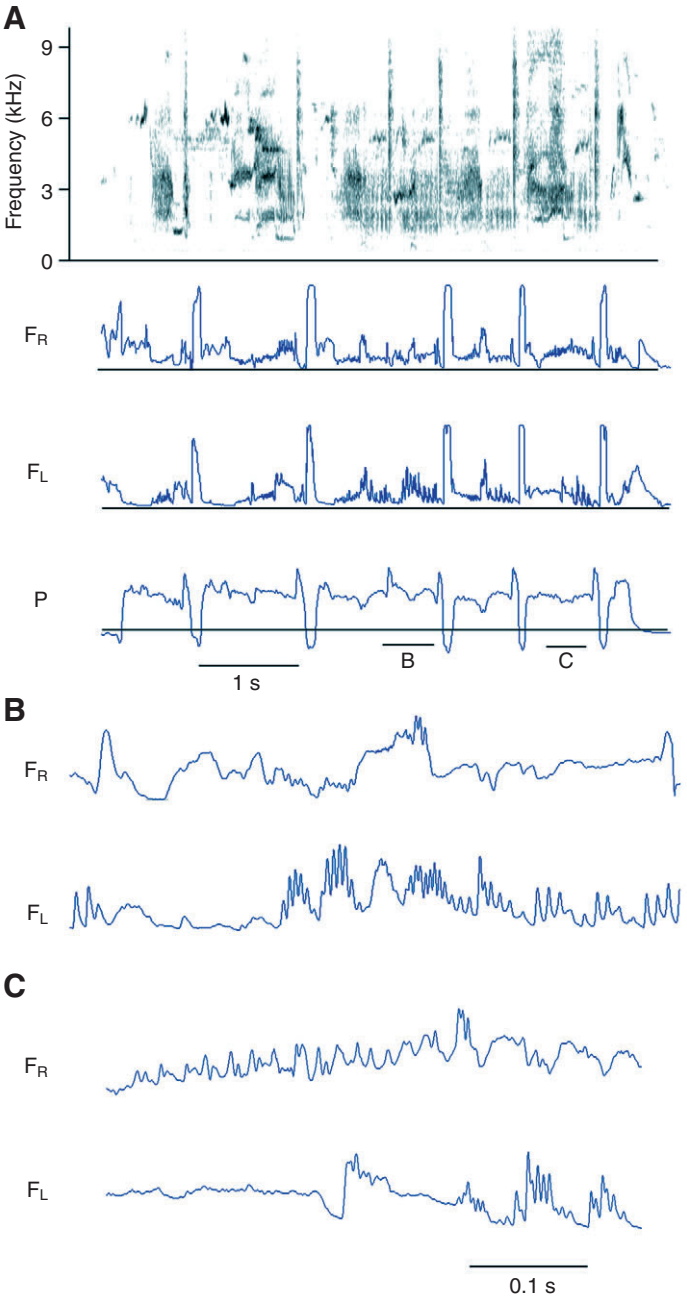

Fig. 4. Segment of starling song illustrating oscillatory modulation of airflow in both sound generators at high rates. (A) Song segment is shown spectrographically (top) together with airflow recordings through the right (F_R) and left (F_L) sound generator, and subsyringeal air sac pressure (P, horizontal line indicates atmospheric pressure). Two segments from A are expanded (B,C) to illustrate the oscillatory modulation of airflow through both sides.

Thus, medial edges ranged in length from 3 to 4.5 mm, with individual fibres of a mean length of 3.75 mm. The lateral edge ranged in length from 6.8 to 8 mm, with fibres measuring a mean length of 3.5 mm; thus this muscle possessed two overlapping fibres with tapering ends arranged serially. Fibres overlapped by approximately 30%. In contrast, syringealis muscles were more square shaped and ranged in length from 3 to 4.2 mm, containing muscle fibres with a mean length of 3.4 mm; their fibres therefore spanned the entire length of the muscle.

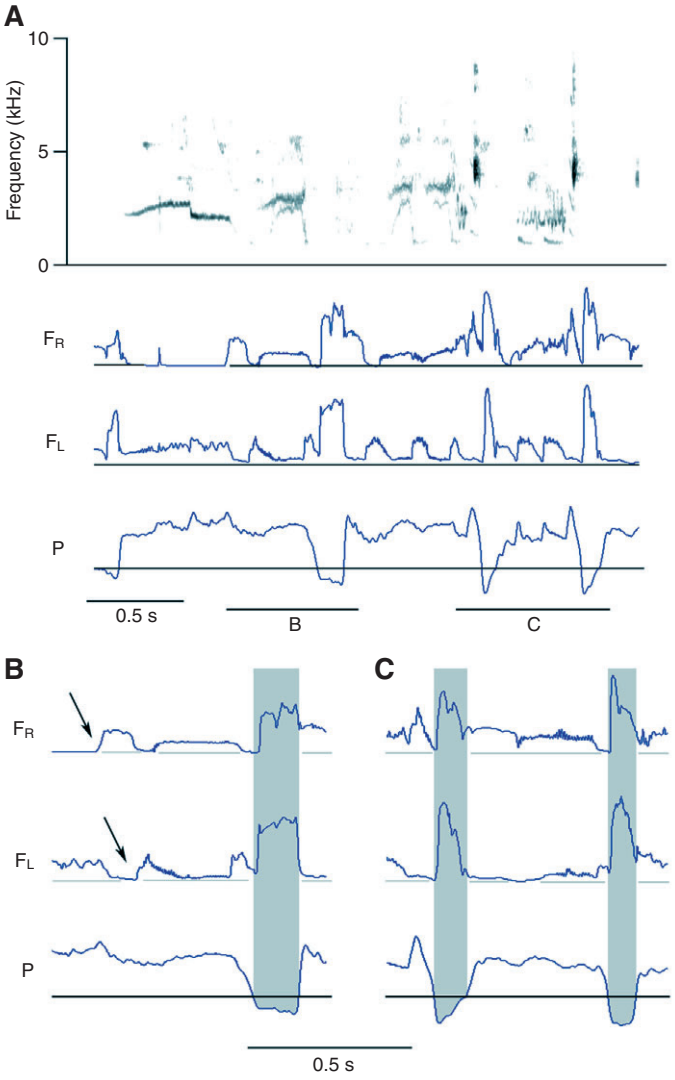

Fig. 5. Song segment with physiological data showing rapid gating of airflow during song and at the transitions between inspiration and expiration. Both gating events involve active control of the syringeal valves. (A) Spectrogram of song with right (F_R) and left airflow (F_L) and subsyringeal air sac pressure (P, pressure values below horizontal line drive inspiration). (B,C) expanded view of segments marked in A to indicate closure and opening (arrows) of the syringeal valve during song and rapid airflow reversal during the switch from expiration to inspiration (shaded columns).

Activation during song

Because muscle architecture suggests that tracheobronchialis muscles are slightly more optimized for speed than syringealis muscles, we used EMG data to test whether the differences in fibre type composition are also represented in their respective use during song. Many syllables of starling song display oscillatory modulation of amplitude (AM) and/or frequency (FM). We measured the period of burst-like EMG patterns during oscillatory acoustic modulation (AM, FM). A representative example of the data is shown in Fig. 7. The shortest period of burst-like muscle activation was similar in all recorded muscles (Table 4). Although there were small differences in means for the different muscles, these can be attributed to generally lower burst rates in some individuals. The fact that syringealis and tracheobronchialis muscles were measured in the same individual and no difference was found suggests that burst-like activation rates do not differ systematically between muscles. Whereas activation patterns indicate similar temporal use for syringealis and tracheobronchialis muscles, this analysis does not allow us to assess whether there might be performance differences between these muscles resulting from the differences in fibre composition.

Table 3. Gating parameters on left and right sides of the syrinx

	Mini-breath flow increase as % maximum (% per ms)		Flow increase as % maximum (% per ms) during song	
Bird	Left	Right	Left	Right
ST1	34.8	34.8	11.4	13.5
ST22	15.5	31.0	16.5	19.2
ST33	15.2	14.8	12.7	–
ST43	32.6	27.4	14.8	–
ST14	–	21.5	–	9.9
Mean	24.5	25.9	13.8	14.2

DISCUSSION

This research shows that intrinsic syringeal muscles of the European starling are heterogeneously composed of superfast and fast oxidative muscle fibres, and therefore provides a morphological explanation for the extremely fast performance of these muscles (Elemans et al., 2008). Although the ratio of these two fibre types varies between tracheobronchialis and syringealis muscles, this specialization is not reflected in a difference in the fastest temporal neural activation pattern between these muscles. Differences in muscle morphology must, however, influence the muscle contraction pattern of different syringeal muscles at these high activation rates.

Fibre type composition of muscles and functional specialization

The heterogeneous make-up of intrinsic syringeal muscles of songbirds was not fully recognized in previous morphological studies. Although measurements on cross-sectional areas of fibres have been published for syringeal muscles in the zebra finch, no distinction between different fibre populations was made (Wade and Buhlman, 2000). In the European starling, fast and superfast fibres do not show much overlap in diameter (approximately 22.5 µm for fast and 37.5 µm for superfast fibres). The fibre type composition of syringeal muscles in male zebra finches is similar to that observed in the European starling (Uchida et al., 2009) and may represent a more general pattern for male songbirds.

The syrinx of ancestral bird groups is thought to have been controlled by two extrinsic syringeal muscle pairs, m. sternotrachealis and m. tracheolateralis (King, 1989). These extrinsic muscles represent the only muscular control in the vocal organ of many extant bird groups, whereas more derived groups have evolved additional, intrinsic muscles. This raises the question of whether superfast fibres are already present in extrinsic muscles or whether they are a specialization of intrinsic muscles. The results of muscle fibre characterization of sternotracheal muscles in ducks indicate that they possess populations of slow (type I) fibres (Lalatta-Costerbosa et al., 1990) and no superfast fibres, although neither antibody characterization nor physiological measurements were performed. The available data suggest the latter interpretation of a modulation of intrinsic muscles is correct, but more research is needed to clarify this issue. Preliminary data from the sternotrachealis muscle in European starlings and other songbirds show the muscle to be largely composed of fast (type IIA) fibres, although some superfast fibres are present as well. There does not

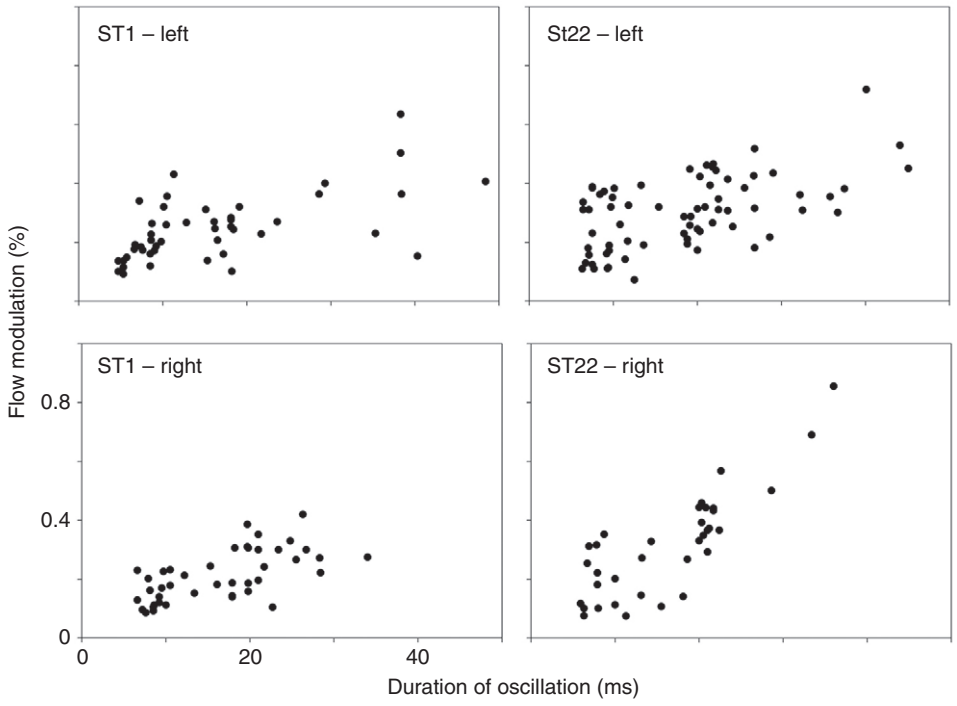

Fig. 6. The depth of flow modulation decreases with increasing modulation rate. Representative data for the left and right sides of two individuals illustrate how modulation depth increases with increasing duration of the flow oscillation. Linear regressions for all data are highly significant (St1 – left: R=0.6, F=24.67, P<0.0001; St1 – right: R=0.63, F=27.1, P<0.0001; St22 – left: R=0.55, F=29.7, P<0.0001; St22 – right: R=0.83, F=73.0, P<0.0001).

appear to be the difference in fibre diameter between fast and superfast fibres that we found in intrinsic syringeal muscles (A.M.U. and R.A.M., unpublished).

The larynx of mammals has an analogous function to the avian syrinx. The five intrinsic muscles function as abductors or adductors during quiet respiration and vocalization, and in controlling sound frequency. Shiotani and colleagues (Shiotani et al., 1999) identified muscles active during quiet respiration as possessing both fast and slow muscle fibres, but those responsible for glottal closure were uniformly fast. The rat thyroarytenoid was found to possess superfast fibres (type IIL).

The avian syrinx likewise contains two fibre types. Although we found no slow fibres, it is possible that the fast (type IIA) fibres are predominantly activated during quiet respiration, whereas superfast fibres are preferentially activated during control of vocal behaviour or rapid gating activity during various other behaviours. At present, it is not known how different fibre types are organized into motor units, and how these units are activated during different behaviours.

Functional considerations of differences between syringeal muscles

The difference in fibre type composition between the syringealis and tracheobronchialis muscles may indicate functional differences during song production. The tracheobronchialis muscles control the movement of the lateral labium in and out of the bronchial lumen (Goller and Suthers, 1996a). Rapid oscillatory gating function of airflow is one mechanism for generating rapid oscillatory modulation of sound amplitude (e.g. Goller and Suthers, 1996a). Starling song contains sound elements with oscillatory, rapid amplitude and frequency modulation. Tracheobronchialis muscles can directly control such acoustic features at frequencies above 200 Hz (Elemans et al., 2008). Interestingly, tracheobronchialis muscles had two fibres serially arranged. Although the functional significance of in-series muscles is unknown (Meyers and Hermanson, 1994), it may relate to the gating function of the tracheobronchialis. Whether differences exist in contraction kinetics between syringealis and tracheobronchialis muscles is not known,

Table 4. Shortest period of burst-like activation (in ms) correlated with sound AM/FM

Starling	vS_L	vTB_L	dTB_L	dS_L	vS_R	vTB_R	dTB_R	dS_R
ST6	–	–	–	5.0	–	–	–	5.0
ST42	5.3	–	–	–	5.3	–	–	–
ST36	5.0	–	5.0	–	5.0	–	–	5.2
ST33	–	5.0	5.0	–	–	5.0	5.0	–
ST60	4.8	–	–	4.8	–	–	–	4.8
ST45	–	5.0	–	5.0	–	5.3	5.3	–
ST63	5.4	–	–	5.4	5.4	–	–	–
ST67	–	–	4.8	4.8	–	–	–	–
ST34	–	4.8	4.8	–	–	4.8	4.8	–
ST39	–	4.8	4.8	–	–	4.8	4.8	–
Mean	5.12	4.9	4.82	5.0	5.23	4.97	4.97	5.0

For each muscle, periodic activation of at least 50 syllables with AM/FM was measured. The reported values therefore represent the minimum of at least 100–600 measurements.

vS_L, ventral syringealis left; vTB_L, ventral tracheobronchialis left; dTB_L, dorsal tracheobronchialis left; dS_L, dorsal syringealis left; vS_R, ventral syringealis right; vTB_R, ventral tracheobronchialis right; dTB_R, dorsal tracheobronchialis right; dS_R, dorsal syringealis right.

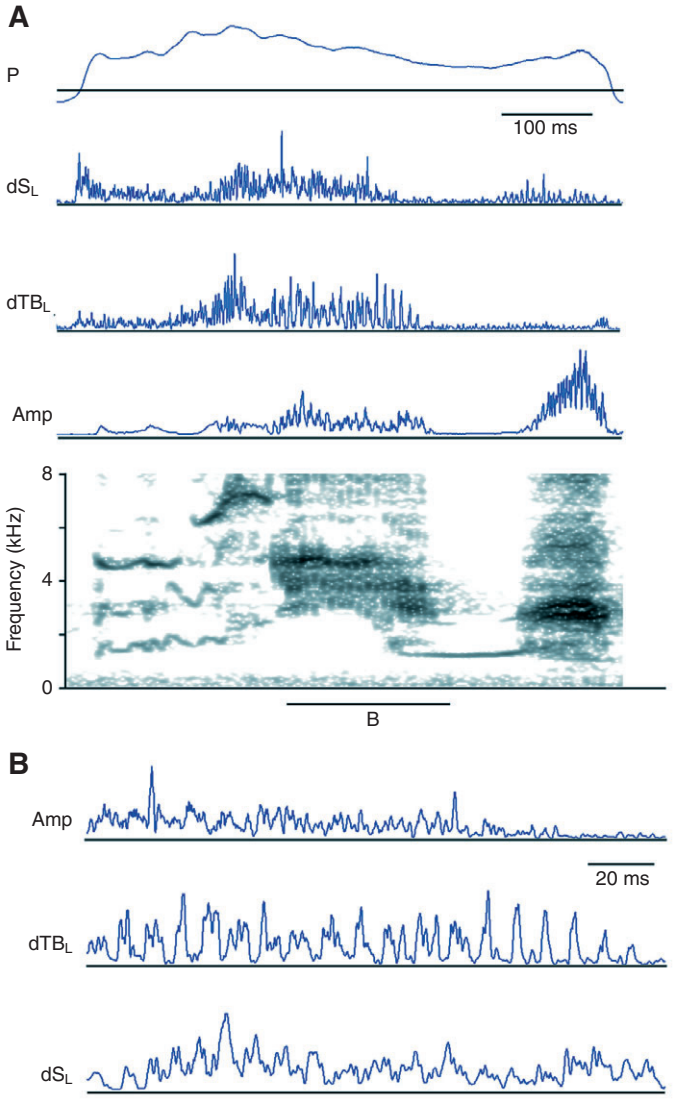

Fig. 7. Example of EMG data illustrating burst-like activation patterns of syringeal muscles during song production in the European starling. (A) One song syllable (illustrated spectrographically at the bottom and as integrated amplitude, Amp) with corresponding EMG patterns in left dorsal m. tracheobronchialis (dTB_L) and left dorsal m. syringealis (dS_L) (rectified and integrated with 2 ms sliding window), and subsyringeal air sac pressure (P). The sound segment with pronounced amplitude modulation in the middle of the syllable is accompanied by burst-like activation in both muscles but no corresponding modulation of air sac pressure. (B) Expanded view of the sound segment indicated in A, with amplitude modulation (Amp).

since a comparison has not been made in physiological studies (Elemans et al., 2008).

The burst-like activation of syringeal muscles correlates well with the contraction characteristics of the tracheobronchial muscle (Elemans et al., 2008). Assuming that this correspondence exists, it is likely that the fastest oscillatory activation cycles do not differ between the syringealis and tracheobronchialis muscles. In fact, in birds where EMG activity was recorded simultaneously from the two muscles, for song syllables with burst-like activation in both muscles these occurred at the same frequency (Fig. 4). Similar activation patterns, however, need not translate into similar biomechanical effects, if muscle contraction kinetics are different as suggested by different fibre type composition. However, it is not known in detail how muscle contraction relates to movement of cartilaginous components of the syringeal framework (Larsen and Goller, 2002), and how this movement affects the vibrating labia. It is therefore not possible with the available data to predict the implications of this difference in fibre type ratio for the detailed control of acoustic parameters.

In the mammalian larynx, muscles regulating the valve action of the vocal folds (posterior cricoarytenoid muscle as abductor and thyroarytenoid muscle as adductor) display faster contraction kinetics than the main muscle involved in frequency control (cricothyroid muscles) (Hoh, 2005). This difference is also reflected in the fibre type composition of these muscles, and substantial differences in fibre composition exist between species (Hoh, 2005). Interestingly, the gating muscles are innervated by the recurrent laryngeal nerve, while the cricothyroid muscle receives innervation from the superior laryngeal nerve. Such a difference in the origin of neural control does not exist in the avian syrinx, where all muscles are innervated by the tracheosyringeal branch of the hypoglossal nerve (CN XII). It would be interesting to know to what degree different nervous supply influences the fibre type composition of the respective laryngeal muscles and their physiological performance (Hoh, 2005).

Lateralization of song production and syringeal muscles

In the European starling, muscles on the left and right sides of the dual sound source do not display differences in fibre type composition, and their maximal, burst-like activation frequency also does not differ. This indicates that the potential for rapid acoustic control is similar on the two sides of the syrinx in this species. However, this does not imply that the two sides are used interchangeably in regard to the acoustic characteristics of the sounds contributed by the respective sound generators. As was found in all other investigated species (Suthers and Goller, 1997; Suthers, 1999; Suthers et al., 1999), the left side of the syrinx contributes, on average, lower frequency sounds than the right side (B.G.C. and F.G., unpublished). Starling song contains many syllables with oscillatory modulation of frequency and amplitude, two sound features that are frequently controlled by direct action of the syringeal muscles. In brown thrashers such modulations are more frequently generated on the right side of the syrinx (Goller and Suthers, 1996a). In European starlings a similar tendency exists (B.G.C. and F.G., unpublished), but this disproportionate use of the right side for high-frequency modulation of sound is not expressed as a difference in fibre type composition of the left and right muscles. Similarly, no difference in twitch half-time was found between the left and right dTB muscles in the starling (Elemans et al., 2008). It is not clear whether differences in muscle performance and cytoarchitecture could be expected even in species with very pronounced lateralization of song production. In the Waterslager canary (*Serinus canaria*), for example, most song syllables are generated on the left side of the syrinx, but in order to do so, the right side needs to be actively closed to airflow. In addition, the right side is abducted to maximize inspiratory airflow during inter-syllable mini-breaths (Suthers, 1992; Suthers, 1997; Suthers and Goller, 1997). Both of these mechanisms require extremely rapid gating activity as sound syllables and corresponding mini-breaths may be as short as 15 ms. Considering this gating activity, it is clear that silencing of one syringeal side during song requires equally rapid muscular control in the gating muscles as muscular control of phonation. This requirement may reduce a specialization of syringeal muscles on the two syringeal sides, but more comparative data are needed to test this interpretation further.

CONCLUSIONS

Syringeal muscles in the European starling are among the fastest vertebrate muscles, but unlike other fast muscles involved in sound generation, they are not uniformly composed of a superfast fibre type. This heterogeneous composition may permit differentiation of possible functions, for example, slower gating activity during quiet respiration, and more rapid control of sound generation. It also suggests a potential for sophisticated neural control of motor units, but the nature of the cytoarchitectural organization into motor units is not known for syringeal muscles.

In comparison to the mammalian larynx, the songbird syrinx is controlled by muscles with faster contraction kinetics. Whether this specialization is a consequence of selective pressures related to vocal behaviour or another unrelated function such as airflow control during other behaviours (flight) is unknown. Whereas different intrinsic muscles with different functional roles display distinctly different fibre composition in mammals, there is much less variation within the syringeal muscles of the starling. Although some muscle-controlled acoustic modulation is lateralized for the left and right sound generators of the syrinx, the muscles do not show a distinct specialization in fibre composition or contractile behaviour (Elemans et al., 2008).

ACKNOWLEDGEMENTS

Experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Utah. Joshua McFarland, Karalee Baquero, April Adair and Daniel Schmutz assisted with the collection and analyses of the histology data. John Cavitt assisted with the statistical analyses. This research was supported by the National Institutes of Health grant DC 04390 (to F.G. and R.A.M.) and by a Weber State Undergraduate Research Scholarship, a Denker's Family Foundation Undergraduate Research Scholarship, and a WSU Undergraduate Research Fellowship (all to A.M.U.). Deposited in PMC for release after 12 months.

REFERENCES

Alipour, F. and Titze, I. (1999). Active and passive characteristics of the canine cricothyroid muscles. *J. Voice* **13**, 1-10.
Alipour-Haghighi, F., Titze, I. and Durham, P. (1987). Twitch response in the canine vocalis muscle. *J. Speech Hear. Res.* **30**, 290-294.
Andersson, M. (1994). *Sexual Selection*. Princeton, NJ: Princeton University Press.
DelGaudio, J. M., Carroll, W. R., Sciote, J. J. and Esclamado, R. M. (1995). Atypical myosin heavy chain in rat laryngeal muscle. *Ann. Otol. Rhinol. Laryngol.* **104**, 237-245.
Elemans, C. P. H. (2004). How do birds sing? Sound analysis – mechanical modeling – muscular control. PhD dissertation. Wageningen University.
Elemans, C. P. H., Mead, A. F., Rome, L. C. and Goller, F. (2008). Superfast vocal muscles control song production in songbirds. *PLoS ONE* **3**, e2581.
Fee, M. S., Shraiman, B., Pesaran, B. and Mitra, P. P. (1998). The role of nonlinear dynamics of the syrinx in the vocalizations of a songbird. *Nature* **395**, 67-71.
Fine, M. L. and Pennypacker, K. R. (1988). Histochemical typing of sonic muscle from the oyster toadfish. *Copeia* **1988**,130-134.
Goller, F. and Suthers, R. A. (1996a). The role of syringeal muscles in gating airflow and sound production in singing brown thrashers. *J. Neurophysiol.* **75**, 867-876.
Goller, F. and Suthers, R. A. (1996b). The role of syringeal muscles in controlling the phonology of bird song. *J. Neurophysiol.* **76**, 287-300.
Gopalakrishnakone, P. (1985). Structure and innervations of the tracheal muscles of the white Pekin duck. *J. Anat.* **140**, 205-219.
Hoh, J. F. (2005). Laryngeal muscle fibre types. *Acta Physiol. Scand.* **183**, 133-149.
King, A. S. (1989). Functional anatomy of the syrinx. In *Form and Function in Birds*, vol. 4 (ed. A. S. King and J. McLelland) pp. 105-192. London: Academic Press.
Lalatta-Costerbosa, G., Scapolo, P. A., Barazzoni, A. M., Petrosinop, G., Clavenzani, P., Lucchi, M. L. and Bortolami, R. (1990). Analysis of the sternotrachealis muscle fibers in some anseriformes: Histochemistry and sex differences. *Am. J. Anat.* **189**, 357-364.
Larsen, O. N. and Goller, F. (2002). Direct observation of syringeal muscle function in songbirds and a parrot. *J. Exp. Biol.* **205**, 25-35.
Martensson, A. and Skoglund, C. R. (1964). Contractile properties of intrinsic laryngeal muscles. *Acta Physiol. Scand.* **60**, 318-336.
McFarland, J. and Meyers, R. A. (2008). Anatomy and histochemistry of hindlimb flight posture in birds. 1. The extended hindlimb posture of shorebirds. *J. Morphol.* **269**, 967-979.
Meijer, A. E. (1968). Improved histochemical method for the demonstration of the activity of α-glucan phosphorylase. *Histochemie* **12**, 244-252.
Meyers, R. A. and Hermanson, J. W. (1994). Pectoralis muscle morphology in the little brown bat, Myotis lucifugus: A non-convergence with birds. *J. Morphol.* **219**, 269-274.
Meyers, R. A. and Stakebake, E. F. (2005). Anatomy and histochemistry of spread-wing posture in birds. 3. Immunohistochemistry of flight muscles and the "shoulder lock" in albatrosses. *J. Morphol.* **263**, 12-29.
Novikoff, A. B., Shin, W. and Druker, J. (1961). Mitochondrial localization of oxidative enzymes: staining results with two tetrazolium salts. *J. Biophys. Biochem. Cytol.* **9**, 47-61.
Rome, L. C. (2006). Design and function of superfast muscles: new insights into the physiology of skeletal muscle. *Ann. Rev. Physiol.* **68**, 193-221.
Rome, L. C. and Lindstedt, S. L. (1998). The quest for speed: muscles built for high-frequency contractions. *News Physiol. Sci.* **13**, 261-268.
Rome, L. C., Syme, D. A., Hollingworth, S., Lindstedt, S. L. and Baylor, S. M. (1996). The whistle and the rattle: The design of sound producing muscles. *Proc. Natl. Acad. Sci. USA* **93**, 8095-8100.
Rome, L. C., Cook, C., Syme, D. A., Connaughton, M. A., Ashley-Ross, M., Klimov, A. and Goldman, Y. E. (1999). Trading force for speed: Why superfast crossbridge kinetics leads to superlow forces. *Proc. Natl. Acad. Sci. USA* **96**, 5826-5831.
Schultz, E., Clark, A. W., Suzuki, A. and Cassens, R. G. (1980). Rattlesnake shaker muscle: I. A light microscopic and histochemical study. *Tissue Cell* **12**, 323-334.
Sciote, J. J., Morris, T. J., Brandon, C. A., Horton, M. J. and Rosen, C. (2002). Unloaded shortening velocity and myosin heavy chain variations in human laryngeal muscle fibers. *Ann. Otol. Rhinol. Laryngol.* **111**, 120-127.
Shiotani, A., Westra, W. H. and Flint, P. W. (1999). Myosin heavy chain composition in human laryngeal muscles. *Laryngoscope* **109**, 1521-1524.
Suthers, R. A. (1992). Lateralization of sound production and motor action on the left and right sides of the syrinx during bird song. In *Proceedings of the 14th International Congress on Acoustics*. Beijing: IUPAP, paper I1-5.
Suthers, R. A. (1997). Peripheral control and lateralization of birdsong. *J. Neurobiol.* **33**, 632-652.
Suthers, R. A. (1999). The motor basis of vocal performance in songbirds. In *The Design of Animal Communication* (ed. M. D. Hauser and M. Konishi), pp. 37-62. Cambridge, MA: The MIT Press.
Suthers, R. A. and Goller, F. (1997). Motor correlates of vocal diversity in songbirds. In *Current Ornithology*, vol. 14 (ed. V. Nolan, E. D. Ketterson and C. F. Thompson), pp. 235-288.
Suthers, R. A. and Hector, D. H. (1985). The physiology of vocalization by the echolocating oilbird, Steatornis caripensis. *J. Comp. Physiol. A* **156**, 243-266.
Suthers, R. A. and Zollinger, S. A. (2008). From brain to song: the vocal organ and vocal tract. In *Neuroscience of Birdsong* (ed. H. P. Zeigler and P. Marler), pp. 78-98. Cambridge: Cambridge University Press.
Suthers, R. A., Goller, F. and Hartley, R. S. (1994). Motor dynamics of song production by mimic thrushes. *J. Neurobiol.* **25**, 917-936.
Suthers, R. A., Goller, F. and Pytte, C. (1999). The neuromuscular control of birdsong. *Phil. Trans. R. Soc. Lond.* **354**, 927-939.
Uchida, A. M., Green, J., Ahmad, S., Goller, F. and Meyers, R. A. (2009). Sexual dimorphism of syringeal muscles in songbirds. *Integrat. Comp. Biol.* **49**, e318.
Wade, J. and Buhlman, L. (2000). Lateralization and effects of adult androgen in a sexually dimorphic neuromuscular system controlling song in zebra finches. *J. Comp. Neurol.* **426**, 154-164.