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Superfast Vocal Muscles Control Song Production in Songbirds

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Abstract

Birdsong is a widely used model for vocal learning and human speech, which exhibits high temporal and acoustic diversity. Rapid acoustic modulations are thought to arise from the vocal organ, the syrinx, by passive interactions between the two independent sound generators or intrinsic nonlinear dynamics of sound generating structures. Additionally, direct neuromuscular control could produce such rapid and precisely timed acoustic features if syringleal muscles exhibit rare superfast muscle contractile kinetics. However, no direct evidence exists that avian vocal muscles can produce modulations at such high rates. Here, we show that 1) syringleal muscles are active in phase with sound modulations during song over 200 Hz, 2) direct stimulation of the muscles in situ produces sound modulations at the frequency observed during singing, and that 3) syringleal muscles produce mechanical work at the required frequencies and up to 250 Hz in vitro. The twitch kinematics of these so-called superfast muscles are the fastest measured in any vertebrate muscle. Superfast vocal muscles enable birds to directly control the generation of many observed rapid acoustic changes and to actuate the millisecond precision of neural activity into precise temporal vocal control. Furthermore, birds now join the list of vertebrate classes in which superfast muscle kinetics evolved independently for acoustic communication.

Introduction

Some of the most complex vocal communication signals in the animal kingdom are produced by songbirds [1], whose songs often contain long sequences of rapidly modulated sound elements [2]. Rapid acoustic modulations (<10 ms) during song can arise from passive interactions between the two independent (i.e. left and right) sound generators in the vocal organ – the syrinx [3] and intrinsic nonlinear dynamics of sound generating structures [e.g. 4–7]. However, many acoustic features of song correlate with neural [8–14] and electromyographic (EMG) activity [15–19], which suggests the possibility of direct neuromuscular control of the syrinx.

Indirect evidence from EMG recordings in brown thrashers (Toxostoma rufum) indicates that sound modulations up to 125 Hz correlate with muscle activity [17]. Furthermore, the variation in temporal characteristics of song in zebra finches (Taeniopygia guttata) correlates with variation in the spiking patterns of neurons in premotor brain nuclei [8,9,11,12,14], which suggests that the temporal precision of the CNS can be expressed at the behavioral level of song production. Both findings are consistent with very fast muscular control of the vocal production system of songbirds. However, to actuate these rapid changes, songbirds would need to have evolved syringleal muscles with superfast contractile kinetics. This rarely evolved trait would enable them to produce positive work over 100 Hz [20]. To our knowledge no direct evidence exists that avian vocal muscles can produce modulations at such high rates.

To assess how high temporal precision in the central premotor song circuits is translated into equally high precision at the behavioral level, we need to make a direct determination of temporal performance limits of vocal muscular control in songbirds. By conducting a series of experiments at different levels of organization, we show that the vocal muscles of songbirds exhibit superfast contractile kinetics and can generate acoustic modulations up to 250 Hz.

Results

European starlings (Sturnus vulgaris) have a complex learned song [21,22], which contains many fast modulations. Some syllables contain amplitude modulated “buzzes” (100–125 Hz) and other modulations up to 200 Hz (Figure 1). In order to test whether these modulations could be the result of direct muscular control, we measured electromyographic (EMG) activity of the syringleal muscles in freely singing starlings (see Supporting Information, Methods S1). The main muscle causing amplitude modulations by regulating airflow is the m. tracheobronchialis dorsalis (dTB) [17]. In vivo recordings of dTB activity in freely singing starlings showed that amplitude modulation of the produced sound was accompanied by synchronized dTB activity, suggesting active control (Figure 1B). The dTB showed activity bursts that correlated with sound amplitude at intervals as short as 4.6 ms, which is equivalent to a repetition rate of 218 Hz (Figure 1C). If syringleal...
Figure 1. Song of starling exhibits fast modulations that correlate with muscle activity. (A) Spectral derivative plot [45] (top), oscillogram of sound and EMG activity of m. tracheobronchialis dorsalis (dTB). (B) Expanded time scale of segment i. The rate of modulation of the sound amplitude is paralleled by peaks in muscle activity (shaded areas). Positive traces; integrated values (time constant 0.2 ms), negative traces; half-wave rectified values. (C) Cross-correlations (R) of integrated sound amplitude and EMG activity signals of segments i and ii show a temporal link between EMG activity and sound modulation. The segments i and ii are indicated in (A). The distance between the local peaks in the bar diagrams equals the period of the signals. The periods are 4.6 and 6.6 ms in segment i and ii respectively, which is equivalent to repetition rates of 218 and 152 Hz. EMG; electromyogram of dTB, Sound; sound oscillogram.

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muscles cause these sound modulations, it requires that they can produce work and modulate syringeal parameters at these high rates.

To determine whether the muscles are indeed capable of this performance, we measured the in vitro performance of syringeal muscles in European starlings and zebra finches. We used isolated fibre bundles of the adductor muscle dTB in starlings and the abductor muscle m. tracheobronchialis ventralis (vTB) in zebra finches.

The twitch half-times measured 3.23±0.44 ms in male starling (N = 12), 2.93±0.79 ms in female starling (N = 3), 3.73±0.68 ms in male zebra finch (N = 8) and 7.08±0.79 ms for female zebra finch (N = 3) at 41.4°C. The twitch half-times did not significantly differ between males and females in starlings (t-test; p = 0.5802, combined measurement: 3.23±0.44 ms, N = 15), but were significantly different between males and females in zebra finches (t-test; p = 0.01; Figure 2A).

In some bird species, song control is lateralized [23–27] with a tendency to predominantly generate amplitude (and frequency) modulations on the right side in the brown thrasher [17]. Therefore we focused on obtaining muscle preparations from the right side of the syrinx (See Supporting Information, Methods S1). However, we also obtained a number of muscle preparations from the left side of the syrinx (three for male starling and two for male zebra finch). The twitch half-times did not differ significantly for starling (t-test; p = 0.499; lumped data males and females) or zebra finch (t-test; p = 0.969; males only).

In starlings, the dTB developed isometric force from 10% to 100% in 1.74±0.32 ms (N = 15). In one preparation, the twitch half-time was as short as 1.6 ms and it developed force from 10% to 100% in 1.03 ms. Twitches were still completely separate at 150 Hz stimulation frequency and tetanic fusion was not complete until 600–800 Hz in all preparations (Figure 2B).

Isometric measurements, however, do not provide evidence whether the muscles can produce work at the high cycle frequencies as suggested by our in vivo measurements. To modulate sound, muscle must be able to perform mechanical work at the modulation frequency. Non-isometric measurements in which we subjected the muscles to various strain cycles and stimulation regimes (the workloop technique [28]) revealed that syringeal muscles indeed produce positive work and power at cycle frequencies up to 250 Hz (Figure 3). These data establish that syringeal muscles have the contractile potential to actuate syringeal elements as fast as 250 Hz. Because measurements on isolated fiber bundles do not take into account additional elasticity and mass of the in situ configuration, we tested whether this extreme modulation performance is attainable by the whole muscle in the intact syrinx. We electrically stimulated the dTB and vTB at different rates and measured their capacity to modulate airflow in anaesthetized male starlings (Methods S1, Figure S1). These measurements showed that also in situ, syringeal gating muscles could modulate syringeal airflow up to 250 Hz (Figure 4A,B). When flow was increased above the phonation threshold, muscle stimulation caused amplitude modulation in the radiated sound (Figure 4C).

### Discussion

We present direct evidence that songbird have superfast syringeal muscles, which can modulate song acoustics up to 250 Hz. The extremely fast performance of isolated muscle fibers in vitro translates into very rapid modulation of syringeal airflow and sound amplitude in the biomechanically relevant setting of the intact syrinx in situ. Both tests indicate clearly that the presumed direct muscular control inferred from EMG activity during spontaneous song in vivo is well within the temporal performance capabilities of syringeal muscles, and therefore most likely indicates direct active neuromuscular control of sound modulation in spontaneously singing birds.

The performance of syringeal muscle ranks them among the fastest known vertebrate muscles [20]. With twitch contraction half-times of 3.23±0.44 ms (N = 15) and 3.73±0.68 ms (N = 8) for the adductor muscle dTB in starling and the abductor muscle vTB in male zebra finch, respectively, these highly specialized muscles attain the fastest measured isometric twitch kinematics of any vertebrate muscle to our knowledge. The isometric twitch characteristics of syringeal muscles in a non-songbird, the ring dove (Streptopelia risoria), were previously shown to be close to other superfast vertebrate muscles [15], but their performance limits (i.e., capability of performing mechanical work at high frequencies) were not explored [16]. With twitch half-times around 10 ms [15], the syringeal muscles in ring doves are much slower than the syringeal muscles of songbirds and can not be fast enough to control the modulation rates we observe in songbirds. Consistent with this, ring doves exhibit modulation rates of only 25 Hz during their vocalizations [16].

The extremely rapid activation and relaxation phases of syringeal muscle contraction require that multiple ultra-structural and molecular systems must be in place and work in concert [20]. It is currently unknown how the extremely rapid kinetics is achieved in syringeal muscles. Because both activation and relaxation are fast, we can expect that the processes of Ca2+ release and cross-bridge attachment during the activation cycle, and Ca2+ reuptake, Ca2+ unbinding from troponin and cross-
bridge detachment during deactivation [20,29,30] are extremely fast. Syringeal muscles operate at a high temperature of 41°C. The expected increase in kinetics induced by these high temperatures may not require as extreme adaptations as found in ectothermic animals, such as toadfish, who call at temperatures from 15–25°C. Nonetheless, syringeal muscles are much faster than locomotory muscles and likely involve novel myosin isoforms.

Superfast muscle is a muscle type that has only sporadically evolved, but appears in some vertebrate classes. The best-documented cases are the swimbladder muscles in various fishes [29–33] and the tail shaker muscles in rattle snakes [29,34]. Fast muscles have been observed in mammals: laryngeal muscle [35], and extraocular muscles e.g. [36,37], but mechanical measurements are yet to place them in the same league as the superfast muscles described above. The presence of superfast muscles in the avian vocal organ adds another independent case where these highly specialized muscles seem to have evolved in a sound production system. The function of the vocal muscles in mammals and birds differs from that of the swimbladder and tailshaker muscles. In mammals and birds, sound is produced by airflow-induced vibration of vocal folds in the larynx or labia in the syrinx, respectively. Vocal muscles of the mammalian larynx and avian syrinx do not generate sound pulses with each contraction, but they adjust vocal parameters that cause modulation of the airflow-induced oscillations of vocal folds and labia.

Cross-bridge kinetics [30] and space constraints at the muscle ultra-structural level dictate a trade-off between force production and maximal attainable frequency at which positive work can be produced [31,38,39]. This trade-off makes only certain biomechanical systems amenable to deriving benefits from superfast muscles. Motor systems under selection for speed therefore need to reduce actuator mass. The energy content of sound waves is low and the production and modulation of sound generally involves manipulation of low masses (e.g. swimbladder, rattle, syringeal and glottal structures), compared with much heavier skeletal elements (e.g. appendages). In contrast to muscles in locomotory systems [40], muscles in sound production systems appear to be optimized for speed and not power [31].

We show that motor performance of syringeal muscles in zebra finches is sexually dimorphic: the twitch characteristics of a main gating muscle vTB are significantly different between females and males, with the vTB of the females being almost two times slower.

![Figure 3. Superfast syringeal muscles produce positive work and power up to 250 Hz in vitro.](image-url)

- (A) Work and (C) power production of male starling m. tracheobronchialis dorsalis (dTB, N = 5). (B) Work and (D) power production of male zebra finch m. tracheobronchialis ventralis (vTB, N = 6). Plotted values are mean ± S.D. The insets show the bird species and the position of the dTB and vTB muscle on a schematic of the songbird’s syrinx (modified after [46]). The arrows indicate which muscle is being tested. (E) Active workloops of starling dTB at 150, 175, 200 and 250 Hz. Dotted vertical lines indicate zero strain (= resting length). Arrows indicate lengthening (pointing right) and shortening (pointing left) portions of the workloop.

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Figure 4. Stimulation of syringeal muscles causes tracheal flow and sound modulation. In situ muscle stimulation of syringeal muscles (A) dTB (N = 3) and (B) vTB (N = 3) modulates tracheal flow up to 250 Hz. Values are mean ± S.D. The insets show the position of the dTB and vTB muscle on a schematic of the songbird’s syrinx (modified after [46]). The arrows indicate which muscle is being tested. (C) Sound amplitude of vocalization during dTB stimulation at 100 Hz. The sound amplitude drops following muscle stimulation (red areas). Black trace; half-wave rectified sound signal; orange trace; integrated sound signal (time constant 2 ms).

This sexually dimorphic performance could arise from any of the above-mentioned muscular (ultra-) structural or molecular systems that affect contraction speed. In starlings however, no sexual dimorphism is found in muscle performance. These results are paralleled by singing behavior of these two species; in zebra finches only the males sing [41,42], whereas in starlings both males and females sing [21].

The neuromuscular control of song production in the syrinx is a well-known example of lateralization behavior [23–27,43]. Despite the low number of preparations, our data suggest that there is no lateralization in syringeal muscle twitch performance in the two investigated species. Therefore both sides of the syrinx seem to have an equal potential to modulate labial vibrations.

Extremely rapid transitions during song - in the order of 1 ms - can be caused by intrinsic dynamics of the syrinx [4]. In this study we provide direct evidence that songbird syringeal muscles are sufficiently fast to actively modulate acoustic parameters at these same high rates. Syringeal muscles of starlings generate full force within 1.74±0.32 ms (N = 15), which allows direct adjustments of vocal output in the millisecond range. In addition, with the use of fast syringeal muscles timing of sound onset can be controlled with high precision. The vocal production system of birds operates with high fidelity and is capable of millisecond temporal precision in portions of the song [8,10–12]. Superfast vocal muscles represent the mechanical actuator to translate the temporal precision in neural motor activity into similar precision in the behavioral output.

Materials and Methods

In vivo muscle activity

Syringeal muscle activity was measured in freely singing male starlings (Sturnus vulgaris) as previously described [16–19,44]. All experiments were in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah, Salt Lake City, USA. Methods are described in detail in the Online Supporting Information, Methods S1.

In vitro muscle performance

The work and power generated by syringeal muscles was determined using the workloop technique on isolated muscle fibre bundles as previously described [16,28,29,31]. In starlings, we isolated fibre bundles along the surface of musculus tracheobronchialis dorsalis (dTB). In zebra finches, we isolated fibre bundles of the musculus tracheobronchialis ventralis (vTB). Experiments were performed at the University of Pennsylvania, in October 2006 (zebra finches) and May 2007 (starlings) according to regulations by the IACUC, University of Pennsylvania, Philadelphia, USA.

In situ muscle stimulation and flow modulation

The effect of direct muscle stimulation (dTB) on syringeal airflow was measured on anaesthetised starlings in situ. Airflow above the syrinx was monitored with a custom-built direction sensitive flow probe, consisting of plastic tubing with two heated microbead thermistors in the lumen of the tube. Experiments were performed at the University of Utah, June 2007. All experiments were in accordance with the IACUC of the University of Utah, Salt Lake City, USA. A detailed description of the Materials and Methods can be found in the Supporting Information. Methods S1.

Supporting Information

Methods S1 Supplemental Information: Materials and Methods.

Figure S1 Flow modulation in the trachea following electrical stimulation of the syringeal muscles.

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Author Contributions

Conceived and designed the experiments: FG CE AM LR. Performed the experiments: FG CE AM LR. Analyzed the data: FG CE AM LR. Contributed reagents/materials/analysis tools: FG LR. Wrote the paper: FG CE AM LR.
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Animals.

Seventeen male and eight female European starlings (*Sturnus vulgaris*) were caught in Salt Lake City, USA in January 2007. All animals were housed individually on a seasonally varied light:dark cycle and fed *ad libitum*. For the *in vitro* muscle experiments, ten male and three female zebra finches (*Taenopygia guttata*) were used. All zebra finches were housed in 45x45x52 cm group cages on a 12h:12h light:dark cycle and fed *ad libitum*.

**In vivo muscle activity**

Syringeal muscle activity was measured in freely singing male starlings. The techniques for measuring muscle activity in freely singing birds have been described in detail [1-5] and will only be summarized here. All experiments were in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah, Salt Lake City, USA. Male starlings were anaesthetized with an intramuscular injection of chloropent (3.4 µl/g). The syrinx was exposed through an incision in the skin and the interclavicular air sac between the clavicles. Up to four pairs of insulated bipolar EMG electrodes (0.025 mm stainless steel, California Fine Wire, CA) were inserted into syringeal muscles and secured to the outermost fascia with cyanoacrylate tissue adhesive (Nexaband, Abbott laboratories, IL, USA). Wires were led out of the air sac and routed subcutaneously to the back. The air sac was then closed with
surgical sutures and Nexaband tissue adhesive. Spontaneous vocalizations were recorded with a microphone in front of the cage. EMG signals from the syringeal muscles were filtered and differentially amplified (Brownlee Precision, Instrumentation Amplifier 410 or 440, CA, USA). All signals were digitized at 32 kHz and recorded using AviSoft Recorder (Avisoft Bioacoustics, Berlin, Germany).

**Data analysis**

Spectral time derivatives were estimated using the methods described in ref. [6]. We used the Matlab code written Sigal Saar and Parta Mitra (available at http://ofer.sci.ccny.cuny.edu/html/sam.html). Sound amplitude and EMG signals were integrated with a time constant of 0.2 ms. We calculated the un-scaled cross-correlation estimates of the latter two signals: $R_{xy}(m) = \sum_{n=0}^{N-m-1} x_{n+m} y^*_n$ (for $m \geq 0$), where $x$ is the integrated sound amplitude signal, $y^*$ is the complex conjugate of the integrated EMG signal, $N$ is the length of the vectors and $m$ is an integer variable ($m = 1, \ldots, 2N-1$) to delay the signals with respect to each other (i.e. the lag variable).

**In vitro muscle performance**

**Muscle preparations**

The work and power generated by syringeal muscles was determined using the workloop technique [7]. Here, we provide a brief description of this technique, our protocol and calculations. Experiments were performed at the University of Pennsylvania, in October 2006 (zebra finches) and May 2007 (starlings) according to regulations by the IACUC, University of Pennsylvania, Philadelphia, USA. Animals were anaesthetized and euthanised using a 70/30% CO$_2$/O$_2$ mixture. The syrinx was
exposed by cutting through the rib cage, isolated and pinned down on Sylgard-covered Petri dishes in oxygenated Ringers solution (recipe cf. [1,8,9]) at room temperature (21°C). In starlings, we isolated fiber bundles along the surface of *musculus tracheobronchialis dorsalis* (dTb). In zebra finches, we isolated fiber bundles of the *musculus tracheobronchialis ventralis* (vTB). In some bird species, song control is lateralized [10-14] and there is a tendency to predominantly generate amplitude (and frequency) modulations on the right side in the brown thrasher (*Toxostoma rufum*) [2]. Therefore we focused on obtaining muscle preparations from the right side of the syrinx. During the experimental protocol, we always kept the left side ready for dissection and oxygenized in case we had time left after the first preparation or something went wrong with the first preparation during the protocol. As such, in starlings (males and females), we obtained twitch data from 12 right and 3 left preparations (all from males). In zebrafinch males, we obtained twitch data from 6 right and 2 left preparations.

*Set-up and data acquisition*

Muscle preparations were mounted in a teflon-coated aluminum test chamber [15,16] that was continuously flushed with oxygenated Ringers solution at 21°C using a peristaltic pump (Rabbit-Plus, Rainin Instrument Co., Woburn, MA). One end of the muscle was mounted to the arm of a servo-motor (Model 6023AFM, Cambridge Technology, Cambridge, MA) using single strands of a 0G silk suture (Ethicon, Somerville, NJ) under a Wild M3 dissection microscope. The other end was mounted on a force transducer (Model 400, Cambridge Technology, Cambridge, MA). The position of the servo and force transducer could be controlled using micromanipulators. Preparations were stimulated with parallel platinum electrodes.
Stimuli were applied with a pulse generator (Puslar 6b, FHC, Brunswick, ME) coupled to a DC power amplifier (Hewlett Packard, Palo Alto, CA). The temperature of the aluminum block with the test chamber was controlled using water-cooled Peltier elements (bipolar controller, Cambion, Cambridge, MA).

Force transducer, servo motor position and stimulation signals were digitized at 40 kHz with AD board PCI-MIO-16E4 (National Instruments, USA). We developed custom software in Matlab 7.3 (The Mathworks) to control the set-up and for data analysis.

**Experimental protocol**

After a preparation was allowed to rest for 10 minutes, tetanic contractions (stimulation frequency 250 Hz, duration 100 ms, pulse duration 1.0 ms) were performed at 2-minute intervals to optimize stimulation parameters and resting length ($L_0$) for maximal force generation. Then the temperature was slowly increased to 40.4 °C over a period of 15 minutes. After another rest period (5-10 minutes), twitch and tetanic (stimulation frequency 600-800 Hz, duration 50 ms, pulse duration 0.1 ms) characteristics were determined.

Muscle preparations were subjected to a series of 5-10 sinusoidal strain cycles around $L_0$. The first and last cycles were omitted from analysis to avoid on- and offset transients. A run consisted of a series with stimulation (i.e. active series), followed by a series without stimulation (i.e. passive series). The performance of every preparation was optimized for power production at cycle frequencies of 10, 20, 40, 60, 80, 100, 125, 150, 175, 200, 225, 250 and 275 Hz by altering strain amplitude, stimulation phase and duty factor. After the optimized settings were obtained for every cycle frequency, we collected the final dataset by repeating the measurements.
for every cycle frequency with the optimized settings. Reference twitches and tetani were obtained every 30 minutes to monitor decay of the preparation. The tetanic tension of a preparation after the final dataset was compared to the first measurement prior to optimization. No preparation decreased more in tension than 34% and the average decrease was $22 \pm 10\%$ (mean ± S.D.) after $142 \pm 16$ minutes.

After testing, non-contractile and dead material was removed under a dissecting microscope. The remaining muscle mass was blotted on Kimwipes to remove any fluid and weighed (model C-35 microbalance, ATI Cahn Instruments, Boston, MA; precision ± 0.1 µg) to determine wet weight. Preparations were desiccated overnight in a small vial with Drierite (Hammond Drierite Company, OH), to determine dry weight.

Data Analysis

We used similar parameters as previous authors [1,8,9,17,18] to characterize the twitch. Maximal isometric stress (MIS) was calculated as $F_{max} / A_{csa}$ of the muscle, where the cross-sectional area $A_{csa}$ was estimated from the resting length $L_0$ and wet weight of the muscle fibers (assuming a constant density of 1060 kg/m$^3$ from [19]).

The instantaneous power was $P_{inst} = F \cdot \frac{dL}{dt}$ [W]. Work per cycle was defined as the area of the work loop: $W = \int F \cdot dL$ [J]. Mean power was calculated as the product of mean net work and cycle frequency: $\bar{P} = \bar{W} \cdot f$ [W].

At cycle frequencies $\geq 100$ Hz, a special precaution was taken to calculate work done by the muscle preparation. The strain wave that is forced on the muscle preparation by the servo motor, travels from the servomotor end to the force transducer end of the muscle preparation at a speed roughly depending on the
Young’s modulus and density of the muscle. Therefore, at very high cycle frequencies (\( \geq 100 \) Hz), the force measured lags slightly behind the applied strain at the servomotor. This phase delay creates the false appearance of additional positive work done by the muscle. Because this phase-lag is present in both active and passive runs, we obtained work done by the active muscle by subtracting the aligned force signals of the passive set from those of the active set in workloops at cycle frequencies \( \geq 100 \) Hz. The active muscle is stiffer than the passive muscle, which leads to a smaller lag in the active trials compared to the passive trials. Therefore the passive trial generates a larger false amount of work than the active trial. This method of subtraction results in an underestimation of work done by the active muscle at cycle frequencies \( \geq 100 \) Hz. Note that at cycle frequencies \(< 100 \) Hz, this phase lag is insignificant (passive work was negative or zero) and no subtraction was made.

**IN SITU FLOW MODULATION**

*Surgical procedures*

Experiments were performed at the University of Utah, June 2007. All experiments were in accordance with the Institutional Animal Care and Use Committee (IACUC) of the University of Utah, Salt Lake City, USA. Three male starlings were anaesthetized with an intramuscular injection of chloropent (3.4 µl/g). A 10 cm silastic tube cannula (OD, 2.16 mm; ID, 1.02 mm; cat. no. 508-005, Dow Corning, MI, USA) was inserted in the anterior thoracic air sac to control flow through the syrinx. The syrinx was exposed by making a 20 mm incision in the skin between the clavicles and opening the interclavicular air sac. Bipolar, teflon-coated silver-wire stimulation electrodes (76 µm diameter without coating, A-M systems Inc., WA, USA) were implanted in the rostral part of the left *musculus*
tracheobronchialis dorsalis (dTB) and left musculus tracheobronchialis ventralis (vTB). The electrodes were glued to the muscle fascia with Nexaband tissue adhesive and routed out of the interclavicular air sac. The air sac was then closed with surgical sutures and tissue adhesive.

Airflow above the syrinx was monitored with a custom-built direction sensitive flow probe, consisting of plastic tubing with two heated microbead thermistors in the lumen of the tube. The trachea was exposed by making an incision in the neck rostral of the right clavicle. The flow probe was inserted in the trachea about 20 mm above the syrinx by removing a small piece of two tracheal rings. The trachea was closed with tissue adhesive and the skin closed with surgical sutures.

Set-up and data acquisition

After surgery, the bird was placed on its back in a pre-shaped bed of modeling clay in the bottom-centre of a 80x60x60 cm box, lined with 3 cm thick sound insulation foam (SONEX, Illbruck Acoustic, MN, USA). The beak was held open at constant angle with a 3 mm diameter tube. Sound was recorded 2-4 cm from the beak opening with a microphone (type 40AG, pre-amplifier type 26AK, power module 12AD, G.R.A.S., Denmark). Moisturized air was supplied through the air sac cannula with a digital mass-flow/pressure controller (Alicat Scientific, Tucson, AZ, USA). In addition, supplied air pressure was measured prior to the cannula connection, 10 cm from the air sac, with a 3.5F Mikro-Tip catheter pressure transducer (SPR524, Millar Instruments, Texas, USA) connected to a pre-amplifier and pressure control unit (PCU-2000, Millar Instruments, Texas, USA). The flow passing each thermistor was measured by a Wheatstone bridge circuit (Hector Engineering, IN, USA), where the current needed to maintain the heated thermistor at a constant temperature is
proportional to the rate of airflow. The muscle stimulation electrodes were connected to two optically isolated, constant current stimulators (DS3, Digitimer Ltd. Hertfordshire, UK). Sound pressure, air sac pressure, the two flow signals and the mass flow and pressure from the flow controller were low-pass filtered at 10 kHz (Brownlee Precision, Instrumentation Amplifier 410, CA, USA) and digitized at 30 kHz with an AD board (PCI-6251, National Instruments, USA). The set-up was controlled with custom written software in Labview (National Instruments).

**Experimental protocol**

Above a certain bronchial pressure and or flow threshold, the labia in the syrinx start self-sustained oscillations [4]. We determined the phonation onset pressure \( p_{on} \) of every bird by stepwise increasing pressure \( p \) in a 6 s block wave (from atmospheric pressure to \( p \), and back to atmospheric pressure). The fundamental frequency of the self-sustained oscillations of the labia was 250-500 Hz. When the muscle modulation is in the order of magnitude of the fundamental frequency of sound, distinguishing muscle modulation from labial oscillation becomes impossible using the radiated sound signal and difficult using the tracheal flow signal. Figure 4c show an example of sound modulation in starling B15, stimulating the dTB at 100 Hz. Therefore, we used a pressure below onset phonation threshold (0.90 \( p_{on} \)) to study flow modulation by the syringeal muscles.

Both dTB and vTB were stimulated using periodic pulse train stimulations (5 - 20 cycles) at cycle frequencies of 20, 40, 60, 80, 100, 125, 150, 175, 200, 225 and 250 Hz, with a tetanic frequency of 600 Hz. Pulse duration was optimized for the 20 Hz cycle frequency and kept constant at 0.5 –1.0 ms for the rest of the protocol. Measurements for every cycle frequency were optimized for modulation depth of the
tracheal flow by optimizing the number of pulses in the pulse train (i.e. duty factor of the cycle) and pulse amplitude (0.5 – 2.0 mA). After the experiment, the cannula, stimulation electrodes and flow probe were removed. The two flow signals from the probe were calibrated by applying known flow amplitudes using the mass-flow/pressure controller (Alicat Scientific, AZ, USA).

Data Analysis

Muscle stimulation results in flow modulation (Figure S1). Stimulating vTB increases flow, while stimulating the dTB reduces flow. Modulation depth per stimulus \((i)\) is defined as: \(D_i = \frac{\text{min}(F_i) - \text{max}(F_i)}{\text{F}_{\text{pre}} - \text{min}(F_i)}\), where \(\text{min}(F_i)\) and \(\text{max}(F_i)\) are the local minima and maxima in the flow signal \((F)\) associated with stimulus \(i\), and \(\text{F}_{\text{pre}}\) is the average flow value of the 50 ms segment prior to stimulation. In figure S1, \(\text{min}(F_i)\) are indicated by blue diamonds, \(\text{max}(F_i)\) by green diamonds and \(\text{F}_{\text{pre}}\) by the red boxes.

The average modulation depth is calculated as: \(\overline{D} = \frac{\sum_{i=1}^{n-2} D_i}{n}\), where \(n\) is the number of stimuli.
FIGURE LEGEND

Figure S1. Flow modulation in the trachea following electrical stimulation of the syringeal muscles. Upper trace, *musculus tracheobronchialis dorsalis* (dTB); lower trace, *musculus tracheobronchialis ventralis* (vTB). Blue diamonds indicate local minima, green diamonds indicate local maxima in the flow signal associated with preceding stimulus. Red boxes indicate flow value prior to stimulation ($F_{pre}$) for dTB and vTB. Orange boxes on top and bottom of figure indicate muscle stimuli for dTB and vTB respectively.

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