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## RESEARCH ARTICLE

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# The hummingbird syrinx morphome: a detailed three-dimensional description of the black jacobin's vocal organ

Amanda Monte[1], Alexander F. Cerwenka[2], Bernhard Ruthensteiner[2], Manfred Gahr[1] and Daniel N. Düring[1,3,4*]

## Abstract

**Background:** The ability to imitate sounds depends on a process called vocal production learning, a rare evolutionary trait. In addition to the few mammalian groups that possess this ability, vocal production learning has evolved independently in three avian clades: songbirds, parrots, and hummingbirds. Although the anatomy and mechanisms of sound production in songbirds are well understood, little is known about the hummingbird's vocal anatomy.

**Results:** We use high-resolution micro-computed tomography (μCT) and microdissection to reveal the three-dimensional structure of the syrinx, the vocal organ of the black jacobin (*Florisuga fusca*), a phylogenetically basal hummingbird species. We identify three features of the black jacobin's syrinx: (i) a shift in the position of the syrinx to the outside of the thoracic cavity and the related loss of the sterno-tracheal muscle, (ii) complex intrinsic musculature, oriented dorso-ventrally, and (iii) ossicles embedded in the medial vibratory membranes.

**Conclusions:** The extra-thoracic placement of the black jacobin's syrinx and the dorso-ventrally oriented musculature likely aid to uncoupling syrinx movements from extensive flight-related thorax constraints. The syrinx morphology further allows for vibratory decoupling, precise control of complex acoustic parameters, and a large motor redundancy that may be key biomechanical factors leading to acoustic complexity and thus facilitating the occurrence of vocal production learning.

**Keywords:** Vocal production, Musculus sternotrachealis (ST), Intrinsic musculature, Vibrato, Evolution

## Background

Vocal production learning in birds -- the rare ability to modify the acoustic parameters of a vocalization as a result of experience with external vocal signals -- holds striking parallels with speech acquisition in humans [1, 2]. Vocal learning evolved independently in songbirds (suborder *Passeri*) [3, 4], parrots (order *Psittaciformes*) [5] and hummingbirds (family *Trochilidae*) [6] due to convergent

neurological shifts [7]. Thus, the brains of avian vocal learners are uniquely specialized, unlike non-vocal-learner species, to perceive, produce and memorize sounds [4, 8, 9]. However, the pressures underlying this convergent evolution remain unknown and attempts to explain the evolution of vocal learning face challenges from the divergences in ecological aspects, such as gregariousness or mating system, of the vocal learners [10, 11].

Efforts to understand vocal learning have concentrated on the neural processes that modulate vocal output with little regard to the biomechanics of sound production in the vocal organ [12]. The vocal organ in birds is the syrinx [13, 14], an avian novelty hypothesized to be

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optimized for birds' particularly long air tracts [15]. The syrinx is located where the trachea bifurcates into the bronchi and is suspended inside the interclavicular air sac [13]. One or two pairs of vibratory membranes are present; depending on where these structures are located, the syrinx can be classified as tracheal, tracheobronchial or bronchial [16]. The syrinx musculature is of two basic types: extrinsic musculature, which is attached outside of the syrinx at one end, and intrinsic musculature, which is attached to the syrinx at both ends [17, 18]. While every bird has extrinsic musculature, not all syringes have intrinsic musculature [18], which varies among birds [13, 17]. For example, gallinaceous species have none, songbird species have from three to five intrinsic muscle pairs [17, 19], while parrot species mainly have two [19, 20].

Syrinx anatomy, in general, is highly variable among and consistent within higher-level taxa, to the extent that syrinx anatomy has been used to guide avian phylogenetic classification [12, 17, 21]. Similarities in gross morphology and its implications for vocal production may help us to understand the morphological basis of vocal learning [19, 22]. Thus, the presence of intrinsic musculature has been hypothesized as a prerequisite and not an adaptation for vocal learning [19, 23], that is, all vocal learners should have intrinsic muscles, but not all species that have intrinsic muscles are vocal learners. Unlike extrinsic muscles, which move the syrinx as a unit [19, 23], intrinsic muscles dissociate the control of tension from the control of amplitude, for example, which in turn affects fundamental frequency [24, 25].

Recent studies indicate that musculature is just one of the variables that define the multi-dimensional parameter space that translates motor commands into vocal output [12, 22, 24, 26]. Many factors, such as syrinx's morphology, physical interaction with the surrounding environment, and neuro-mechanic activity, contribute to the creation of a large acoustic space that is highly redundant in terms of control parameters [22]. When a certain sound is produced, the bird has more degrees of freedom than necessary to reach that specific target [22]. This motor control redundancy allows specific vocal parameters, such as frequency, to be achieved by multiple combinations of, for example, expiratory air pressure and muscle activity [22]. The availability of multiple motor commands for a certain acoustic target may simplify the trial-and-error learning process and is hypothesized as necessary for the development of vocal learning [22].

To approach vocal learning from the biomechanical perspective, the syringes of vocal learners need to be systematically compared. Among avian vocal learners, hummingbirds are the most basal taxon and phylogenetically distant from parrots and songbirds [6, 8, 27, 28], and the only group in which not all species have the ability of vocal learning [8, 29]. The acoustic features of their vocalizations vary substantially within the group [30, 31], ranging from simple vocalizations to acoustic performances that are above the known perceptual limits of birds [32, 33]. Currently, we lack a detailed description of the hummingbird syrinx in basal clades and, therefore, insights into the biomechanics of hummingbirds' peculiar vocalizations.

Here we use micro-computed tomography (μCT) and microdissection to resolve the detailed structure of osseous and cartilaginous elements and vibratory soft tissues of the black jacobin (*Florisuga fusca*) syrinx. The black jacobin belongs to the clade Topazes (tribe *Topazini*), relatively basal among hummingbirds [34]. It can vocalize on high fundamental frequency with harmonics over the human audible range [33]. Our results provide fundamental insights into the biomechanics of sound production in hummingbirds and the anatomical factors facilitating the emergence of vocal learning in birds.
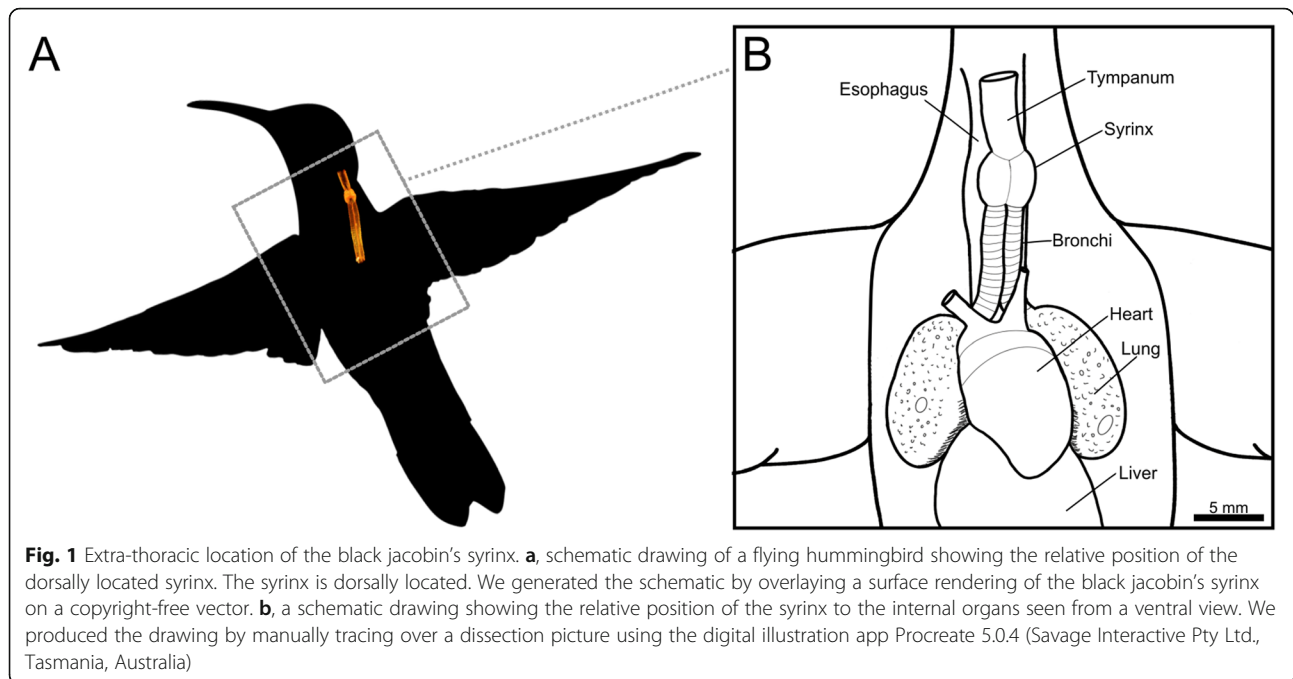
## Results

### General anatomy of the black jacobin's syrinx

The black jacobin has a tracheobronchial syrinx that is located where the trachea bifurcates into the two primary bronchi, approximately 9.4 mm cranially distant from the heart and outside of the thoracic cavity (Fig. 1). The trachea is a long, funnel-shaped tube that extends along the cervical column whose diameter is reduced to around ¼ of its original size when proximal to the syrinx. From the syrinx on, the bronchi run parallel for about 1.3 mm, separating when inside the thorax. The bronchi are enveloped by a multi-layered membrane that keeps them tightly together. Parts of the trachea, bronchi, and the syrinx, are all tightly packed by this multi-layered membrane, most likely an evagination of the clavicular air sac membrane [35].

### The osseous and cartilaginous syringeal structures of the black jacobin

The syrinx of the black jacobin is composed of cartilaginous tracheal rings, an osseous tympanum and modified bronchial half-rings, two of which are partially ossified (Fig. 2a and c). The trachea consists of complete cartilaginous rings (T1 to Tn), each of which is thinner in its dorsal part and narrower towards the tympanum. The tympanum is a cylindrical osseous element likely formed by the fusion of tracheal and bronchial rings; this fusion forms the tympanum in other tracheobronchial syringes, for example, that of the zebra finch syrinx [36]. Internally, the tympanum body is a relatively uniform tube with an ossified lamella in its caudal part that projects medially into the air passage. Externally, the ventral part of the tympanum body presents a squared expansion that, together with the internal lamella, constitutes the pessulus. The pessulus separates a symmetrical pair

**Fig. 1** Extra-thoracic location of the black jacobin's syrinx. **a**, schematic drawing of a flying hummingbird showing the relative position of the dorsally located syrinx. The syrinx is dorsally located. We generated the schematic by overlaying a surface rendering of the black jacobin's syrinx on a copyright-free vector. **b**, a schematic drawing showing the relative position of the syrinx to the internal organs seen from a ventral view. We produced the drawing by manually tracing over a dissection picture using the digital illustration app Procreate 5.0.4 (Savage Interactive Pty Ltd., Tasmania, Australia)

of horizontal ridges that delimit medially the two main cranio-caudal concavities to which muscles are attached (Fig. 2b). The U-shaped dorsal part of the tympanum is formed by two solid expansions in each of the lateral caudal edges and a medial concavity that extends horizontally along the entire surface as a muscle attachment site. In the caudomedial part, a pair of rounded osseous elements, the tympanic ossicles (*ossicula tympanica*), are embedded in an extension of the most medial part of the vibratory membranes (Fig. 2d).

Caudally to the tympanum, bronchial half-rings (B1 to Bn) extend for around 12.3 mm until reaching the lungs. Only the first two pairs are partially ossified (B1 and B2); the others are cartilaginous. The first pairs (B1 to B3) are highly modified compared to the other bronchial half-rings (Fig. 2a and b). Each ring of the B1 pair is composed of a ventral spherical ossification, and a cartilaginous arc projects both dorsally along the caudal part of the tympanum and caudally in relation to the B2 pair. The B2 pair is located in the dorsal part of the syrinx, in a transverse plane, each of which has round edges and a concavity towards the ventro-lateral part of the syrinx; a cartilaginous projection extends in the same shape into the caudal direction, almost reaching the B1 cartilaginous arc. Each of the B3 pairs is a cartilaginous arc-shaped half-ring whose concavity extends toward the lumen of the bronchus. Slightly medial in relation to the B1 arc, each pair has ventral and dorsal extremities that serve as attachments for one of the vibratory membranes.

### The syringeal muscles of the black jacobin
All syringeal muscles of the black jacobin are paired (Fig. 3). There is one pair of extrinsic, the *musculus*

*tracheolateralis* (tracheolateral muscle; TL) and at least three pairs of intrinsic syringeal muscles: *musculus syringealis cranialis* (cranial syringeal muscle; CrS), *musculus syringealis centralis* (central syringeal muscle; CeS), and *musculus syringealis caudalis* (caudal syringeal muscle; CaS) (Table 1).

The extrinsic TL is composed of a few sparse sheets of muscle fibers attached to the cranial part of the trachea. It extends caudally alongside the lateral part of the trachea until reaching the cranial end of the tympanum (Fig. 3e-g).

All intrinsic muscles are oriented ventro-dorsal. They attach ventrally to the tympanum and dorsally to some of the modified bronchial half rings. The CrS, the largest muscle, is responsible for just over 50% of the intrinsic musculature volume and follows the typical cardioid contour of the dorsal syringeal surface (Fig. 3c and d). The CrS caudal attachment site is in the ventrocranial head of the modified bronchial half-ring B2. A few muscle fibers of the CrS are attached via connective tissue to the tympanic ossicles. The CeS comprises about one-third of the intrinsic musculature volume placed mainly in the lateral part of the syrinx (Fig. 3f). The caudal attachment site of the CeS is located on the lateral extent of half-ring B2 and includes its cartilaginous expansion. The CaS makes the remaining of the intrinsic musculature volume and runs mainly ventrally (Fig. 3a and b). The attachment sites of the CaS are located at the most caudal concavities of the pessulus and along the lateral outline of the cartilaginous extension of the half-ring B1.
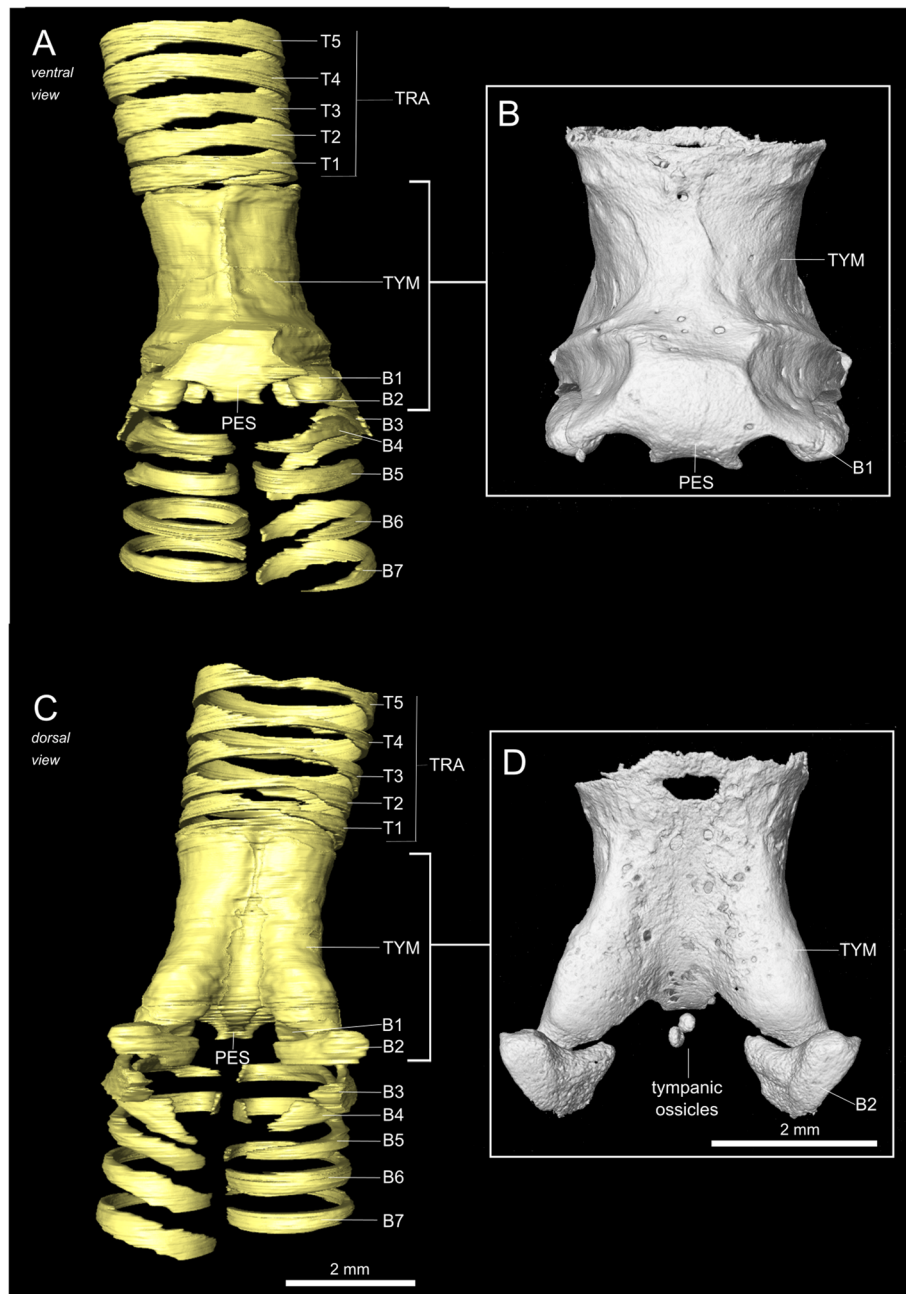
Monte *et al. BMC Zoology*      (2020) 5:7

Page 4 of 15

**Fig. 2** The syringeal osseous and cartilaginous elements of the black jacobin. 3D visualizations of μCT data. **a** and **c**, surface renderings of the contrasted sample; **b** and **d**, volume renderings of the non-contrasted sample. **a** and **b**, ventral view; **c** and **d**, dorsal view. In **d**, the tympanic ossicles. T1 to T5, tracheal rings; TYM, tympanum; B1 to B7, bronchial half-rings and PES, pessulus

## Vibratory membranes of the black jacobin's syrinx

The syringeal vibratory membranes are composed of a pair of lateral labia (LL), each labium located in the lateral part of each side of the syrinx, and a pair of medial labia (ML) that continue into the medial tympaniform membrane (MTM). ML and MTM form the medial vibratory mass (MVM) in the medial part of the syrinx caudal to the tympanum (Fig. 4a). The LL is placed parallel to the ML and extends cranially over the tympanic lumen and caudally among the half-rings B1 to B3 (Fig. 4a). The LL has around 45% of the volume of the ML and is ventrally attached to the pessulus, dorsally to B2 and laterally to the medial part of the B1 (Fig. 4a). The MVM constitutes a continuous mass of vibratory tissue reduced to just over one-third in thickness from the cranially located ML to the MTM. The ML is

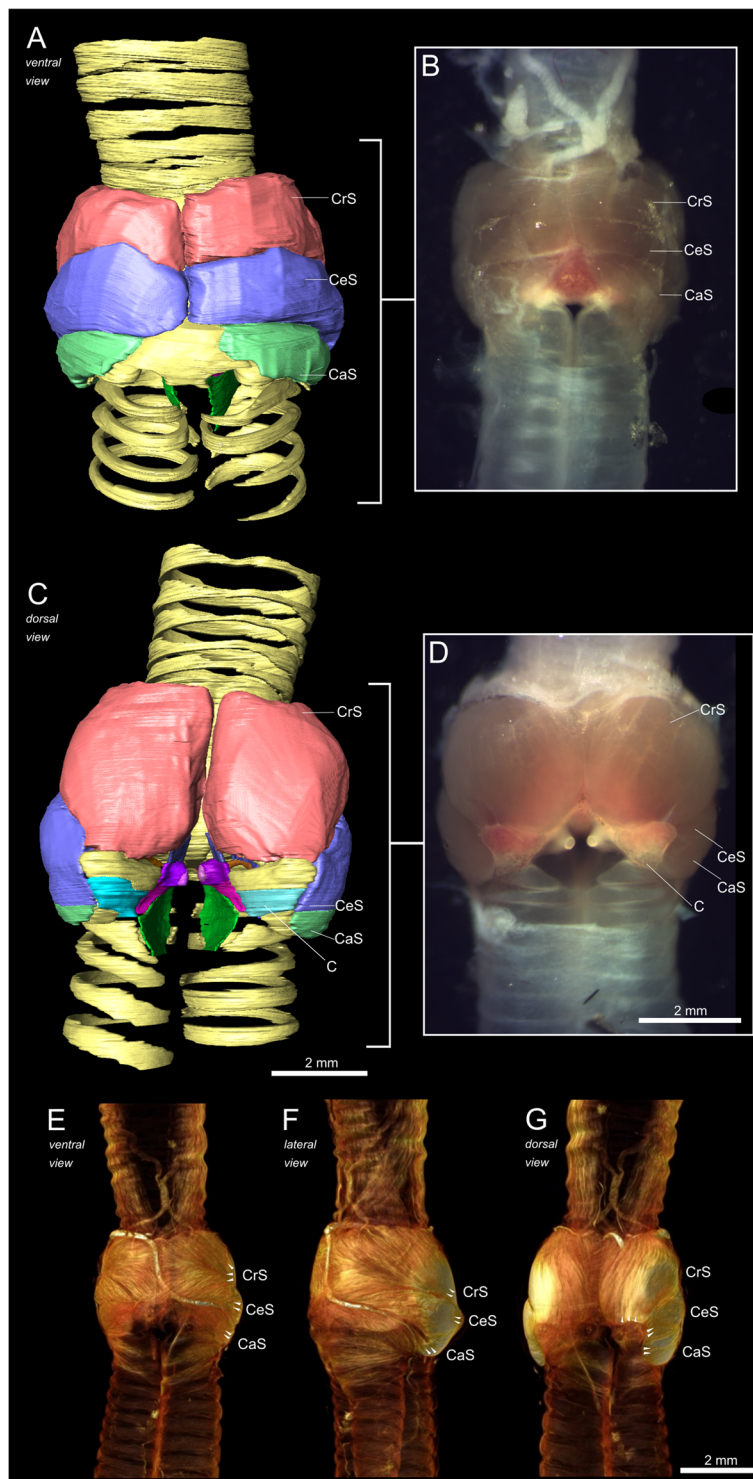**Fig. 3** Musculature of the black jacobin's syrinx. **a** and **c**, surface renderings; **b** and **d**, dissected syrinx and **e**, **f** and **g**, volume renderings. **a**, **b** and **e**, ventral view; **c**, **d** and **g**, dorsal view and **f**, lateral view. The arrows indicate the orientation of the fibers. CrS, cranial syringeal muscle; CeS, central syringeal muscle; CaS, caudal syringeal muscle and C, cartilage

**Table 1** Anatomical structures of the black jacobin syringes described in this study

| English term | Abbreviation | Latin term | Source | Brief anatomical description | Figure(s) |
|---|---|---|---|---|---|
| lateral labium | LL | *labium laterale* | [36] | Paired soft tissue. Projects from between the caudal tympanum and half-ring B1 into the syrinx air passage. | Figure 4a |
| medial labium | ML | *labium mediale* | [36] | Paired soft tissue. Projects from the medial part of the syrinx into the air passage. Forms a tissue continuum with the medial tympaniform membrane. | Figure 4a |
| medial tympaniform membrane | MTM | *membrana tympaniformis medialis* | [36] | Paired connective tissue element. Suspended between the ventro-dorsal extremities of the bronchial half-rings B1 and B3. Forms a tissue continuum with the medial labium. | Figure 4a |
| tracheolateral muscle | TL | *musculus tracheolateralis* | [36] | Paired muscle. Forms an extended band along the trachea. Attaches caudally to the syrinx and cranially to the larynx. | Figure 3e-g |
| cranial syringeal muscle | CrS | *musculus syringealis cranialis* | present study | Paired muscle. Attaches to tympanum and the cranial part of the half-ring B2. | Figure 3 |
| lateral syringeal muscle | CeS | *musculus syringealis centralis* | present study | Paired muscle. Attaches to tympanum and the central part of the half-ring B2. | Figure 3 |
| caudal syringeal muscle | CaS | *musculus syringealis caudalis* | present study | Paired muscle. Attaches to tympanum and the lateral part of the half-ring B1. | Figure 3 |

cranioventrally attached to the pessulus and dorsally attached to the half-ring B2. In the craniodorsal part, the ML thickens nearly 5-fold in an extension that embeds the tympanic ossicles and projects to the air passage (Fig. 4b and c). This extension, which comprises just over 30% of the total volume of ML and the tympanic ossicles comprise a further 5%, connects to a muscle via a thin ligament such as was reported previously for hummingbirds [35]. The MTM is ventro-dorsally attached to the medial edges of the bronchial half-ring B3.

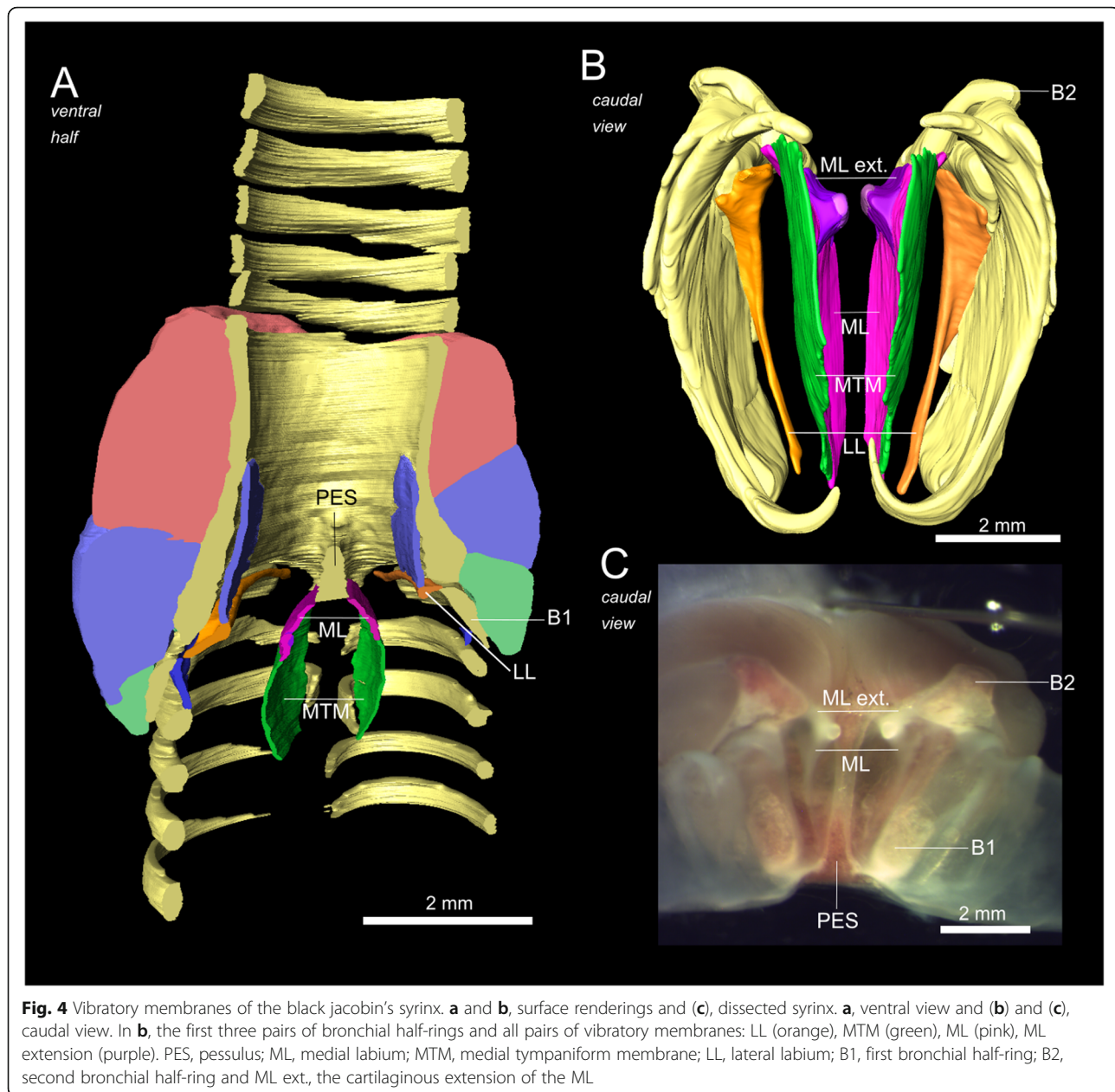## Hypothetical biomechanics of the black jacobin's syrinx

To explore potential general mechanisms of adduction, abduction, and stretching of the sound-producing elements, we carefully micro-dissected the black jacobin's syrinx under a stereo microscope. During manual manipulation of the musculature around the ML, we identified a potential mechanism, which in sequential muscle activation seems responsible for the adduction of the LL and stretching of the ML and LL. Applying an increasing amount of force to the caudal part of the cartilaginous arc extending from B1, the attachment site of CaS, resulted in an inward rotation of B1 and caused first partial and then complete adduction of the LL. A craniad force applied to the head of B2, the attachment site of the CrS, resulted in the dorso-ventral stretching of the ML and LL (Fig. 5a). Considering the anatomical disposition of the CeS, a lateral force applied on the lateral part of the B2, the attachment site of CeS, may result in outward rotation of the B2 and cause the abduction of the ML. Thus, each of the three intrinsic muscles seems to be involved in one of the main tasks controlling sound production in the syrinx: the CaS on adduction, which closes the bronchial lumen; the CeS on abduction, which opens the bronchial lumen; and the CrS, which controls the tension of ML and LL (Fig. 5b). Nonetheless, these

suggestions should be confirmed by a paradigm that ensures muscle specificity such as the ex vivo paradigm of the syrinx described in [22].

## Spectral analysis of the black jacobin's vocalization

The black jacobin vocalizes with a fundamental frequency (F0) that ranges from 1.8 to 11.8 kHz ($n = 105$ recordings with a total of 1242 motor units, so-called syllables). We identified three types of vocalizations with distinct spectral structure: low-pitched vocalization with an F0 average of 1.8 kHz ($\pm$ 0.5, $n = 66$ syllables); click-like chirps with an F0 average of 7.9 kHz ($\pm$ 1, $n = 148$ syllables); and high-pitched vibratos with an F0 average of 11.8 kHz ($\pm$ 0.4, $n = 1028$ syllables) (Fig. 6a). The human voice from the lowest suitable phonation to the highest falsetto reaches 0.08 to 0.7 kHz for males and 0.1 to 1.1 kHz for females [37].

The black jacobin's most frequent type of vocalization is the high-pitched vibrato composed of syllables repeated in groups with up to three repetitions; the vibrato has a fundamental frequency of around 12 kHz with harmonics reaching the ultrasonic range of humans [33]. Each vibrato syllable is composed of fast oscillations with periodic changes of the fundamental frequency (Fig. 6b) and an average duration of 95.8 $\pm$ 35 ms ($n = 18$ syllables). Within a syllable, the difference between the highest and the lowest modulation of frequency, known as the vibrato extent, ranged from 0.7 to 3 kHz (average 1.5 $\pm$ 0.5 kHz, $n = 401$ crest-trough pairs, Fig. 6b) with a periodicity of around 2.4 ms ($\pm$ 1.4 ms, $n = 401$). Thus, the black jacobin can produce syllables that change their fundamental frequency at an average rate of 233.2 Hz ($\pm$ 37.5, $n = 18$ syllables). For example, Whitney Houston produced an average vibrato rate of 5.1 Hz ($\pm$ 0.6, $n = 12$ vibratos) with a vibrato extent of 0.05 kHz ($\pm$ 0.01) (Fig. 6c). The human vibrato rate is similar between different music
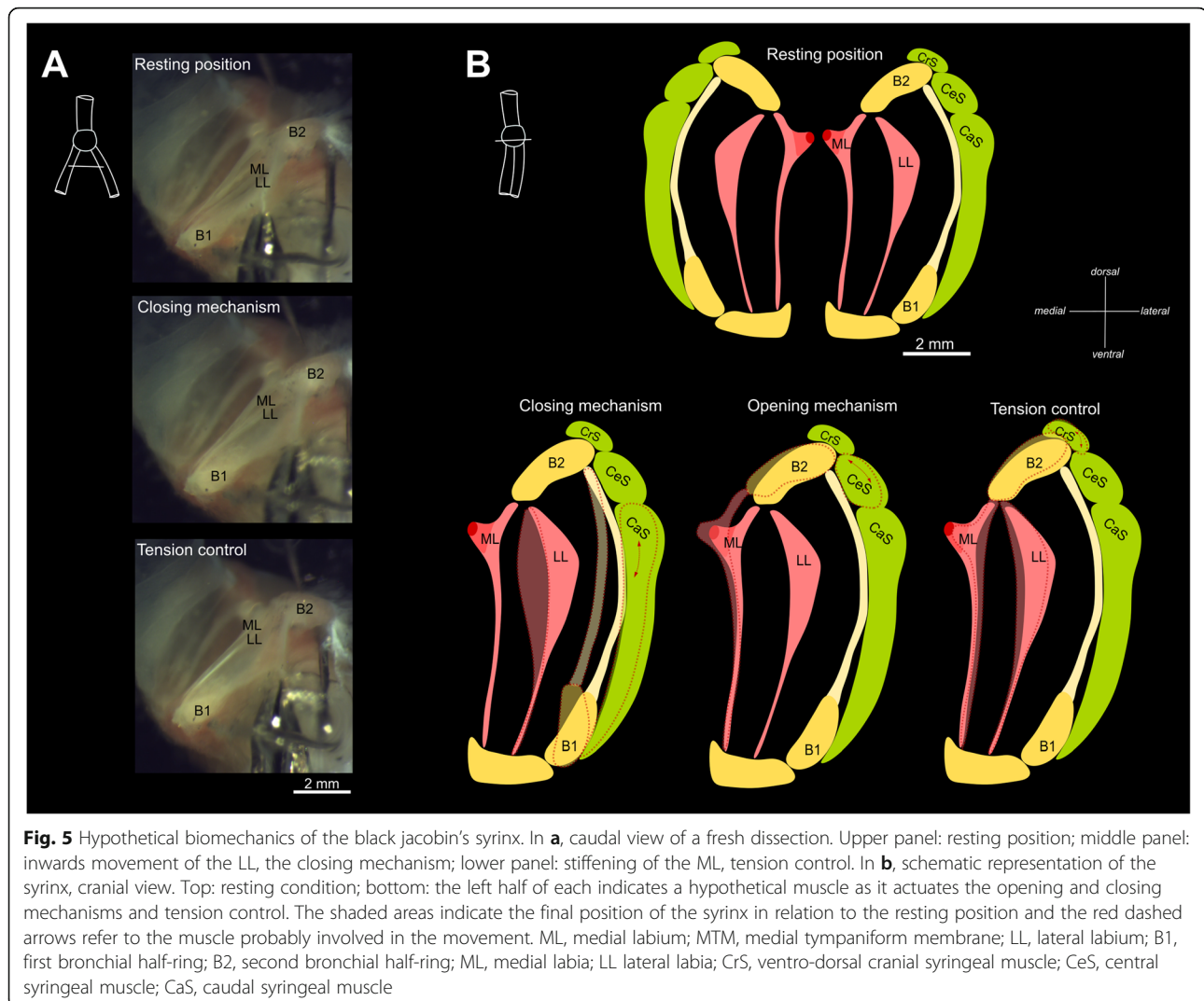
**Fig. 4** Vibratory membranes of the black jacobin's syrinx. **a** and **b**, surface renderings and (**c**), dissected syrinx. **a**, ventral view and (**b**) and (**c**), caudal view. In **b**, the first three pairs of bronchial half-rings and all pairs of vibratory membranes: LL (orange), MTM (green), ML (pink), ML extension (purple). PES, pessulus; ML, medial labium; MTM, medial tympaniform membrane; LL, lateral labium; B1, first bronchial half-ring; B2, second bronchial half-ring and ML ext., the cartilaginous extension of the ML

genres such as opera (5.8 Hz ± 0.4, $n$ = 5 singers), rock (5 Hz ± 0.6, $n$ = 5 singers) or Brazilian country music (6 Hz ± 0.5, $n$ = 5 singers) [38]. This means black jacobin's vibrato rate surpasses that of a human singer by more than 45-fold. Within birds, vibratos are reported to be produced by only a few species [30, 39], but no quantification of vibrato rate or extent is available. For comparison, we quantified the vibrato rate of a songbird, Eurasian skylark (*Alauda arvensis*). The skylark's vibrato rate is almost 17-fold higher than that of a human singer, with an average of 89.7 Hz (± 14.4, $n$ = 10 syllables), and the vibrato extent averaged 8-fold larger, 0.4 kHz (± 0.02) (see Methods for details). No

vibrato reported to date combines such a fast rate and wide extent as that of the black jacobin (Fig. 6c).

## Discussion

Here we present the first detailed description of the vocal tract of a basal hummingbird; a species with the potential to illuminate how vocal learning has evolved. We identified a tracheobronchial syrinx located in the dorsal part of the neck. The syrinx appears to be comprised of heavily modified osseous structures, intrinsic syringeal musculature with a particular ventro-dorsal orientation and a pair of vibratory membranes in each of

**Fig. 5** Hypothetical biomechanics of the black jacobin's syrinx. In **a**, caudal view of a fresh dissection. Upper panel: resting position; middle panel: inwards movement of the LL, the closing mechanism; lower panel: stiffening of the ML, tension control. In **b**, schematic representation of the syrinx, cranial view. Top: resting condition; bottom: the left half of each indicates a hypothetical muscle as it actuates the opening and closing mechanisms and tension control. The shaded areas indicate the final position of the syrinx in relation to the resting position and the red dashed arrows refer to the muscle probably involved in the movement. ML, medial labium; MTM, medial tympaniform membrane; LL, lateral labium; B1, first bronchial half-ring; B2, second bronchial half-ring; ML, medial labia; LL lateral labia; CrS, ventro-dorsal cranial syringeal muscle; CeS, central syringeal muscle; CaS, caudal syringeal muscle
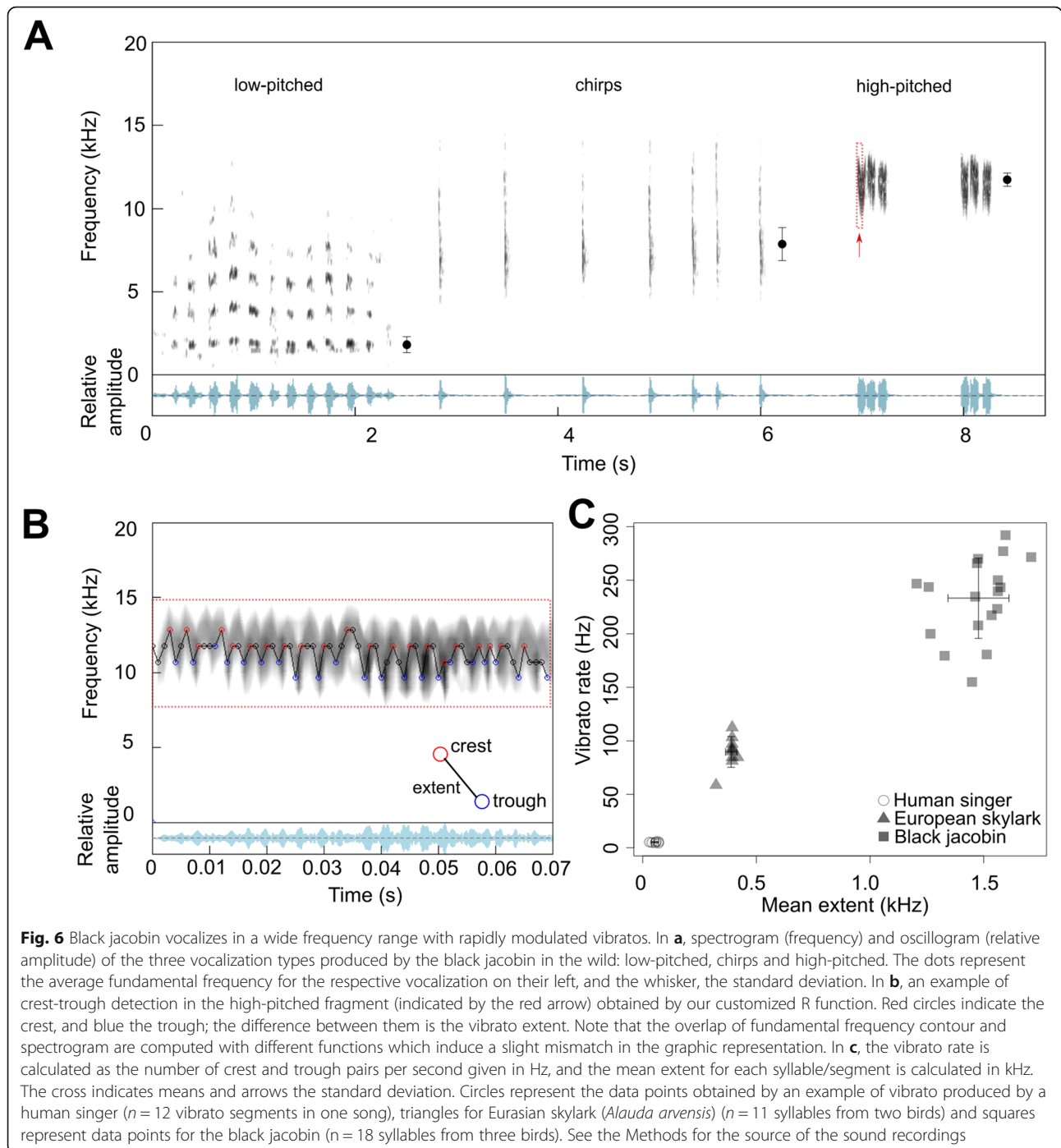
the sides of the syrinx. Further, we find one ossicle embedded in each of the medial vibratory membranes. This peculiar syringeal morphology allows the black jacobin to produce a vibrato that challenges the known limits of this acoustic feature.

Hummingbirds (family *Trochilidae*) are grouped with swifts (family *Apodidae*) and treeswifts (family *Hemiprocnidae*) in the order Apodiformes [27]. Within hummingbirds, a comparative study that included the syrinx of the clades Hermits, Mangoes and Brilliant suggested that adjacent bronchi, highly modified bronchial half-rings, intrinsic muscles and tympanic ossicle are unique to hummingbirds within Apodiformes [35]. A recent study that investigated the syrinx of the clade Bees, relatively recent radiation within hummingbirds [34], found also specialized pairs of accessory cartilages, one of them embedded in the medial vibratory membranes, and intrinsic muscles [40]. Similarly to hummingbirds, the syrinx of the swifts is tracheobronchial with an osseous

tympanum [41]. However, the synrix of the swifts is placed inside of the thoracic cavity, have two pairs of extrinsic muscles (tracheolateralis and sternotrachealis) and no intrinsic muscles [35, 41–43]. Given the relatively basal placement of Topazes within hummingbirds [34] and the consistency of these characters in the hummingbird species reported to date, we suggest that the extrathoracic syrinx, heavily modified bronchial half-rings, tympanic ossicles, more than one pair of intrinsic muscles, lack of sterno-tracheal muscle and two pairs of vibratory membranes are synapomorphies of the family.

## Decoupling from physiological noise and syrinx stabilization without ST muscles

The black jacobin's syrinx is located outside of the thoracic cavity, in contrast to most of the birds that have their syringes inside the thoracic cavity [17], except for the roseate spoonbill (*Platalea ajaja*) [42]. Hummingbirds are highly specialized for hovering: unsurprisingly,

**Fig. 6** Black jacobin vocalizes in a wide frequency range with rapidly modulated vibratos. In **a**, spectrogram (frequency) and oscillogram (relative amplitude) of the three vocalization types produced by the black jacobin in the wild: low-pitched, chirps and high-pitched. The dots represent the average fundamental frequency for the respective vocalization on their left, and the whisker, the standard deviation. In **b**, an example of crest-trough detection in the high-pitched fragment (indicated by the red arrow) obtained by our customized R function. Red circles indicate the crest, and blue the trough; the difference between them is the vibrato extent. Note that the overlap of fundamental frequency contour and spectrogram are computed with different functions which induce a slight mismatch in the graphic representation. In **c**, the vibrato rate is calculated as the number of crest and trough pairs per second given in Hz, and the mean extent for each syllable/segment is calculated in kHz. The cross indicates means and arrows the standard deviation. Circles represent the data points obtained by an example of vibrato produced by a human singer (*n* = 12 vibrato segments in one song), triangles for Eurasian skylark (*Alauda arvensis*) (*n* = 11 syllables from two birds) and squares represent data points for the black jacobin (n = 18 syllables from three birds). See the Methods for the source of the sound recordings

its flight muscles make up 25 to 30% of its body weight, a ratio that is more than that of any other bird family [44]. The hummingbird's enlarged flight muscles are combined with an enlarged heart, comprising about 2.5% of its body mass, which beat rate is the highest among birds [44, 45]. The syrinx location outside of the thoracic cavity potentially alleviates spatial constraints caused by the enlarged flight muscles and avoids mechanical disturbances from the cardiac muscles. Thus, we

speculate that an extrathoracic syrinx may have allowed hummingbirds to evolve control over its syringeal biomechanics despite their unique adaptations for the hovering flight.

The black jacobin's syrinx lacks the sterno-tracheal muscle (ST), in contrast to almost all other birds. Further exceptions are the nearly non-vocal New World vultures and the vocalizing tinamous Darwin's Nothura (*Nothura darwinii*) [46]. The function of ST is unclear,

Monte *et al. BMC Zoology*     (2020) 5:7

Page 10 of 15

in species in which intrinsic muscles are absent it is hypothesized to function as syringeal adductor [43, 47–49]. For example in the tracheal syrinx of pigeons, the shortening of the ST brings its cartilages closer together, thereby closing the syringeal lumen [23]. The adduction of the labia is crucial for sound production in general as it facilitates the build-up of the phonation threshold pressure (PTP), which is necessary for sound onset [50]. Surprisingly, in species with intrinsic syrinx muscles, adduction is achieved by intrinsic musculature rather than ST and ST function is attributed to syringeal stabilization [17, 51]. Similarly, and congruent with our observations, the closing mechanism in black jacobins is probably realized through intrinsic musculature. Darwin's nothura, which also lacks ST, has the membrane of the interclavicular air sac more caudal than in the other tinamous species and it is proposed to also stabilize the syrinx [46]. Black jacobins seem to obtain syrinx stabilization through tight wrapping of the syrinx in several layers of soft tissue. These layers create a rigid frame that might also compensate for the lack of rib cage protection. While protecting the syrinx from its immediate environment, this tight wrapping also keeps the syringeal elements inside flexible and potentially aids in maintaining the differential pressure necessary for the onset of sound production [18, 22]. The most external of these layers may be an evagination of the interclavicular membrane that cranially encloses the syrinx within the interclavicular air sac, which has also been reported in other hummingbird species [35].

The syrinx displacement may also have had implications for muscle orientation. The intrinsic muscles of the black jacobin's syrinx are oriented dorso-ventrally, while the intrinsic muscle fibers of most bird taxa run cranio-caudally, for example, the ventral syringeal muscle of songbirds [13, 16, 36]. Because all of the black jacobin's intrinsic muscles are ventrally attached to the tympanum, but each of them is dorsally attached to a different point, they run dorso-ventrally on different angles. The general dorso-ventral orientation with differences in angulation might allow the black jacobin to control the mobile syringeal elements despite the lack of lateral stabilization provided by the STs in other taxa.

The extrathoracic disposition of the syrinx and accompanied absence of STs in hummingbirds [35, 40, 52], might have been one of the driving pressures for the evolution of intrinsic muscles, a key prerequisite of vocal learning.

### Tympanic ossicles
Although cartilaginous formations were found embedded in the vibratory membranes of songbirds [36], tympanic ossicles have not been reported in any species other than hummingbirds [35, 40, 52]. The origin of tympanic ossicles is uncertain. Due to their medial position and

proximity to the tympanum, they might be either modified bronchial half-rings or have originated from a tracheal ring. In humans, the prevalence of a small sesamoid bone in the knee has increased worldwide in the past century, probably as a dissipative response to increased mechanical forces due to the enlargement of leg bones and muscles [53]. Similarly, increased tension in the labia might have led to the formation of tympanic ossicles in the black jacobin's syrinx.

In addition to direct muscular activity, stiffness of vocal tissues depends on the elastic properties of the tissue itself [24, 54]. In songbirds, cartilage embedded in the medial labia (ML) both aids in the dissipation of the tension, avoiding rupture under high stress and modifies the elastic properties of the syrinx [36]. In particular, the cartilage that connects with the muscle potentially supports a more gradual bending mechanism, which in turn allows uncoupling the control of amplitude and frequency [36]. Similarly, this might be the function of the cartilaginous extension in the dorsal part of the ML and its embedded ossicles, the tympanic ossicles, in the black jacobin. This extension is connected by a thin strip of connective tissue to a few muscle fibers of the larger syringeal muscle; given this arrangement, direct muscular control of the extension seems likely.

The tympanic ossicles may contribute to achieving the black jacobin's high fundamental frequency: they cause high local density and prevent an entire part of the ML from vibrating at all, thus shortening its length and increasing the fundamental frequency. In other words, the tympanic ossicles could be used as a secondary mechanism to gradually increase ML stiffness and reduce ML length. It is therefore likely that the cartilaginous extension of the ML in the black jacobin both shifted the elastic properties of the ML towards the optimal for high fundamental frequency by increasing ML density towards the muscle attachment site that directly controls ML stiffness, and shortened the vibratory part of the ML.

### Extreme vocal performance
Black jacobins produce particularly rapidly-modulated vibrato sounds [33]. The black jacobins' vibratos oscillate periodically up and down with a frequency bandwidth of up to 3 kHz at a rate of about 250 Hz. This fast vibrato rate can be compared to that of other extreme vocal performances, such as of starlings (*Sturnus vulgaris*), a songbird whose muscle activity in the syrinx produces changes in sound amplitude at a repetition rate of 218 Hz [55]. The musculature of the songbird's syrinx belongs to a special class of muscles, called superfast muscles [56, 57], and can produce work at cycling limits of approximately 90 Hz to 250 Hz [58]. In vitro preparations revealed that the superfast songbird muscles in the syrinx have the potential to function at cycle frequencies

as fast as 250 Hz [55]. Although direct electromyographic recordings of the syringeal musculature would be needed to confirm that the black jacobin's vibrato rate of 250 Hz is a direct result of muscular control, this extremely fast performance suggests that the black jacobin's syringeal muscles produce work on the upper limit of the superfast muscle activity reported to date [55] and that black jacobins may have muscle properties comparable to those of songbirds.

### Biomechanics of sound production and implications for vocal learning in hummingbirds

Parrots and songbirds, two vocal learners, have a tracheal and a tracheobronchial syrinx, respectively, both with intrinsic musculature [17, 20, 36, 59]. The black jacobin's syrinx, like that of all the other hummingbird species reported so far [35, 40, 52], is tracheobronchial, with three pairs of intrinsic muscles that are as complex as those of songbirds [40]. The black jacobin's multiple intrinsic muscles attach in close proximity to movable elements of its syringeal osseous elements (modified bronchial half-rings) to which the vibratory membranes (medial labia or lateral labia) are attached via cartilaginous extensions. These muscles seem to operate consecutively. For example, both lateral and medial labia are attached to the bronchial half-ring B2, where two large muscles are attached. At its cranial surface is the cranial syringeal muscle (CrS), and at its lateral part, the central syringeal muscle (CeS). Given the location and orientation of each muscle, we speculate that various amounts of contraction of each muscle might contribute gradually to distinct functions, such as the abduction of the ML and the stretching of the labia. Since the position and tension of the labia are directly related to distinct acoustic parameters, multiple muscles contributing to the same function creates redundancy in possible motor commands controlling acoustic parameters such as fundamental frequency. When the brain has multiple, rather than a single motor command available to achieve a certain vocal output, a redundant control space may simplify trial-and-error attempts during imitation in the vocal production learning process [22].

Hummingbirds and songbirds converge in their syrinx morphology, while parrots produce learned vocalizations with seemingly less complex syringeal musculature [40]. However, parrot's lingual articulation introduces a hitherto overlooked level of complexity to their vocal production system [60]. Syrinx muscle complexity alone does not correlate with vocal learning [61]. Nevertheless, the presence of intrinsic musculature when combined with further specializations leading to acoustic complexity may facilitate the evolution of neurological structures associated with vocal learning. Thus, we speculate that the more degrees of freedom are provided to the motor redundancy by peripheral adaptations for vocal production, the more likely a species is to follow on to the next evolutionary step towards the evolution of vocal learning.

## Conclusions

Here we present the first high-resolution morphome of an ancestral hummingbird syrinx, the black jacobin's. We suggest that the absence of sterno-tracheal muscle, presence of tympanic ossicles and dorso-ventral muscle orientation are all present synapomorphies within hummingbirds. These characteristics might have evolved concomitantly with the displacement of the syrinx out of the thorax, as an operational solution to reduce interference of the syrinx with the enlarged heart and flight muscles. The vocal motor control redundancy emerging from the hummingbird's syrinx morphology may have facilitated the evolution of vocal learning in hummingbirds.

## Methods

### Tissue collection and preparation

The black jacobins ($n = 3$) were captured with a hummingbird-specific "Ruschi trap" [62] in the park of the National Institute of the Atlantic Forest (former Professor Mello Leitão Museum), Espírito Santo State, Brazil, in accordance with the Brazilian Institute of Environment and Renewable Natural (IBAMA) regulations under the Biodiversity Information and Authorization System (SISBIO) license number 41794–2.

After capture, two males were deeply anesthetized with an intramuscular injection of ketamine hydrochloride (Cetamin, Rhobifarma Indústria Farmacêutica Ltda, SP, Brazil) in the concentration recommended by the manufacturer (15 mg/kg). This method was the preferable alternative given the limitations in the field and the particular anatomy of the hummingbirds. No signs of distress were observed in the birds during and after the injection. When the birds were completely unconscious, they were perfused through a cardiac injection with the following sequence of solutions: 0.5 ml heparin-natriun anticoagulant, 0.9 saline buffer and 4% paraformaldehyde fixative. After the perfusion, the syrinx was dissected and stored in the fixative for 24 h and then stored in 0.1 M phosphate-buffered saline (PBS) in solution with 0.05% sodium acid until use. We used both fixed syringes for micro-computed tomography, one stained for the visualization of soft tissues and the other without the staining procedure for clear visibility of the ossified structures. Both syringes were dissected with a large part of the esophagus and bronchi as close as possible to the beak and lungs, respectively, to access the syrinx structures integrally. A third male black jacobin received an anesthetic overdose (ketamine hydrochloride: 15 mg/kg), when the bird was unconscious, the euthanasia was

confirmed by decapitation, the syrinx immediately dissected and cryopreserved at – 80 °C until use. This syrinx was micro-dissected. The euthanasia method used in this study followed the guidelines n°. 13, 20 Sep 2013 of the Brazilian National Council on the Control of Animal Experiments (CONCEA).

### Micro-dissection

The cryopreserved syrinx was thawed gradually. First, at – 20 °C for 1 h followed by 24 °C during the time of manipulation. For manipulation, the syrinx was pinned down on a glass Petri dish covered by black dissecting pan wax and filled with 0.1 M PBS. We disassembled the syrinx under an MZ75 stereomicroscope (Leica Microsystems, Germany) equipped with an ISH500 5.0 MP camera (Tucsen Photonics, China).

The syrinx was inspected ventrally and dorsally; the main musculature and ossifications matched with the μCT-based reconstruction. The difference in the density of adjacent soft tissues was noted by a comparison of their light reflection. We sectioned the muscles at their tympanic insertion site and noted the general orientation of fibers. We repositioned the syrinx caudally, centering it where the bronchia bifurcated, with bronchi angled at 180° exposing the vibratory membranes. The mobile structures in which the vibratory membranes were attached were noted. With a pin, we applied gentle force to each of these mobile structures and photographed the effect of the applied force on the vibratory membranes (See Fig. 5a).

### Micro-computed tomography

The micro-computed tomography (μCT) scans of isolated syringes (two males) was conducted at the Zoologische Staatssammlung München (Munich, Germany) using a phoenix nanotom m cone beam μCT scanner (GE Measurement and Control, Wunstorf, Germany) with down to 3.1 μm voxel size.

One syrinx was scanned without staining to access the anatomy of the ossified structures as a fourfold multiscan with the following parameters: 100 kV source voltage, 170 μA source current, 0.1 mm aluminum filter, 500 ms exposure time, 3.1 μm isotropic voxel resolution, 1000 projections over 360° with three averaged images per rotation position, and a total of 132 min scan time, using a molybdenum target. The second syrinx was stained with a contrast agent to image soft tissues. It was placed inside a glass vial with 0.1% Lugol's solution (Sigma Aldrich). The vial was placed on a tube roller for 48 h. The stained syrinx was scanned for 48 min using the following parameters: 80 kV source voltage, 180 μA source current, 0.1 mm copper filter, 500 ms exposure time, 3.6 μm isotropic voxel resolution, 1440 projections over 360° with three averaged images per rotation position, using a tungsten ("standard") target. The volume

reconstructions were performed using the software phoenix datos2×   (GE Sensoring & Inspection Technologies GmbH, Germany).

### Three-dimensional reconstruction and nomenclature

The annotation was performed onto the μCT-based syringeal dataset of a black jacobin adult male. We identified the recognized musculature, ossification, cartilaginous pads, and vibratory membranes. The visualization procedures including volume rendering and manual segmentation for surface rendering and relative quantifications were done with the software Amira 6.1 (Thermo Fisher Scientific, Massachusetts, USA).

The syrinx structures were defined by the consensus of the microdissection and the μCT data. The nomenclature was given following the same procedure used in songbirds [36]. In the case of lacking analogous structures in songbirds, we proposed a nomenclature following the same procedure used in non-passerines to name syringeal muscles according to their topographic position [63] (Table 1). We named the tracheal rings T1 to Tn starting from tympanum and moving toward the larynx. We present a conservative number of intrinsic muscles due to their delineation. We delineated the intrinsic syringeal muscles by aggregating fibers that were oriented at the same angle and defined their differential attachment sites based on both microdissection and the μCT scans. The extrinsic musculature was not traced in the 3D reconstruction due to its undetermined tracheal insertion but is partially shown. The vibratory membranes we found are analogous to those described in songbirds [36].

### Sound analysis

First, we investigated the fundamental frequency of the black jacobin's vocalizations in the wild. Observations and recordings were made in the Professor Mello Leitão Museum (Santa Teresa, Espirito Santo state, Brazil) at a feeding point where every day wild hummingbirds visit feeders that contain 25% sugar water solution. The observations were made over 9 days from November to December 2013, and over 15 days from September to October 2015 during the black jacobin's breeding season [64]. Black jacobins were observed continuously for 1 h a day on the dates mentioned above; observations were made sometime between 6:30 and 11 a.m. for a total of approximately 24 h. The sampling method was ad libitum [65], according to which the most conspicuous occurrences of the vocal behavior were recorded for the first black jacobin spotted at the feeding point until the individual had left the place. The black jacobins were not individually marked, but the high abundance of the species at the feeding point [66] and the fact that recordings were obtained over two non-consecutive years make it unlikely that the observations were biased toward a

few individuals. Recordings were made 3–10 m from the individuals with a *Marantz PMD 671* (Marantz, New York, USA) solid-state recorder connected to a Sennheiser MKH 70/P48 (Sennheiser, Wedemark, Germany) directional microphone in a 48 kHz sampling rate wave file. We obtained 105 recordings totaling 5 h. We isolated the black jacobin's vocalizations and calculated the fundamental frequency for each of their syllables (vocal units) using the packages "Seewave" [67] and "WarbleR" [68] in R 3.5.0 [69]. The recordings are not public due to storage reasons but are available from the corresponding author upon request.

Second, we focused on the most common vocalization of the black jacobin. This vocalization is composed of syllables with continuous and regular fast modulations in fundamental frequency [33]. Given the periodicity of these modulations, we classified the syllables as vibratos. Vibrato is a demanding vocal task produced by opera singers and characterized by periodic pitch fluctuation [70]. The accuracy of the vocal performance can be quantified in terms of four parameters: rate, extent, regularity, and waveform [70]. Here we measured two features of the black jacobin's vibrato: the rate that was measured by the number of oscillations per second and the extent that was the depth of the oscillations. We measured the vibrato based on and adapted from [71]. All calculations were performed on the platform R 3.5.0 [69]. For the calculations, syllables were selected from full recordings using the function autodetect() of the package "WarbleR" [68]. For each syllable, a spectrogram (Hanning windows) was generated with the function spectro() of the package "Seewave" [67]. The adequated windows length was selected manually per syllable and varied from 220 to 360. Then the fundamental frequency contour of each unit was identified with the function dfreq() of the package "Seewave" [67]. The crest-trough pairs were detected using a customized R script named "vibrato_scan.R" which is available in the following GitHub repository: https://github.com/amandamonte/black_jacobin.git. The vibrato rate given in Hz was calculated by:

$$\sum \frac{Nct}{T}$$

Where Nct is the total number of crest-trough pairs detected per vocal unit, and T is the total duration of the unit in seconds.

The vibrato extent given in Hz was obtained per crest-trough pair by the difference between the frequency of the crest and the frequency of the trough. The values were presented as means (± standard deviation, sample size).

We analyzed three high-quality sound recordings and 18 syllables with the highest quality obtained from three black jacobins. These recordings were kindly provided by Chirstopher Olson, who obtained them using an Avisoft CM16/CMPA ultrasound microphone (2–250 kHz range) coupled to an UltraSoundGate 416H amplifier recorder at the frequency rate of 214 kHz. To have something to compare with the black jacobin, we analyzed the soundtrack "I will always love you" performed by Whitney Houston (© Sony Music, 1992) and selected 12 fragments in which the singer produces a vibrato as an example of a human singer (frequency rate of 44.1 kHz). As an example of a vibrato produced by a songbird, we analyzed two recordings of the Eurasian skylark (*Alauda arvensis*) obtained from the Xeno-canto collaborative bird sound collection (https://www.xeno-canto.org/), catalog numbers XC401962 and XC417772 uploaded by Karl-Birger Strann and Jarek Matusiak, respectively (frequency rate of 48 kHz). The vibrato examples of both the human singer and songbird were analyzed following the same parameters as the black jacobin recordings, except for the windows length (Eurasian skylark: 120 to 240 and Whitney Houston: 1200 to 1600).

Monte *et al. BMC Zoology*          (2020) 5:7

Page 14 of 15

## Ethics approval and consent to participate

The samples for this study were collected with permission from the direction of the National Institute of the Atlantic Forest (former Professor Mello Leitão Museum), Espírito Santo State, Brazil. All procedures were in conformity with the normative instruction n° 03/2014 of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and the Brazilian Institute of Environment and Renewable Natural (IBAMA) that regulates the collection of biological material for scientific and didactic purposes in the context of higher education under the Biodiversity Information and Authorization System (SISBio) license number 41794–2.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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## Publisher's Note