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Original Article

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Evolution of the syrinx of Apodiformes, including the vocal-learning Trochilidae (Aves: Strisores)

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ABSTRACT

The vocal organ of birds, the syrinx, represents a key innovation in the evolutionary history of vertebrate communication. Three major avian clades: passerines, parrots, and hummingbirds, independently acquired both specialized syringeal structures and vocal-production learning, between which a functional relationship has been proposed but remains poorly understood. In hummingbirds, the syrinx has never been studied comparatively alongside non-learning relatives in the parent clade Strisores. Here we describe the anatomy of the syrinx in three swift species using enhanced-contrast computed tomography, which reveals structures previously unreported in the clade. We also tested for correlations between syringeal and acoustic traits in a sample of hummingbirds and swifts using phylogenetically informed regressions. The swift syrinx presents lateral labia located on the first pair of bronchial half-rings, which are present in hummingbirds and may be ancestral to Strisores. The further enlarged lateral labia of hummingbirds are found to be significantly correlated to the reduction in length of their trachea and m. tracheolateralis. Acquisition of intrinsic muscles and loss of the sternotrachealis muscle co-occur with these shifts. We recover a significant negative correlation between tracheal elongation and maximum vocalization frequency, suggesting that tracheal shortening in hummingbirds facilitated the acquisition of high-frequency vocalizing.

Keywords: birds; comparative anatomy; hummingbirds; phylogenetic regressions; Strisores; swifts; syrinx; vocal production

INTRODUCTION

The syrinx, a vocal organ unique to birds that plays a prominent part in their evolution and life history, presents a wide range of morphological variation within Aves (Ames 1971, King 1989, Clarke *et al.* 2016, Düring and Elemans 2016, Kingsley *et al.* 2018). In the 19th century, this variation was extensively described by anatomists in the context of avian taxonomy (e.g. Cuvier 1795, Yarrell 1830, Garrod 1873, Müller 1878, Forbes 1880, 1882, Beddard 1886, 1898, Gadow 1892, Pycraft 1900). Starting in the 1970s, a series of studies (Gaunt and Wells 1973, Gaunt *et al.* 1982, Gaunt 1983, Gaunt and Gaunt 1985a) compared primarily external syringeal morphology of various avian species with acoustic data of their calls, hypothesizing a functional link between diversity in syrinx anatomy and vocal production.

The structure of the syrinx is defined by a series of anatomical modifications of the tracheobronchial junction (Beddard 1898, Ames 1971, King 1989, Clarke *et al.* 2016, Düring and Elemans 2016, Kingsley *et al.* 2018). These include the acquisition, between adjacent pairs of cartilagineous rings (tracheal or bronchial, depending on the clade—Suthers 2001, Mindlin and Laje 2005), of vocal folds, specialized tympaniform membranes, which in many birds are associated with labia (King 1989, Baumel and Witmer 1993, Düring and Elemans 2016, Kingsley *et al.* 2018). Labia are capable of generating sound through self-sustained oscillations induced by the airflow, analogous to laryngeal vocalizations in mammals (Goller and Larsen 1997a, b, 2002, Elemans *et al.* 2008, 2015, Riede and Goller 2010a, Düring and Elemans 2016). Properties of such vocalizations (e.g. frequency, amplitude, duration) are locally controlled by two tracheal paired muscles, the m. tracheolateralis (TL) and the m. sternotrachealis (ST), which can alter the length and cross-sectional shape of the trachea in antagonistic action, thus affecting the position and tension of the vibrating vocal

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folds (Gaunt 1983, Brackenbury 1989, Mindlin and Laje 2005, Elemans *et al.* 2006, Riede *et al.* 2006, 2019, Riede and Goller 2010b).

In addition to these mechanisms, which are probably ancestral to Aves (Clarke *et al.* 2016, Kingsley *et al.* 2018), three major avian clades—i.e. oscine songbirds (suborder Passeri), parrots (order Psittaciformes), and hummingbirds (family Trochilidae)—have independently acquired vocal learning, i.e. the cognitive ability to learn and reproduce vocalizations (Kroodsma and Miller 1996, Nowicki and Searcy 2004, Podos and Warren 2007), controlled by complex neural mechanisms (Jarvis 2004, 2019, Beecher and Brenowitz 2005, Brenowitz and Beecher 2005). These three clades present multiple pairs of syringeal intrinsic muscles (IM)—the number and position of which varies between clades—which have been experimentally shown to contribute to the 'fine tuning' of vocalizations through local modifications of syringeal transsectional geometry (complementing those of the ST and TL in the trachea) and abduction/adduction of the labia (Goller and Suthers 1996a, b, Suthers 2001, Larsen and Goller 2002, Mindlin and Laje 2005, Elemans *et al.* 2008, 2009, Riede and Goller 2010b, Düring and Elemans 2016). Over the past two decades, many studies have linked acoustic parameters of vocal production [e.g. fundamental frequency (F0), peak frequency, bandwidth, song duration] to morphoanatomical features in oscine passerines, including body mass (Brumm 2009, Mason and Burns 2015, Pearse *et al.* 2018, Mejías *et al.* 2020, Demery *et al.* 2021), beak gape (Hoese *et al.* 2000, Goller *et al.* 2004, Podos *et al.* 2004, Riede *et al.* 2006, 2016, Ohms *et al.* 2010), and beak dimensions (Podos 2001, Podos and Nowicki 2004, Podos *et al.* 2004, Huber and Podos 2006, Derryberry *et al.* 2012, 2018, Langin *et al.* 2017, García and Tubaro 2018, Porzio *et al.* 2019, Mejías *et al.* 2020, Demery *et al.* 2021). By contrast, syringeal correlates of vocal production, acquired through histology or X-ray computed tomography (CT; Düring *et al.* 2013)—e.g. tracheal length (Daley and Goller 2004, Riede *et al.* 2006, 2019, Ohms *et al.* 2010); labia size, volume, asymmetry, and inner structure (Riede and Goller 2010a, 2014, Riede *et al.* 2010, Düring *et al.* 2017)—have only been studied in a select few passerine species through experimental stimulation/ablation of structures of interest to test their effect on acoustic patterns, and never in a comparative statistical framework. The impact of syrinx morphology and presence/absence of IM on fine tuning in vocal learners is thus poorly understood (Riede and Goller 2014, Düring and Elemans 2016).

Among known vocal learners, hummingbirds in particular have been comparatively understudied, especially regarding the evolution of their distinctive syrinx morphology. Most hummingbird species studied in the context of vocal production (18 out of >350—Billerman *et al.* 2022, Duque and Carruth 2022, Monte *et al.* 2023) have been confirmed as vocal learners (Nottebohm 1972, Gaunt *et al.* 1994, Ficken *et al.* 2000, Ferreira *et al.* 2006, Baptista and Schuchmann 2010, Araya-Salas and Wright 2013, Araya-Salas *et al.* 2019, Johnson and Clark 2020, 2022, Duque and Carruth 2022). A recent literature review proposed song production as ancestral to hummingbirds, but found that 7 out of 78 species mentioned in this context were described as 'non-singing', which the authors interpreted as indicative of a potential loss of vocal learning (Monte *et al.* 2023). The cerebral cortex of at least four vocal-learning hummingbird species presents specialized vocal nuclei (Gahr 2000, Jarvis *et al.* 2000) analogous to those associated with vocal learning in oscine passerines and parrots (Jarvis 2004, 2019). However, research on the acquisition of a specialized syrinx morphology associated with advanced control of vocal production has focused almost exclusively on passerines (ten Cate 2021), with a few studies also including parrots (Nottebohm 1976, Gaunt and Gaunt 1985b, Larsen and Goller 2002).

The first formal description of the general structure of the hummingbird syrinx, based primarily on four species (Zusi 2013; see also: Müller 1878), described a number of diagnostic characters, several of them unique among birds (syrinx located in the neck rather than in the thoracic cavity; bronchi bound together by a surrounding evagination of the interclavicular air sac; absence of the ST; a medial labium with two embedded ossicles connected to the IM; enlarged, dorsally flattened first bronchial half-rings). Two subsequent studies (Monte *et al.* 2020, Riede and Olson 2020) confirmed these results using high-resolution CT scans of syringes from one and four hummingbird species, respectively, and described three pairs of IM in all sampled species. Both studies hypothesized that the relative organization, synergetic action, and ossicle attachment of IM, combined with shortening of the trachea and increased collagen content of the labia, contributed to the ancestral acquisition of vocal learning in hummingbirds (Monte *et al.* 2020, Riede and Olson 2020). In both cases, however, the authors remained cautious regarding the evolutionary mechanisms and exact role of these traits in the acquisition of vocal learning.

One of the main difficulties of understanding the evolution of the specialized hummingbird syrinx comes from Trochilidae being one of eight families within Strisores, a large clade (~600 species—Billerman *et al.* 2022) defined only comparatively recently in its modern phylogenetic sense (Mayr 2010, 2011, Chen *et al.* 2019, Kimball *et al.* 2019). Phylogenetic relationships within Strisores, and the position of Strisores within Aves, are still debated among ornithologists (Chen *et al.* 2019, White and Braun 2019, Chen and Field 2020, Braun and Kimball 2021), although there is a general consensus on the monophyly of Apodiformes (hummingbirds, swifts, and treeswifts—Hackett *et al.* 2008, Jetz *et al.* 2012, Ksepka *et al.* 2013, Yuri *et al.* 2013, Prum *et al.* 2015, Reddy *et al.* 2017, Chen *et al.* 2019, Kimball *et al.* 2019, White and Braun 2019, Kuhl *et al.* 2021). Furthermore, the syringeal morphology of most non-hummingbird Strisores has been poorly studied, whether entirely undocumented (potoos, treeswifts) or only described in 100+-year-old taxonomic descriptions for a handful of species (frogmouths, owlet-nightjars: Beddard 1886; nightjars: Cuvier 1795, Beddard 1886, Marshall 1905). In the remaining two families, the oilbird (*Steatornis caripensis* Humboldt 1817) and the swifts (~110 species—Billerman *et al.* 2022), select species (oilbird, swiftlets *Aerodramus* spp. and *Collocalia troglodytes* Gray 1845) have been the subject of functional studies on vocal production due to their unique ability among birds to produce echolocation calls (review in: Brinkløv *et al.* 2013). In both groups, a sequence of antagonistic actions from two muscles—the ST to initiate phonation, and either the TL (swifts) or a pair of IM (oilbird) to terminate it—has been experimentally identified as the main sound-generating mechanism (Suthers and Hector 1982, 1985). In swifts, this model, determined from observations in one species (Suthers and Hector 1982), was later refined using a sample of nine species (Thomassen 2005). Clicks produced by echolocating swifts were described as distinct from calls of non-echolocating swifts, but no significant difference in labia morphology was found between echolocators and non-echolocators (Thomassen 2005), and the syrinx morphology of non-echolocating swifts has never been described. Since then, despite an increasing interest in Strisores' phylogeny (Mayr 2010, Päckert *et al.* 2012, Ksepka *et al.* 2013, Chen *et al.* 2019, Chen and Field 2020) and the evolutionary radiation of Trochilidae (McGuire *et al.* 2007, 2014, Bleiweiss 2009, Parra *et al.* 2010, Tripp and McDade 2013, Licona-Vera and Ornelas 2017, Barreto *et al.* 2023), the combined lack of morphological data and phylogenetic comparative framework has prevented the study of the hummingbird syrinx in an evolutionary context within Apodiformes and Strisores.

In this study, we provide the first description of the internal, as well as external, morphology of syrinx in three species of swifts (Apodiformes: Apodidae) and analyse the variation/covariation of syringeal morphological characters in a sample of hummingbirds, swifts, and a nightjar—the latter two clades being closely and distantly related to hummingbirds among Strisores, respectively (Chen *et al.* 2019). We also test for correlations between these traits and acoustic parameters for these same species in a phylogenetic comparative framework, and propose functional hypotheses on characteristics of the vocal tract that may have influenced the variation of vocal production within Strisores. Finally, we discuss the evolution of these characters in the context of the ancestral Strisores syrinx and its impact on the diversification of life history strategies in hummingbirds.

MATERIALS AND METHODS

All birds included in this study ($N = 21$; Supporting Information, Table S1) are fluid-preserved specimens sampled from natural history collections at the Field Museum of Natural History (FMNH; Chicago, IL, USA) and the Texas Memorial Museum (TMM; Austin, TX, USA). Specimens of *Oreotrochilus chimborazo* Delattre and Bourcier 1846 were collected by Carlos A. Rodríguez-Saltos and Fernanda G. Duque under permit MAE-DNB-CM-2015-0017 by the Ecuadorian Ministry of Environment and transported to the US in accordance with appropriate protocols and United States Department of Agriculture permit (#129771) to be permanently reposited at the Center for Behavioral Neuroscience, Georgia State University. The sample includes 16 hummingbirds, four swifts, and one nightjar (Supporting Information, Table S1). The dissection and fixation of excised syrinxes can cause damage to their structure and affect the validity of syringeal morphoanatomical measurements (e.g. tracheal diameter, bronchial length; Bilger *et al.* 2020). When possible (Supporting Information, Table S1), we scanned whole-body specimens to create three-dimensional, high-resolution images of all syrinxes using diffusible iodine-based, contrast-enhanced computed tomography (diceCT) at the High-Resolution X-ray Computed Tomography Facility at The University of Texas at Austin (UTCT), following standard recommendations and guidelines (Bilger *et al.* 2020; see also: Gignac and Kley 2014, Li *et al.* 2015, 2016, Gignac *et al.* 2016). All specimens were stained in I_2E—100% EtOH with 0.5–3.75% iodine, depending on specimen size—for 7–10 days prior to being scanned and de-stained (Gignac *et al.* 2016, Bilger *et al.* 2020). Scans of swift specimens were processed and segmented in AVIZO 2022.2 (Thermo Fischer Scientific, Waltham, MA, USA) and DRAGONFLY 2022.2 (Object Research Systems, Montréal, QC, Canada) for specimen visualization and description. Quantitative traits were measured on all scans in ImageJ2 2.9.0/1.53t (Schroeder *et al.* 2021).

The seven morphoanatomical continuous traits (Fig. 1; Supporting Information, Tables S1, S2) included in statistical analyses are:

- Body mass (g), taken from Dunning (2008).
- Distance TL–labia (μm): distance between the distal end of the TL on the trachea and the attachment point of the lateral labium on the tracheal wall, measured in coronal view and averaged for left and right side (Fig. 1D).
- TL cross-sectional area (μm^2): average of six measurements of cross-sectional area (CSA)—three measurements taken at the proximal end, middle, and distal end of the TL, respectively, for both left and right side (Fig. 1B).
- Labia length (μm), measured in coronal view from the attachment point of each lateral labium on the tracheal wall to its protruding tip in the tracheal lumen, averaged for left and right labium (Fig. 1D).
- Tracheal elongation ratio (%): ratio of the tracheal length (in μm; Fig. 1A) over the total length of the vocal tract (trachea + bronchi, in μm; Fig. 1A).
- Tracheal diameter (μm): average of three measurements at the proximal end, middle, and distal end of the trachea, respectively, of average diameter (i.e. average of major and minor axis of the tracheal cross-section as estimated by the 'Fit ellipse' routine in ImageJ—see Fig. 1B).
- IM CSA (μm^2): average of six measurements of CSA—three measurements taken at the proximal end, middle, and distal end of IM, respectively, for both left and right side (Fig. 1C); measured only in hummingbirds, as other species do not have IM.

Audio recordings of Strisores were automatically downloaded from xeno-canto (http://www.xeno-canto.org/) using R package 'warbleR' (Araya-Salas and Smith-Vidaurre 2017). For each species in our morphological dataset, we downloaded all recordings rated 'A' (highest quality rating on xeno-canto) and that had no background species listed in their metadata. In each recording, we extracted vocalizations and measured acoustic features using the function *threshold_detection* in R package 'bioacoustics' (Marchal *et al.* 2022). We selected vocalizations that had a signal-to-noise ratio (SNR) of at least 10 dB (Marchal *et al.* 2022). Because xeno-canto instructs contributors to check that the focal species is found at the beginning of the recording, we extracted vocalizations only from the first 10 s of each recording. Furthermore, we removed vocalizations with a SNR lower than 5 dB of the SNR of the vocalization with the highest SNR. In a random sample of recordings, this threshold was sufficient to

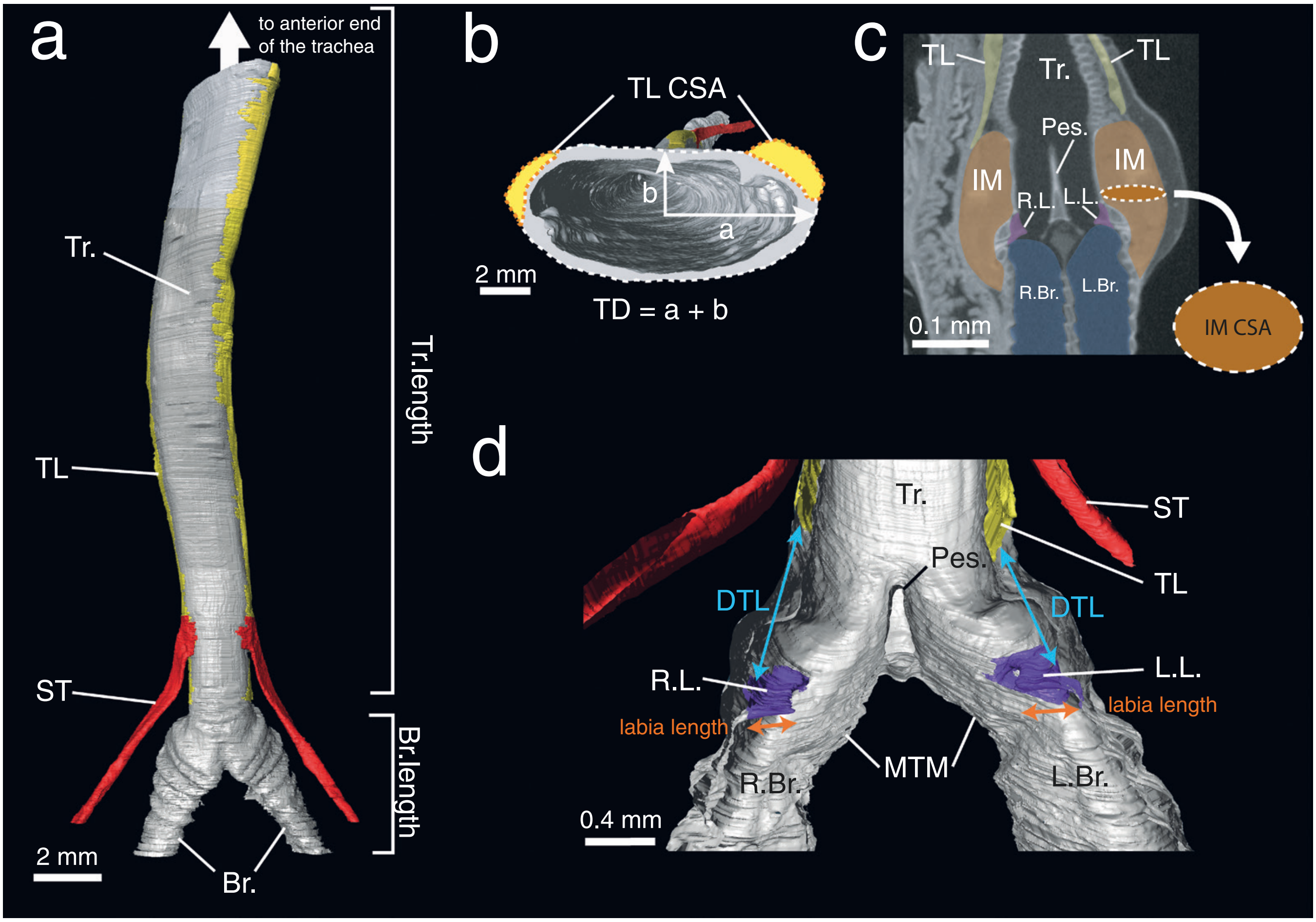

Figure 1. Three-dimensional morphology of the syrinx in a swift, *Apus affinis* (A, B, D), and a hummingbird, *Oreotrochilus chimborazo* (C), visible with diceCT, showcasing morphanatomical traits measured for this study. Colour-coding in all panels is: trachea and bronchi, light grey; sternotrachealis, red; tracheolateralis, yellow; labia, purple. In (C), bronchi are coded in dark blue and intrinsic muscles in light orange. A, ventral view of the segmented trachea and bronchi, showcasing the variables used to calculate the tracheal elongation ratio (=Tr.length/(Tr. length + Br.length)). B, transverse section of the segmented trachea in anterior view, showcasing the cross-sectional area of the tracheolateralis (orange dashed outlines) and the tracheal diameter (white arrows: semi-major and semi-minor axes, a and b, in the white dashed outline). C, coronal section of the trachea and bronchi in ventral view, showcasing the intrinsic muscle cross-sectional area (black arrows, semi-major and semi-minor axes, a and b, in the dark orange circle with white dashed outline). D, coronal section of the segmented trachea and bronchi in ventral view, showcasing distance TL–labia (blue arrows) and labia length (orange arrows). Abbreviations: Br., bronchi; Br.length, bronchial length; DTL, distance tracheolateralis–labia; IM, intrinsic muscle; IM CSA, intrinsic muscle cross-sectional area; L.Br., left bronchus; L.L., left labium; MTM, medial tympaniform membrane; Pes., pessulus; R.Br., right bronchus; R.L., right labium; ST, sternotrachealis; TD, tracheal diameter; TL, tracheolateralis; TL CSA, tracheolateralis cross-sectional area; Tr., trachea; Tr.length, tracheal length.

remove background vocalizations. Five acoustic variables (see full description in the 'Introduction to bioacoustics' R vignette) were used: note duration, peak frequency (i.e. the frequency at maximum amplitude), maximum vocalization frequency, minimum vocalization frequency, and vocalization frequency bandwidth, averaged for each specimen in the sample. While such a sample does not encompass the whole vocal repertoire of species in our sample, it includes the largest amount of acoustic data available for these species, allowing testing for correlations between these traits and syringeal morphological correlates.

A supertree of species in our sample was assembled in MESQUITE 3.70 (Maddison and Maddison 2021) using the topology and calibrations of Chen *et al.* (2019). Topology and calibrations for less inclusive clades were taken from other references (Apodidae: Thomassen *et al.* 2005, Tietze *et al.* 2015; Trochilidae: McGuire *et al.* 2014), with formal definitions for clades within Strisores following McGuire *et al.* (2009) and Chen and Field (2020). Our sample includes two species for which two specimens were sampled: one male and one female for *O. chimborazo*, and one adult and one subadult for *Chaetura pelagica* (L. 1758). High-frequency vocalizations are known to differ between sexes in *O. chimborazo* (Duque and Carruth 2022), and age has been proposed to influence syrinx structure in swifts (Thomassen 2005). In order to consider intraspecific variability in our analysis, we treated each specimen as a separate terminal taxon with a divergence time of 0.1 Myr between two specimens in the same species.

All statistical analyses were performed in R 4.3.1 (R Core Team 2023) on natural log-converted traits (Gingerich 2000). We analysed our set of morphoanatomical traits with phylogenetic generalized least squares (PGLS) regressions (e.g. Symonds and Blomberg 2014) using a lambda model (Freckleton *et al.* 2002) in 'caper' (Orme *et al.* 2023). We performed simple regressions (i.e. one predictor per model), as our relatively low sample size would probaby result in overfitting for multiple regression models (Mundry 2014). To overcome this limitation and investigate causal relationships between our variables, we paired the response and predictive variables in each model to test explicitly stated functional hypotheses based on previous literature (18 simple regressions; Table 1). Normality and homoscedasticity of residuals in our models were checked using Shapiro–Wilk normality tests and residuals versus fitted values plots, respectively, following Mundry (2014). Visual representation of inferred causal relationships between pairs of variables (based on significant PGLS regressions; see Results) was made using a directed acyclic graph (DAG), a common tool in graph theory in which each vertex represents an individual variable and each edge represents an inferred causal relationship (i.e. a regression model), figured as an arrow directed at the response variable of each regression (e.g. Pearl 2009, Laubach *et al.* 2021).

We also investigated the effect of morphological variables on acoustic traits in a PGLS framework. To avoid model overfitting, and because several morphological traits were highly correlated (Fig. 3; Table 2), we opted to perform principal component regressions. Prior to performing principal component analysis (PCA; e.g. Salgado Kent *et al.* 2022), we estimated missing data in the morphological dataset (7.8% missing) using *imputePCA* in 'missMDA' (Josse and Husson 2012, 2016). Given the small size of our dataset ($N = 17$) and recent problems identified with using individual PC scores obtained from phylogenetic PCA (Uyeda *et al.* 2015), we ran ordinary PCAs on the acoustic and completed morphological datasets using *prcomp*. Acoustic PC1 and PC2 explained 68% and 20% of the variance in our acoustic dataset, respectively, and morphological PC1 and PC2 explained 54% and 28% of the morphological variance, respectively (Supporting Information, Fig. S1). We performed PGLS regressions of each acoustic trait (acoustic PC1 and PC2) on each morphological trait (morphological PC1 and PC2), for a total of four PGLS regressions (see Table 3 for results). Traits that were identified as contributing to PCs for which regressions were significant (Supporting Information, Fig. S1B, D) were then regressed on each other in individual PGLS regressions.

Ancestral state reconstructions (ASR) were compiled using *contMap* in 'phytools' (Revell 2012, 2013) for anatomical traits that showed a good fit for a Brownian Motion model; model fit was checked by estimating Pagel's lambda (Pagel 1999) in 'phytools' and performing model selection using *fitContinuous* in 'geiger' (Pennell *et al.* 2014), following Wilson *et al.* (2022). For traits averaged for both sides of the body (distance TL–labia, TL CSA, labia length, IM CSA), difference between left and right measurements was tested using phylogenetic paired *t*-tests in 'phytools' to control for lateral asymmetry (Riede and Goller 2010a, Prince *et al.* 2011).

Table 1. List of causal relationships between pairs of morphoanatomical variables tested using PGLS in this study, with corresponding functional hypotheses. Abbreviations: BM, body mass; DTL, distance tracheolateralis–labia; IMCSA, intrinsic muscle cross-sectional area; LL, labia length; TD, tracheal diameter; TER, tracheal elongation ratio; TLCSA, tracheolateralis cross-sectional area (see text for definitions).

Tested causal relationships	Proposed hypotheses
DTL~BM TLCSA~BM LL~BM TER~BM TD~BM IMCSA~BM	Body mass has a strong, albeit differential influence on acoustic parameters and morphoanatomical measurements of vocal folds (and associated muscles) in vertebrates (e.g. Riede and Brown 2013, Riede and Goller 2014). We expect morphoanatomical traits to be positively correlated with body mass (Riede and Brown 2013) and acoustic traits to be negatively correlated with it (Mason and Burns 2015, Pearse *et al.* 2018, Mejías *et al.* 2020).
DTL~TLCSA TLCSA~TER DTL~TER IMCSA~TLCSA	Hummingbirds have been suggested to use interclavicular air sac, TL, and IM to stabilize the syrinx (Monte *et al.* 2020). This might result in a TL that is thicker to increase stability, but also shorter to make room for the large IM, resulting in a positive correlation between TLCSA and TL shortening, as well as a link between IMCSA and TLCSA. In this hypothesis, a short trachea (high TER) would result in high TLCSA and a higher DTL.
LL~DTL LL~IMCSA IMCSA~DTL	In hummingbirds, IM ensure abduction and adduction of the labia (Riede and Olson 2020), so we expect LL and IMCSA to be correlated. A higher DTL may allow for larger IM, which in turn affects LL; we also test for a direct effect of DTL on LL as a control for that hypothesis.
TLCSA~TD LL~TD IMCSA~TD	A wider trachea with larger tracheal rings may require a thicker TL to modify its length during vocalization and stabilize its position (Riede *et al.* 2006, Riede and Olson 2020). Since LL and IM are involved in fine song tuning in hummingbirds (Monte *et al.* 2020, Riede and Olson 2020), TD may also affect their biomechanical resistance through allometric constraints.
IMCSA~TER LL~TER	Tracheal shortening in hummingbirds has been proposed to be an adaptation for high frequency vocalizing associated with well-developed intrinsic musculature for fine modulation of fundamental frequency (Riede and Olson 2020). The high tensile forces required by this mechanism could constrain the structure of the vocal folds, resulting in thicker and longer lateral labia.

Table 2. Effect size and *P*-value of PGLS regression models built between select pairs of variables measured in our study. For regressions of acoustic traits on morphoanatomical traits, only traits recovered as contributing to significantly correlated principal components (see text) were included. Abbreviations of morphoanatomical traits: BM, body mass; DTL, distance tracheolateralis–labia; IMCSA, intrinsic muscle cross-sectional area; LL, labia length; TD, tracheal diameter; TER, tracheal elongation ratio; TLCSA, tracheolateralis cross-sectional area (see Main Text for definitions). Abbreviations of acoustic traits: fmax_song: maximum vocalization frequency; fpeak_mean: peak frequency; range_song: vocalization frequency bandwidth (see introduction vignette of R package ‘bioacoustics at https://cran.r-project.org/web/packages/bioacoustics/vignettes/introduction.html.).

Regressions of morphoanatomical traits only

Regression formula	Pseudo R-squared	*P*-value	
DTL~BM	0.0483	.1780824	
TLCSA~BM	0.5708	.0000453	***
LL~BM	0.1662	.0377686	*
TER~BM	−0.0593	.7517170	
TD~BM	0.7875	.0000001	***
IMCSA~BM	0.4123	.0058657	**
DTL~TLCSA	0.3132	.0060599	**
LL~DTL	0.0740	.1299169	
DTL~TER	0.4662	.0021327	**
IMCSA~DTL	0.5839	.0008855	***
TLCSA~TER	−0.0429	.5675364	
TLCSA~TD	0.5743	.0000656	***
IMCSA~TLCSA	0.4257	.0049952	**
LL~TER	0.3585	.0065616	**
LL~TD	0.1461	.0539913	
LL~IMCSA	0.6011	.0004149	***
IMCSA~TER	−0.0247	.4170967	
IMCSA~TD	0.5474	.0015014	**

Regressions of acoustic traits on morphoanatomical traits

Regression formula	Pseudo R-squared	*P*-value	
fmax_song~TER	0.2417	.0360931	*
range_song~TER	0.2006	.0534005	
fpeak_mean~TER	0.1532	.0827486	
fmax_song~TD	0.0194	.2789385	
range_song~TD	−0.0291	.4507599	
fpeak_mean~TD	0.1297	.1024637	

Table 3. PGLS regression models built between pairs of morphological PC traits and acoustic PC traits. See Supporting Information, Figure S1 for details on principal components scores and variable loadings.

Regression formula	Estimate	Adjusted R-squared	*P*-value	
Acoustic PC1 ~ Morphology PC1	0.16 ± 0.26	−0.04	.55	
Acoustic PC1 ~ Morphology PC2	−0.75 ± 0.32	0.22	.03	*
Acoustic PC2 ~ Morphology PC1	0.43 ± 0.14	−0.06	.77	
Acoustic PC2 ~ Morphology PC2	0.11 ± 0.20	−0.05	.59	

RESULTS

General description of the swift syrinx

Syrinx morphology shows very little variation between all three sampled species of swifts (Table 1)—*Apus affinis* (Gray 1830), *Co. pelagica*, and *Collocalia esculenta* (L. 1758); therefore, the following anatomical description applies to all of them unless mentioned otherwise. Syrinx anatomical terminology generally follows King (1989), Düring and Elemans (2016), Kingsley *et al.* (2018), Monte *et al.* (2020), and Riede and Olson (2020); colour codes for segmented syrinx scans in Figures 1–6 follow Kingsley *et al.* (2018). The general structure of the syrinx in all sampled hummingbird species matches previously published descriptions of hummingbird syrinxes (Zusi 2013, Monte *et al.* 2020, Riede and Olson 2020).

The swift syrinx is located at the tracheobronchial junction (TBJ), which occurs within the thoracic cavity at the level of the sternum, as documented in most birds (e.g. Düring and Elemans 2016). The trachea has an elliptical cross-sectional shape that remains constant in size and aspect ratio through its entire length (Figs 1A, B, 2A–C, J–L; Supporting Information, Table S1). Cartilaginous tracheal rings are highly contiguous, making them hard to distinguish from one another in the scans (Fig. 2M–O); their cross-sectional shape is elongated along their anteroposterior axis, but progressively becomes more even for the last four to five tracheal rings above the TBJ. The tympanum (i.e. a series of contiguous, partially fused tracheal rings above and at the TBJ; e.g. King 1989) differs between species. In *A. affinis* and *Co. esculenta*, it consists of two enlarged tracheal rings (T0 and T1) with an elongated, slightly triangular cross-sectional shape, well distinct from each other in cross-section (Fig. 2A, C, D, F, M, O). Conversely, the tympanum in *Ch. pelagica* is less conspicuous, with the last tracheal rings being more similar to more craniad rings in both cross-sectional shape and degree of fusion, and a well-defined membrane separates the last tracheal ring from the first bronchial half-rings (Fig. 2B, E, N). In their medial portion, the rings that constitute the tympanum fuse to form a well-developed pessulus (Fig. 2D–F, J–O).

In *Co. esculenta*, the pessulus protrudes slightly more cranially than in the other two species (Fig. 2O), giving it a pointed cross-sectional shape in coronal view that resembles the elongated pessulus found in some hummingbirds (Riede and Olson 2020). In our sample, four hummingbird species not closely related to each other—*Mellisuga minima* (L. 1758), *O. chimborazo*, *Selasphorus heloisa* Lesson and Delattre 1839, and *Topaza pella* (L. 1758)—present such an elongated pessulus (Fig. 1C), suggesting that this trait appeared several times independently among Apodiformes and does not correspond to a major evolutionary shift in vocal production mechanism or learning for either swifts or hummingbirds.

The two bronchi are tubular structures with an elliptical cross-sectional shape, the major axis of which is oriented towards the ventral end of the pessulus (Fig. 2J–L). In transverse view, this results in an angle between the two elliptical bronchi, which varies between species (*A. affinis*: 72.59°; *Ch. pelagica:*

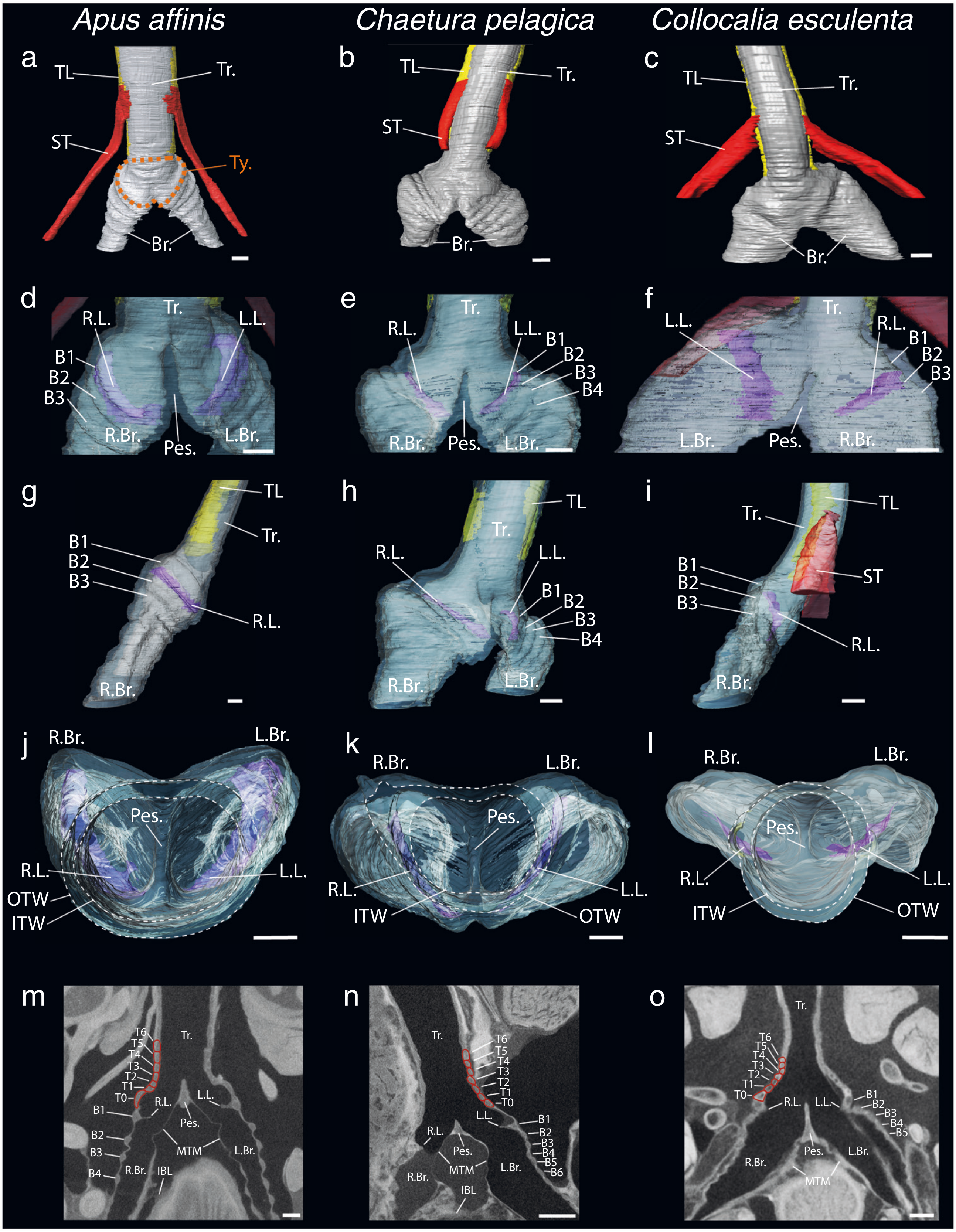

Figure 2. Three-dimensional morphology of the syrinx in sampled swift species visible with diceCT, showcasing morphoanatomical variation of the swift syrinx. Each column showcases a different species (column 1: *Apus affinis*; column 2: *Chaetura pelagica*; column 3: *Collocalia esculenta*). Colour coding in a–l (segmented syrinxes) is: trachea and bronchi, light grey; sternotrachealis, red; tracheolateralis, yellow; labia, purple. The sternotrachealis is not shown in G, E, and H to improve visibility of the tympanum and labia. Following Clarke *et al.* (2016), tracheal rings are numbered from T0 (at the TBJ) up to the anterior end of the trachea, while bronchial rings are numbered from B1 (right

102.8°; *Co. esculenta*: 120.9°; Fig. 2J–L). Cartilaginous structures in the bronchi consist of lateral half-rings (about 10–15 from the TBJ to the lungs; Fig. 2G–I, M–O), as documented in most birds (e.g. King 1989). Unlike tracheal rings, bronchial half-rings are separated from each other and have a well-defined round-to-elliptical cross-sectional shape (Fig. 2M–O). Half-rings are adjacent to each other along the anteroposterior axis, but in *Ch. pelagica* the first four half-rings (B1–B4) in each bronchus form a cluster that aligns in the mediolateral axis on each side, resulting in each bronchus slightly protruding laterally before going back to an anteroposterior orientation in an L-shaped arrangement (Fig. 2B, E, H). All half-rings look similar to one another except for the first pair (B1), which is slightly larger and more triangular in cross-sectional shape than subsequent ones (Fig. 2M–O). One angle in this triangular shape protrudes towards the bronchial lumen and supports one lateral labium on each first half-ring (Fig. 2M–O).

Both lateral labia are long, thin membranes of soft tissue that protrude into the bronchial lumen, and their attachment site consists of the entire inner side of their respective half-ring (Fig. 2D–L). The medial portion of both bronchi is occupied by an evenly thin medial tympaniform membrane (MTM) that attaches to the pessulus and bronchial half-rings (Fig. 2M–O). No medial labia are present, but in *A. affinis* the MTM shows conspicuous local folding that forms a pair of laterally protruding sections that strongly resemble labia (Fig. 2M). These folded sections, however, are located below the lateral labia, at the level of the second pair of bronchial half-rings, and it is not clear whether they would come in contact with the labia during phonation. The interbronchial ligament connects the two bronchi on the caudal end of the MTM at the level of half-rings B5–B6 (Fig. 2M, N).

The musculature in the swift syrinx is similar to that of other birds that do not have vocal production learning: IM are absent and the TL and ST muscles are well-developed. The TL consists of two laterally flattened muscle bands that run on the left and right sides of the trachea, respectively, through its entire length (Figs 1A, B, 2A–C); its distal ends are located right above the TBJ, one to two rings away from the tympanum (Figs 1A, 2A–C, G–I). The ST attaches on top of the TL on each side of the trachea 10–12 rings away from the tympanum and drifts away from the trachea to insert on the craniolateral processes of the sternum (Figs 1A, 2A–C, G, I). The ST is on average five to seven times thicker than the TL in cross-section, which is also the case in the nightjar species in our sample and congruent with previous descriptions of both muscles in other Strisores, e.g. swifts (Suthers and Hector 1982, Thomassen *et al.* 2005), oilbird (Suthers and Hector 1985), and nightjars (Cuvier 1795, Marshall 1905).

Statistical analyses

Twelve out of the 18 regressions between anatomical traits were recovered as significant (Table 2), allowing us to map inferred causal relationships as a DAG between all seven variables (detail in Fig. 3). Significant correlates of labia length, a key parameter in hummingbird vocal innovation (Riede and Olson 2020), were identified as body mass (pseudo $R^2 = 0.167$; $P = .038$; positive correlation), tracheal elongation ratio (pseudo $R^2 = 0.359$; $P = .007$; negative correlation), and IM CSA (pseudo $R^2 = 0.601$; $P < .001$; positive correlation). No significant lateral asymmetry was detected for distance TL–labia ($t = 0.471$; $P = .644$), TL CSA ($t = 0.571$; $P = .575$), or IM CSA ($t = 1.866$; $P = .083$). A significant lateral asymmetry was detected for labia length ($t = 2.209$; $P = .041$), but that difference was recovered as non-significant when tested only in hummingbirds ($t = 0.682$; $P = .509$) or in swifts ($t = 0.522$; $P = .694$), suggesting it to be driven by the difference in labia length between hummingbirds and swifts in our sample.

Phylogenetic principal component regressions revealed a significant negative correlation between morphological PC2 and acoustic PC1 (pseudo $R^2 = 0.2249$; $P = .031$; see Fig. 7; Table 3). Variables that contributed most to morphological PC2 were identified as tracheal elongation ratio (41%) and tracheal diameter (32%), while those that contributed most to acoustic PC1 were identified as maximum vocalization frequency (28%), peak frequency (26%), and vocalization frequency bandwidth (22%—see Supporting Information, Fig. S1B, D). However, out of the six PGLS regressions of individual acoustic traits on individual morphological traits, only one was recovered as significant: that of maximum vocalization frequency on tracheal elongation ratio (pseudo $R^2 = 0.2417$; $P = .036$; see Table 2).

Pagel's lambda was high ($\lambda \geq 0.99$) and significant ($P < .05$) for five out of seven anatomical traits: body mass, TL CSA, tracheal diameter, tracheal elongation ratio, and labia length. A Brownian Motion model was selected as the best fit for tracheal elongation ratio and tracheal diameter, while a lambda model was selected for labia length, an Ornstein–Uhlenbeck model for body mass, and a rate trend model for TL CSA (Supplementary Code). We performed Brownian Motion ASR on all five traits with significantly high lambda values (Fig. 4), since more complex models might be overparameterized for our sample size (Mundry 2014, Cooper *et al.* 2016a, b, Wilson *et al.* 2022). Evolutionary patterns are most conspicuous in major clade comparisons: a strong dichotomy of ancestral states between hummingbirds and swifts can be observed for body mass (decrease in hummingbirds; Fig. 4A), tracheal elongation ratio (decrease in hummingbirds; Fig. 4C), TL CSA (increase in hummingbirds;

below the TBJ) down to the posterior end of the bronchi. A–C, whole syrinx in ventral view. D–F, close-up of the tympanum in ventral view, rendered slightly transparent to show the labia; the tympanum of *Co. esculenta* in (F) is shown in dorsal view, as the angle of insertion of the labia on the first bronchial half-rings prevents their visualization in ventral view. G–I, lateral view (right side) of the syrinx, rendered slightly transparent to show the labia; the syrinx of *C pelagica* in (H) is shown in anteromedial view so that the labia are not hidden by the laterally protruding bronchial half-rings (see text). J–L, anterior view of the two bronchi, rendered slightly transparent to show the labia and pessulus; cross-section of the overlying trachea is shown by white dashed lines. M–O, coronal section of syrinxes in ventral view; the contour of tracheal rings is delimited in dark red. Abbreviations: Br., bronchi; IBL, interbronchial ligament; ITW, inner tracheal wall; L.Br., left bronchus; L.L., left labium; MTM, medial tympaniform membrane; OTW, outer tracheal wall; Pes., pessulus; R.Br., right bronchus; R.L., right labium; ST, sternotrachealis; TL, tracheolateralis; Tr., trachea; Ty, tympanum. Scale bar: 0.5 mm.

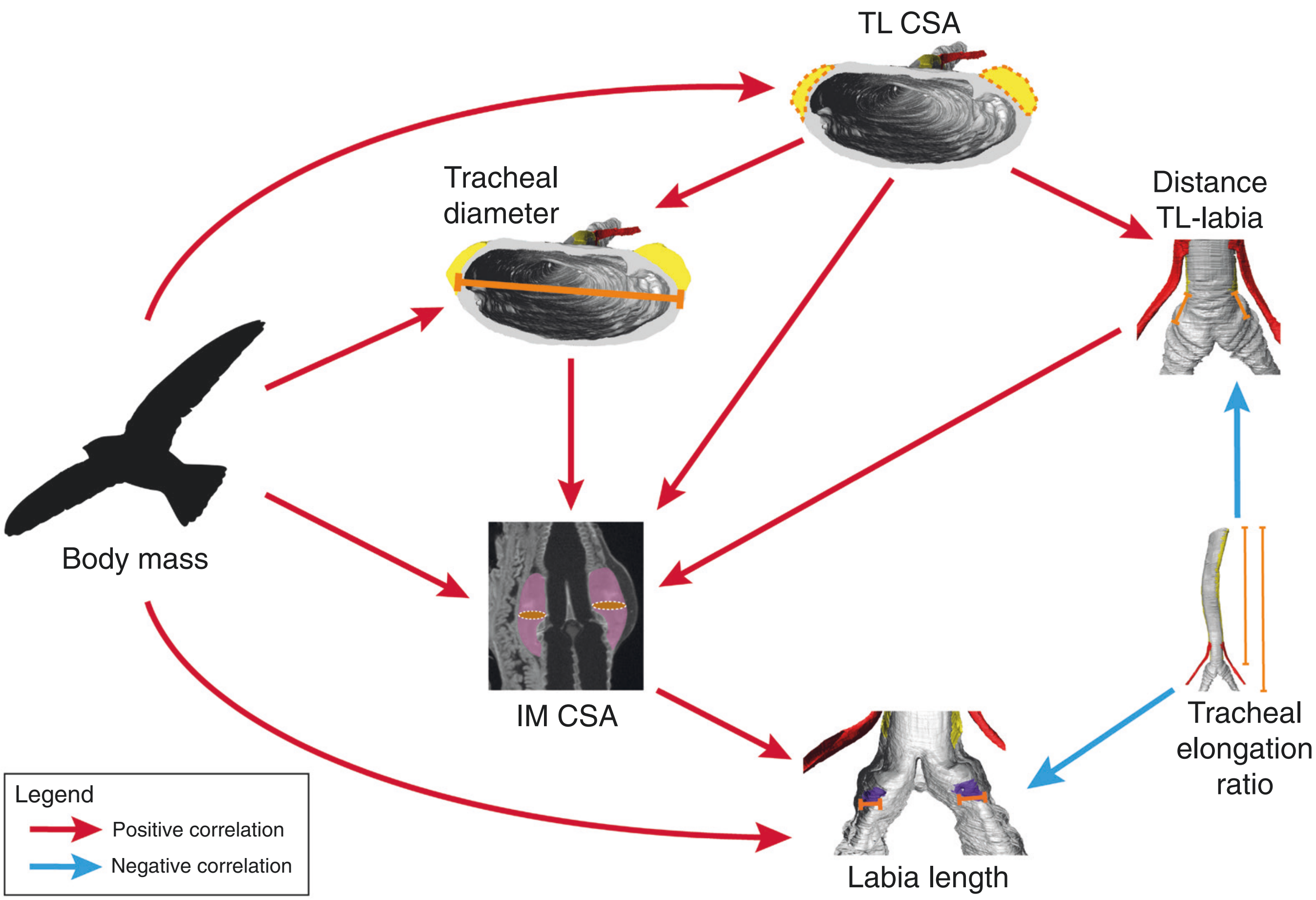

Figure 3. Significant regression models between morphoanatomical variables in our dataset, mapped as a directed acyclic graph (DAG). Each red or blue arrow represents a simple PGLS regression model (see text) corresponding to an inferred causal relationship between two variables (Table 1). All syringeal measurements are represented using orange symbols on top of views of the segmented syrinx of *Apus affinis*, except IM CSA on top of a coronal cross-section of the syrinx of *Oreotrochilus chimborazo* in ventral view. The pseudo R-squared and *P*-value for each model are given in Table 2. Silhouette of *A. affinis* under Creative Commons license (CC0 1.0) from PhyloPic (www.phylopic.org). Abbreviations: CSA, cross-sectional area; IM, intrinsic muscles; TL, tracheolateralis.

Fig. 4E), and labia length (increase in hummingbirds; Fig. 4F). ASR confirm our results for PGLS regressions on body mass (Fig. 3; Table 2): traits significantly influenced by body mass (tracheal diameter, TL CSA, and labia length) all show an evolutionary pattern very similar to that of body mass (Fig. 4A)—particularly within hummingbirds, with an increase of trait value in the largest sampled species *Eutoxeres condamini* (Bourcier 1851), *Patagona gigas* (Vieillot, 1824), and *T. pella* (Fig. 4B, E, F; Supporting Information, Table S1). Conversely, tracheal elongation ratio, a dimensionless trait not influenced by body mass (Fig. 3), does not show any conspicuous pattern within hummingbirds (Fig. 4C), but does exhibit a significant difference between swifts and hummingbirds ($t = 11.47$; $P < .001$; Fig. 4D), probably independent of that found in body mass for the same groups ($t = 3.291$; $P = .022$).

Patterns at less inclusive levels of the tree can also be observed: bee hummingbirds (Mellisugini) show a decrease in body mass compared to other hummingbirds (Fig. 4A), which is recovered for tracheal diameter (Fig. 4B), TL CSA (Fig. 4E), and, to a lesser extent, labia length (Fig. 4F), but not for tracheal elongation ratio (Fig. 4C). The 'Andean clade' (*sensu* McGuire *et al.* 2009) also shows a conspicuous decrease in tracheal diameter and TL CSA (Fig. 4B, E). Finally, all three sampled species in genus *Phaethornis* show much shorter labia than all other hummingbirds, comparable in length to those of swifts and nightjars (Fig. 4F, G; Supporting Information, Table S1). A *t*-test for difference in labia length between swifts and hummingbirds is only marginally significant ($t = 2.520$; $P = .045$). When correcting for body mass by performing the test on the ratio (labia length/body mass), the difference between swifts and hummingbirds is much more pronounced ($t = 6.371$; $P < 0.001$); all *Phaethornis* species are grouped with other hummingbirds, but still show lower values of the ratio compared to them (Fig. 4H).

DISCUSSION

The swift syrinx and the evolution of the syrinx in Strisores

The structure of the swift syrinx is similar to that documented for several non-hummingbird Strisores, and many of its characteristics are probably ancestral to the clade: a conspicuous medial tympaniform membrane present between bronchial half-rings, lateral labia on the first pair of bronchial half-rings,

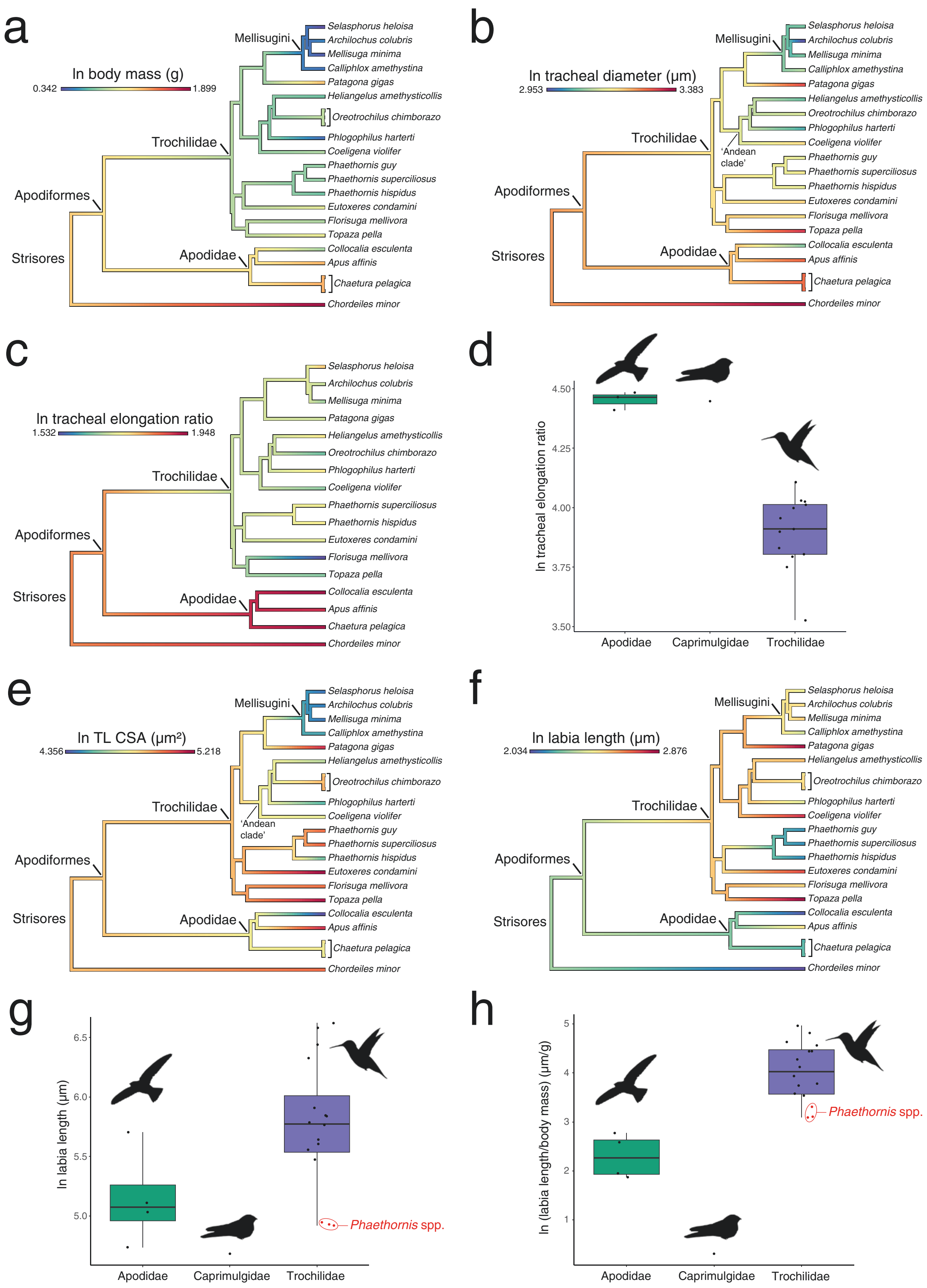

Figure 4. Ancestral state reconstructions for select morphoanatomical traits in our dataset. Reconstructions (A, B, C, E, F) showcase similarities between evolutionary patterns of significantly correlated traits (see text; Fig. 3). Reconstructions were performed for the five traits that showed a significant phylogenetic signal (see text), including: A, body mass; B, tracheal diameter; C, tracheal elongation ratio; E, TL CSA; f, labia length. Clades discussed in the text are labelled. Species for which two specimens are included as terminal taxa (i.e. *Chaetura pelagica* and *Oreotrochilus chimborazo*; see text) are labelled with a vertical bracket connecting both specimens. D, boxplot of tracheal elongation ratio

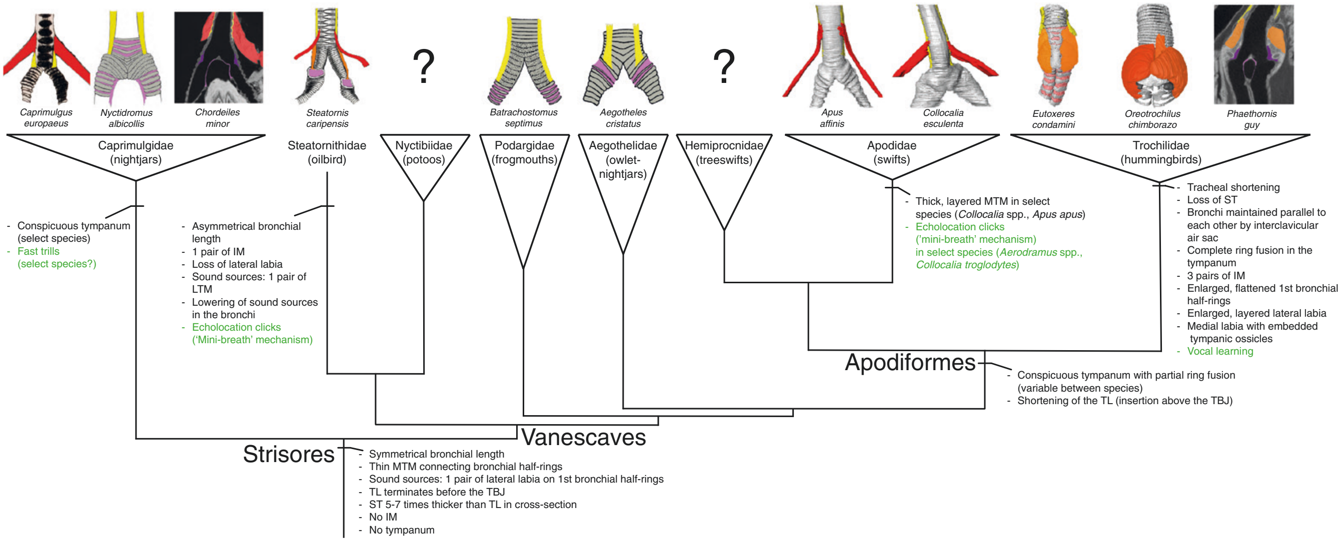

Figure 5. Proposed evolutionary scenario for syrinx evolution in Strisores. Topology follows Chen *et al.* (2019); branch lengths do not represent temporal or character evolution information. Proposed synapomorphies for each clade are listed on the branch leading to that clade, with morphological apomorphies in black and acoustic apomorphies in green. Some syrinx illustrations are modified from previous references: *Caprimulgus europaeus*: Cuvier (1795); *Steatornis caripensis*: Garrod (1873); *Aegotheles cristatus, Batrachostomus septimus, Nyctidromus albicollis*: Beddard (1886). Syrinxes of *Eutoxeres condamini* and *Oreotrochilus chimborazo* were segmented and rendered by C. Urban. Colour coding for syrinxes is: trachea and bronchi, light grey; sternotrachealis, red; tracheolateralis, yellow; membranes, pink; intrinsic muscles, orange; labia, dark purple. Clades discussed in the text are labelled. Syrinxes of Nyctibiidae and Hemiprocnidae, undocumented in the literature, are indicated by question marks. Abbreviations: IM, intrinsic muscles; LTM, lateral tympaniform membrane; MTM, medial tympaniform membrane; ST, sternotrachealis; TBJ, tracheobronchial junction; TL, tracheolateralis.

and well-developed TL and ST (Cuvier 1795, Beddard 1886, Marshall 1905, Suthers and Hector 1982, Thomassen 2005). The oilbird—the only non-Apodiformes Strisores' clade for which functional syrinx morphology has been documented—shows a bilaterally asymmetrical syrinx with a pair of IM, with sound sources consisting of two lateral tympaniform membranes located further down the bronchi (Suthers and Hector 1985). This represents an autapomorphic specialization linked with their use of echolocation for nocturnal foraging, as well as, potentially, intraspecific communication (Suthers and Hector 1985, Brinkløv *et al.* 2013). Conversely, the presence of a well-defined tympanum with partial ring fusion, some of which completely fused as observed in *Ch. pelagica* (Fig. 2B, N), seems to be unique to Apodiformes, as is the shortening of the TL to insert right above it. The variable degree of tympanal ring fusion among swifts might be an important factor to understand the acquisition of a highly fused tympanum in hummingbirds, and quantitative analysis of tympanum cartilage development is likely to clarify the ontogenetic and phylogenetic significance of this trait in the context of vocal production.

Apart from the work of Suthers and Hector (1982, 1985) and Thomassen (2005), previous studies on the vocal organ of non-hummingbird Strisores did not describe its internal syringeal anatomy nor the microstructure of its membranes (e.g. Beddard 1886), meaning that the sound sources of Strisores outside hummingbirds have only been documented in the oilbird and echolocating swiftlets. Thomassen (2005), using a sample of four echolocating and five non-echolocating swift species, did report a variation in thickness of the MTM: very thin in echolocating *Aerodramus* species and the non-echolocating *Hydrochous gigas* (Hartert and Butler 1901) and *Raphidura leucopygialis* (Blyth 1849), and much thicker in non-echolocating *Collocalia* species and *Apus apus* (L. 1758), with a denser layer of collagen and elastin. Thomassen (2005) suggested that a thin MTM might, therefore, be advantageous for echolocation, but not necessary, since the oilbird can produce echolocating clicks and shows a thick MTM (Suthers and Hector 1985). In our sample, *A. affinis* and *Ch. pelagica* present a thin MTM (Fig. 2M, N), while *Co. esculenta* shows the denser and thicker MTM described by Thomassen (2005) in this species (Fig. 2O). Our results confirm that among described swift species, a thicker MTM microstructure is only present in *Collocalia* and *A. apus*, while a thin MTM is found in all other swifts. This suggests that the thinner MTM documented in echolocating swiftlets is probably not an adaptation to echolocation and may instead be ancestral to Apodidae, which would be congruent with the lack of phylogenetic signal in swiftlet echolocating calls (Thomassen and Povel 2006). The thicker MTM in *Collocalia* spp. and *A. apus* probably represents

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distribution for each sampled family of Strisores; G, boxplot of labia length distribution for each sampled family of Strisores, showing the increased size of hummingbird labia compared to those of swifts and nightjars, except for three species in genus *Phaethornis* (red; see text). H, same as (G) but compiled as a ratio (labia length/body mass) to correct for the influence of the latter on the former. All taxa silhouettes under Creative Commons license (CC0 1.0) from PhyloPic (www.phylopic.org). Abbreviations: CSA, cross-sectional area; TL, tracheolateralis.

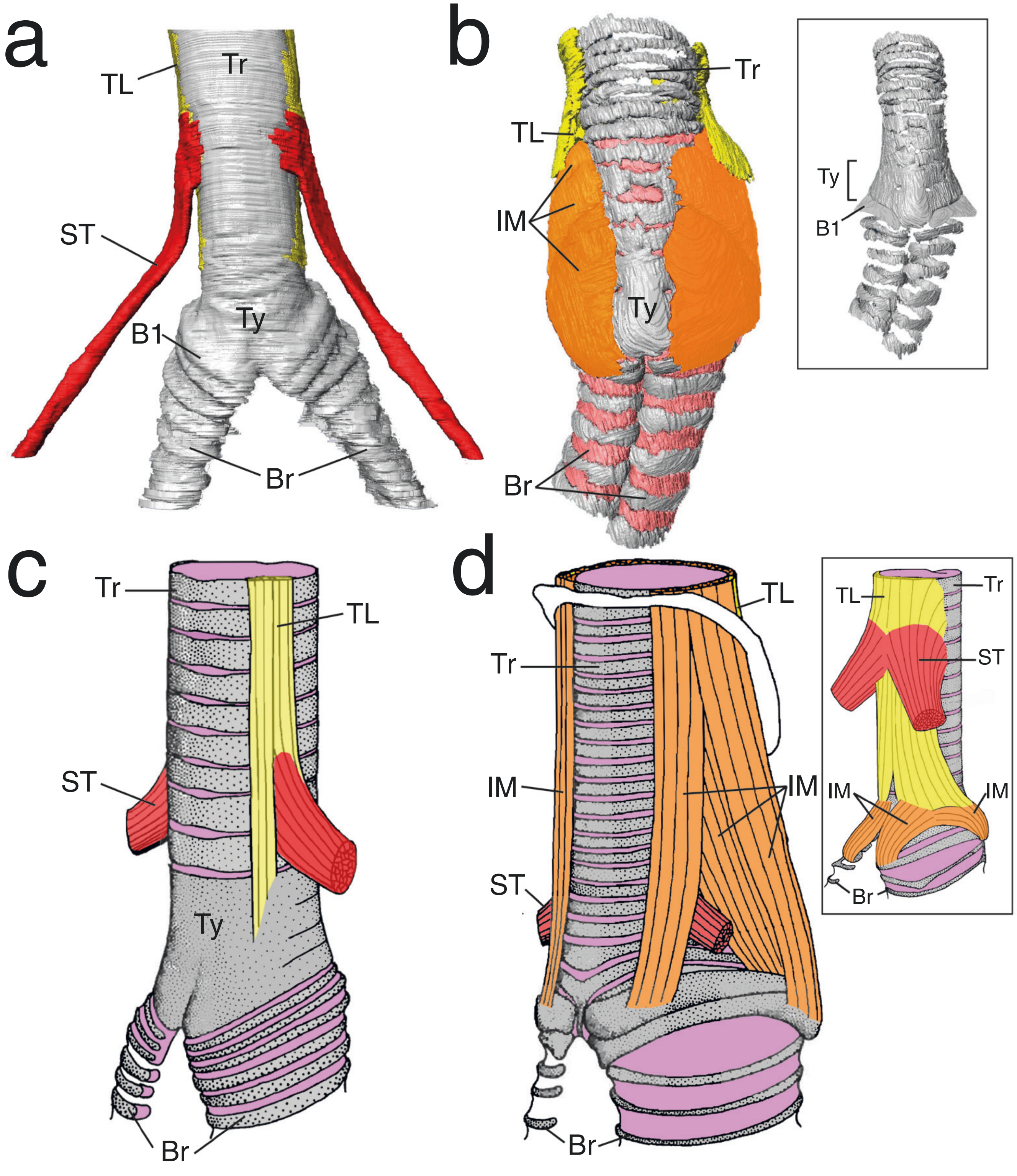

Figure 6. Differences in syrinx morphology between Strisores and Passeriformes. Syrinxes are shown in ventral view (top row) or in ventrolateral (left) view (bottom row). Syrinx of *Eutoxeres condamini* was segmented and rendered by C. Urban. Colour coding for syrinxes is: trachea and bronchi, light grey; sternotrachealis, red; tracheolateralis, yellow; membranes, pink; intrinsic muscles, orange. A, segmented syrinx of *Apus affinis* (this study). B, segmented syrinx of *Eutoxeres condamini* (this study); insert shows the same syrinx without muscles or membranes to showcase the structure of the tympanum and bronchial half-rings. C, illustration of syrinx of *Acanthisitta chloris* Sparrman, 1787 (modified from Ames 1971). D, illustration of syrinx of *Menura novaehollandiae* Latham, 1801 (modified from Ames 1971); the white ring-like structure around the upper part of the trachea is an aponeurosis connecting the trachea to the oesophagus (Ames 1971). The insert shows an illustration of the syrinx of *Corapipo leucorrhoa* (Sclater, 1863) (modified from Ames 1971). Abbreviations: B1, first bronchial half-ring; Br, bronchi; IM, intrinsic muscles; ST, sternotrachealis; TL, tracheolateralis; Tr, trachea; Ty, tympanum.

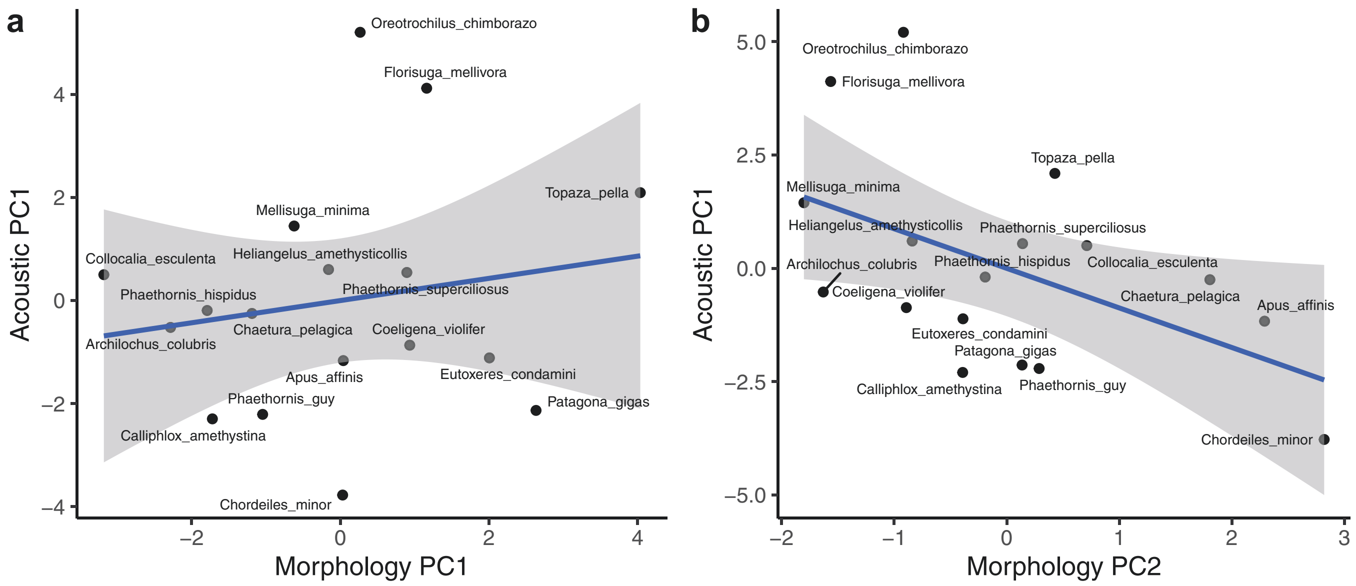

Figure 7. Relationship between acoustic parameters and syrinx morphology in Strisores. Plots show the relationship between acoustic PC1 and morphological PC1 (A) and PC2 (B). The relationship between acoustic PC1 and morphological PC2 is significant (slope = –0.75, lambda < 1e–7, sig2 = 0.04, *P* = .03) while that between other PC traits pairs was not (*P* > 0.05; see Table 3 for detail). Morphological PC1 explained variation in labia length, IM CSA, TL CSA, and distance TL–labia (see text for definitions), while PC2 primarily explained variation in tracheal diameter and tracheal elongation ratio. Acoustic PC1 primarily explained variation in maximum vocalization frequency, vocalization frequency range, and peak frequency (see Supporting Information, Fig. S1).

two independent acquisitions of the trait. Such a structure could provide a structural advantage for sustained high-frequency vocalization, since *A. apus* and other species in the same genus have been documented to produce relatively long calls (~400 ms) at 5.6 kHz (Malacarne *et al.* 1989, Grieve and Kirwan 2012, Marais *et al.* 2021), i.e. within the frequency range of echo clicks produced by echolocating swiftlets (Thomassen and Povel 2006). Accordingly, in hummingbirds, local thickening of the MTM to form a medial labium with tympanic ossicles has been proposed to facilitate high-frequency vocalizing through increased stiffness (Monte *et al.* 2020). However, it is worth noting that the bronchial morphology of swifts can show extensive intraspecific variation (Thomassen 2005), which might also affect differences in MTM thickness. Furthermore, the phylogeny of Apodidae is unresolved and has not been critically reassessed in over a decade (Lee *et al.* 1996, Price *et al.* 2004, Thomassen and Povel 2006, Päckert *et al.* 2012, Tietze *et al.* 2015). Preliminary assessments of the ancestral condition for syringeal traits in swifts and associated functional hypotheses regarding vocalization are highly conditional on future results regarding topology and calibration of the Apodidae tree.

We document for the first time the presence of well-developed labia (range of 113.7–299.6 μm; Supporting Information, Table S1) in three species of swifts, each belonging to one of the three tribes in subfamily Apodinae: Apodini (*A. affinis*), Chaeturini (*Ch. pelagica*), and Collocaliini (*Co. esculenta*). This is congruent with the results of Thomassen (2005), who reported a wide range for labia volume in nine species of swifts (*N* = 16). Zusi (2013), referring to the lateral labia of hummingbirds as lamellae, considered them to be distinct from those of swifts and coded them as absent in *A. affinis* and *Ch. pelagica*. However, while the structure of hummingbird lateral labia—enriched in collagen and supported by a highly modified first bronchial half-ring [B1 in Riede and Olson (2020)]—is distinct from that of other Strisores, their position and attachment in the syrinx are similar to those of swifts in our sample and in Thomassen (2005) [although the lateral labia in *Aerodramus spodiopygius* (Peale 1848) are positioned slightly more craniad and partially attach to the posterior end of the tympanum—Suthers and Hector (1982)], and lateral labia could thus be homologous structures in swifts and hummingbirds. Conversely, the medial labium with embedded ossicles found in hummingbirds is absent in swifts, and is probably an autapomorphy of Trochilidae among Strisores.

The inner structure of hummingbird labia (a lamina propria composed mostly of collagen, lined by a thin outer epithelium; Riede and Olson 2020) is very similar to the thickened MTM described in *Co. esculenta* and *A. apus* (Thomassen 2005). Despite these two anatomical structures being involved in different vocalization mechanisms during sound production, their similar microstructure shows that the acquisition of collagen-enriched laminae in sound-producing structures is not exclusive to hummingbirds among Strisores, and thus might not necessarily be linked to high-frequency vocalizing, as was previously proposed in songbirds (Riede and Goller 2010a, 2014) and hummingbirds (Riede and Olson 2020). Additionally, *Chordeiles minor* (Forster 1771), the nightjar sampled in the present study, also presents conspicuous lateral labia on the inner side of the first two pairs of bronchial half-rings (Fig. 5), very similar in structure to those of swifts. Considering that nightjars (Caprimulgidae) are the earliest-diverging family within Strisores (e.g. Chen *et al.* 2019), a pair of lateral labia on the first pair of bronchial half-rings probably represents the ancestral sound source in Strisores, which would then have been lost in the oilbird and highly modified in hummingbirds. Further descriptions of syrinx anatomy in other Strisores families are required to test this hypothesis.

In addition to their sound sources, the similarity in extrinsic syringeal musculature between swifts and nightjars suggests that the documented sequence of syringeal muscular activity for sound production in swifts may also be similar between the two clades, and possibly ancestral to Strisores. The general model of alternate action of ST ('on' muscle, first click) and TL ('off' muscle, second click) proposed by Gaunt and Gaunt (1985a) for avian vocalization is similar to the vocalization sequence described by Suthers and Hector (1982) and refined by Thomassen (2005) for swiftlet echolocation calls, and could thus be generalized to other Strisores groups. Unusual call types requiring fast oscillations of the labia have been documented in non-hummingbird Strisores, such as long, fast trills (up to 8 min) in the nightjar *Caprimulgus europaeus* L. 1758 (Hunter 1980), or high-frequency repetition of echolocation clicks in the oilbird (Suthers and Hector 1985). Such calls have been proposed to require very fast antagonistic action of ST and TL, as documented for 'mini-breath' mechanisms found in several songbirds and in the oilbird (Brackenbury 1978, 1989). While the fast trills identified in *Ca. europaeus* are not associated with a 'mini-breath' mechanism (Hunter 1980), such a rapid production of trills can be associated with superfast vocal muscles (Elemans *et al.* 2008), a mechanism probably widespread in birds, including clades unable to use mini-breaths (e.g. doves—Elemans *et al.* 2004). The ancestral presence of an enlarged ST in Strisores may thus have facilitated fast muscular contraction cycles that quickly increase and decrease airflow by abducting and adducting the lateral labia (Thomassen 2005), which is crucial for echolocation in oilbird and swiftlets (Brinkløv *et al.* 2013).

In this context, the thin labia visible in *Chordeiles minor* and most swifts would be capable of sustaining moderately fast oscillations and produce simple calls for extended periods of time, but could not produce complex calls at higher F0 that require a wider vocal range (as documented in hummingbirds; Duque and Carruth 2022). Such calls can be achieved by layering the inner structure of the labia, with a lamina propria composed of fibrous proteins (collagen and/or elastin) and glycosaminoglycans that help to reduce mechanical stress, and a surrounding thinner epithelium that can oscillate independently to reach higher frequencies (Riede and Goller 2010a). A significant positive relationship between layering of the labia and F0 range was documented for the medial labia of passerines (Riede and Goller 2014). Such a mechanism is generally associated with fine muscular control by IM for precise abduction of the labia into the lumen to reach specific oscillating frequencies, as documented in vocal learners. While this strategy has been hypothesized to be present in hummingbirds based on similar layering of their labia and precise control of their abduction by IM (Monte *et al.* 2020, Riede and Olson 2020), the ancestral presence of such structures in non-learning avian clades and its role in the subsequent acquisition of vocal learning has not been assessed. The thick, layered structure of the MTM in some swifts, as documented by Thomassen (2005) and in the present study, even though not used in high-frequency vocalizing, suggests that such specialized structures were probably acquired at more inclusive levels of the avian tree than previously suggested. Future studies documenting the histological structure of labia in non-learning avian clades might improve our understanding of the acquisition of vocal learning and high-frequency vocalizing among Aves.

Implications for the evolution of the hummingbird syrinx

The significant relationships we identified between pairs of syringeal traits in our sample provide a framework to describe the possible evolutionary pathway for the acquisition of the hummingbird syrinx (Fig. 5). The main direct predictors of increased labia length in hummingbirds are lower body mass, higher IM CSA, and tracheal shortening. This is consistent with the hypothesis of displacement of the syrinx in the neck through tracheal shortening being a major factor for the acquisition of three pairs of well-developed IM, which finely abduct/adduct larger, more rigid labia adapted to complex high-frequency vocalizations (Monte *et al.* 2020, Riede and Olson 2020).

Interestingly, tracheal shortening is also correlated with a higher distance of TL–labia (which in turn correlates with IM CSA; Fig. 3) and with a higher maximum vocalization frequency (Table 2; Fig. 7; Supporting Information, Fig. S1). Tracheal length has long been known to be a major influence on the fine-tuning of bird song: a longer vocal tract can be 'tuned' by adjusting its length and diameter through combined action of ST and TL (and IM, if present) to match its resonance frequency with the F0 produced by the sound sources (Daley and Goller 2004, Riede *et al.* 2006, 2019). Tracheal shortening is associated with an increase in F0 in chickens and budgerigars (associated with syringeal muscle control in the latter), but not in zebra finches, in which it only causes an increase in nonlinear phenomena (i.e. subharmonics, frequency jumps—Riede *et al.* 2019). The authors hypothesized that the stronger control of F0 in zebra finches was due to the layered structure of their labia (Riede *et al.* 2019), which is also present in hummingbirds (Riede and Olson 2020). Therefore, the peculiar strategy of hummingbirds to reduce tracheal length by displacing the syrinx craniad should, in theory, result in a decreased ability to match formants with F0 and increased nonlinear phenomena, which is not the case (Riede and Olson 2020, Duque and Carruth 2022). Instead, the shortening of the trachea and loss of the ST provide additional space for an increased tracheal diameter and a thicker, shorter TL, resulting in an increase in available space on the surface of the tympanum. This available space allows for the development of large dorsoventrally oriented IM, which (i) stabilize the syrinx in the throat (in conjunction with the invaginated interclavicular air sac) and (ii) finely adjust the position of lateral labia through ligaments. Additionally, embedded tympanic ossicles in the medial labia locally control their tension and help to adjust the frequency of their oscillations (Monte *et al.* 2020). Therefore, the hummingbirds compensate for the shortening of their vocal tract by developing extreme stabilization of their sound sources and highly specialized labia structure and motor control, resulting in decreased 'acoustic chaos' and increased vocal range that facilitates high-frequency vocalizing. This is further supported by the significant correlation between maximum vocalization frequency and tracheal elongation ratio.

This strategy of simplifying the anatomy of the vocal tract (shorter trachea and TL, loss of ST) to achieve greater song complexity is analogous to the loss of air sacs and vocal membranes in the human larynx, which suppressed the generation of subharmonics and chaos used by non-human primates to produce louder calls, but shifted vocal production to a more stable sound source (the vocal folds) that enhanced production of formant frequencies through more complex motor control (Fitch *et al.*

2002, Riede and Brown 2013, Nishimura *et al.* 2022). The reduction of anatomical structures involved in producing pseudoformants ('chaos') can thus give rise to vocal complexity through acquisition of specialized structure and motion control of the sound sources. The advantages of producing complex tonal songs can represent a strong selection pressure for such morphological changes in vocal learners, as recently shown in both hummingbirds and passerines, although the importance of the upper vocal tract filter and its potentially less prominent role in hummingbirds due to trachea shortening remains to be investigated (Faiß *et al.* 2022).

This proposed evolutionary scenario, however, is far from complete and requires further testing, as shown by the almost complete lack of significant relationships between anatomical and acoustic traits in our dataset (only one significant correlation). While this could be due to our relatively low sample size, it could also be explained by the influence on acoustic traits of other anatomical parameters we did not account for, especially those associated with the specialized physiological strategy of hummingbirds. To sustain the energetically expensive hovering flight strategy associated with their nectarivorous diet, hummingbirds have acquired the highest known metabolic rates among vertebrates (Bicudo *et al.* 2002, Hargrove 2005, Shankar *et al.* 2020), achieved through increased muscular heat production from highly hypertrophied pectoral muscles (~30% of body mass—Suarez 1992) and heart (2.1–2.5% of body mass—McNab 2019), the largest of any bird in relation to body mass. Additionally, these enlarged pectoral muscles show very high vascularization and mitochondria content (Mathieu-Costello *et al.* 1992), resulting in increased aerobic capacity (Suarez *et al.* 1990, Nespolo *et al.* 2018). The increased space occupied by the hypertrophied heart and highly vascularized lungs, associated with constraints on the size of the rib cage to support the enlarged pectoral musculature, may have been major selective pressures on syrinx displacement into the throat, which could be tested in future studies by adding quantitative traits measured on the heart and pectoral muscles of Strisores to our phylogenetic framework. We also did not consider the effect of confounding variables (e.g. body mass for labia length ~IM CSA—Fig. 3; Table 1, 2), since our low sample size precluded the use of multimodel causal inference, e.g. through structural equation modelling (von Hardenberg and Gonzalez-Voyer 2013, Thorson *et al.* 2023). Performing such analyses on a larger sample of Strisores syrinxes would improve and clarify causal inferences made from our DAG (Uyeda *et al.* 2018, Laubach *et al.* 2021).

Additionally, our sample does not account for the high ecological disparity of hummingbirds, a clade that experienced accelerated diversification (McGuire *et al.* 2014) associated with colonization of new habitats and subsequent adaptation to highly varied ecological niches (e.g. linked with altitudinal gradient; Parra *et al.* 2010, Licona-Vera and Ornelas 2017, Duque *et al.* 2021, Barreto *et al.* 2023). These habitat-specific adaptations resulted in many specialized call types and strategies, as exemplified by high-frequency vocalizing species with specialized hearing (Duque *et al.* 2018, 2020, 2021, Duque and Carruth 2022), clades that repeatedly evolved complex song patterns associated with elaborate courtship displays (Clark 2011, Clark *et al.* 2018), or even potential loss of singing abilities (Monte *et al.* 2023). In this context, morphological adaptations of syringeal structure that would reflect these evolutionary strategies are probably specific to less inclusive hummingbird clades, and would require much more comprehensive sampling within Trochilidae to be adequately tested for and characterized. Future studies are likely to identify such patterns by investigating specific hummingbird clades known for specialized vocal production strategies (review in: Duque and Carruth 2022) using a rigorous quantitative phylogenetic framework. Such studies would require comprehensive sampling of vocal repertoires for each sampled species in order to identify vocalization patterns associated with vocal learning, which only represent a fraction of the vocal production in vocal learners and can be highly variable between populations of a given species (e.g. Araya-Salas and Wright 2013, Lattenkamp *et al.* 2021). While some acoustic traits (e.g. F0, bandwidth, tonality) have been documented to vary through ontogeny in vocal-learning vertebrates (e.g. bats—Prat *et al.* 2017, Lattenkamp *et al.* 2021), additional acoustic data are needed to adequately characterize this variation and to identify correlates of vocal learning in hummingbirds.

Other aspects of syrinx evolution in Strisores are also difficult to assess due to the lack of data for other families within the clades. A well-defined tympanum associated with a short TL, for example, can be hypothesized as a synapomorphy of Apodiformes, since oilbird, frogmouths, owlet-nightjars, and most nightjars do not present a conspicuous tympanum and their TL comes directly in contact with the tympaniform membranes or first bronchial half-rings (Cuvier 1795, Beddard 1886, Marshall 1905; Fig. 5). If correct, this hypothesis would support the apomorphic tympanum of Apodiformes as having facilitated the later acquisition of a highly fused tympanum and even shorter TL in hummingbirds. Similarly, descriptions and drawings in Beddard (1886) suggest that the sound sources in frogmouths and owlet-nightjars might be located further down the bronchi than those of Apodiformes (Fig. 5). If correct, this hypothesis would imply that Vanescaves ancestrally had labia further down the bronchi, meaning that the position of the labia on the first bronchial half-rings in Apodiformes would be apomorphic and non-homologous to that of nightjars. This would also suggest that the facilitating mechanisms to increase available space on the syrinx for development of IM are opposite between oilbird (displace the sound sources further down the bronchi to make space below the TBJ) and hummingbirds (shorten the TL to make space above the TBJ). However, apart from the oilbird, available descriptions of the syrinx in non-Apodiformes Strisores are from 100+-year-old references with inconsistent terminology, scarce illustrations, and no information on the structure and location of the sound sources. Furthermore, the lack of data for potoos and treeswifts, two clades with crucial phylogenetic positions within Strisores (Chen *et al.* 2019; Fig. 5), prevent any ASR of such traits for the whole group. A reassessment of syrinx morphology in non-Apodiformes Strisores is required to further understand syringeal synapomorphies of Apodiformes.

The presence of a well-defined tympanum with paired IM in the hummingbird syrinx has recently been proposed as evolutionary convergence with the passerine syrinx (e.g. Riede and Olson 2020). The morphology of syringeal muscles in passerines, however, is distinct from that of hummingbirds. Within passerines, the TL usually inserts on the tympanum or first bronchial half-rings (Ames 1971; Fig. 6C, D insert), in contrast to the much more cranial termination in hummingbirds (Fig. 6B). The high diversity in number and position of IM in passerines (Ames 1971, Warner 1972, King 1989; Fig. 6C, D) further contrasts with their highly conserved and distinct morphology in the hummingbird syrinx (three pairs of IM in a craniocaudal sequence—Zusi 2013, Monte *et al.* 2020, Riede and Olson 2020; Fig. 6B). This may be due to the well-documented recent (~20 Mya) and rapid (average of 0.23 species/Myr) diversification of hummingbirds, most of which occurred in its initial 10 Myr burst (McGuire *et al.* 2014). Highly specialized ancestral innovations of the hummingbird syrinx (Fig. 5) were probably acquired during this short initial burst, with limited subsequent diversification in less inclusive clades (McGuire *et al.* 2014, Harvey *et al.* 2017, Barreto *et al.* 2023). Conversely, estimated time-trees for extant passerines (Passeriformes: ~85–47 Mya; Passeri: ~60–38 Mya—Ericson *et al.* 2014, Oliveros *et al.* 2019) recover low early diversification rates, with most major rate shifts occurring over the last 10 Myr (Oliveros *et al.* 2019). This lower initial selective pressure may have resulted in the many syringeal morphologies found in passerines (Fig. 6C, D), with independent selective pressures on vocal production in less inclusive clades. This hypothesis, however, requires further anatomical and bioacoustic comparisons to properly assess similarities and differences in the independent acquisition of complex intrinsic syringeal muscles and vocal learning between songbirds and hummingbirds (Riede and Olson 2020, Faiß *et al.* 2022).

We did not focus on describing the syrinx anatomy of hummingbirds in our sample for this study, as their characteristics did not differ from the general descriptions available in the literature. The surprisingly short labia of species in genus *Phaethornis* (Fig. 4F–H; Supporting Information, Table S1), however, suggests that departures from this classic model might be present in some groups. When looking at the morphology of their labia in coronal view, all *Phaethornis* specimens show labia with a more rounded outer shape and relatively minimal protrusion into the bronchial lumen, and the first pair of bronchial half-rings is reduced and less laterally elongated compared to other hummingbirds (Fig. 5). We do not observe these differences in *E. condamini*, the other hermit species in our sample. While this distinct morphology could also be explained by post-mortem deformation, as previously hypothesized for some configurations of hummingbird labia (Riede and Olson 2020), the high similarity between all three species suggests that it might be a real pattern, potentially corresponding to distinct vocal production strategies in *Phaethornis*. A previous study on another species in this genus, *P. longirostris* (Delattre 1843), reported a high turnover of simple single-note songs produced by males during lekking behaviour (Araya-Salas and Wright 2013), but did not document song patterns specific to *Phaethornis* among hermits, a clade in which lekking is widespread (Araya-Salas and Wright 2013), and song learning reported in all documented species (Monte *et al.* 2023). Investigating the inner structure of the labia and bronchial half-rings in this genus and other hermit hummingbirds using histology and microscopy may help to characterize this potential difference in syrinx morphology and to expand our understanding of the diversification of vocal production strategies in hummingbirds.

CONCLUSION

The swift syrinx highlights several structural characteristics that probably represent the ancestral vocal production strategy for Strisores, but it also showcases structures that probably represent synapomorphies of Apodiformes, particularly its tympanum and vocal folds. Our statistical analyses also suggest that the shortening of the trachea and TL observed in hummingbirds (probably in conjunction with their displacement of the syrinx in the throat) facilitated their acquisition of high-frequency vocalizing. The influence of such traits on the acquisition of vocal learning in hummingbirds, however, remains to be investigated in a comparative context. A comprehensive analysis of all major Strisores' groups in a morphoanatomical and acoustic context, to reconstruct detailed evolutionary scenarios for each of them, is yet to come and would benefit from the combined use of diceCT imaging and phylogenetic comparative methods, as used in the present study. Additional methods, such as comparative histology and advanced microscopy techniques that complement CT data (Riede and Goller 2010a, 2014, Riede and Olson 2020), or direct observation of syringeal movements through endoscopy (Goller and Larsen 1997b, Larsen and Goller 2002), could also be used in such a framework to investigate evolutionary innovations and to provide a more detailed picture of the evolution of syrinx anatomy in both learning and non-learning Strisores. Such studies are greatly needed to bridge the gap between morphological diversity of sound sources and known patterns of acoustic diversification, particularly in non-learning avian species, and will represent the next step in deciphering the evolution of vocal production in birds.

SUPPLEMENTARY DATA

Supplementary data are available at *Zoological Journal of the Linnean Society* online.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DATA AVAILABILITY

All quantitative data generated for this study are available in the Supporting Information, Tables S1 and S2. The R code, data, and phylogenetic tree used for analyses are available on GitHub at https://github.com/LucasLegendre/Strisores_syrinx_project. Scans of specimens sampled for this study are available on Open Science Framework at https://osf.io/aw8fz, except for the two *Oreotrochilus chimborazo* specimens, which are part of the supplementary material of a forthcoming study.

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