# Basis of the vocal communication in hummingbirds

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# Author contributions

The doctoral research presented here was conducted at the Max Planck Institute for Biological Intelligence (former Max Planck Institute for Ornithology, Seewiesen). The study was carried out from September 2013 until November 2021; the thesis writing was concluded in Mai 2023. For all studies (**Chapters 1-5**), I performed the fieldwork, data collection, part of laboratory work, data analysis and writing of the manuscripts under the guidance of my supervisor Prof. Dr. Manfred Gahr, who fund the project, contributed to conceiving the study, scientific discussion and writing, except for the **Chapter 2** which was conducted under the expertise of Dr. Daniel N. Düring. Dr. Maria Luisa da Silva co-supervised the studies of **Chapter 1, 3, 4 and 5**, contributed to fieldwork and helped with the acquisition of permits necessary to perform fieldwork in Brazil in all studies. Alexander F. Cerwenka and Dr. Bernhard Ruthensteiner contributed to data acquisition (µCT scan of the vocal organ) in **Chapter 2**. Dr. Albertine Leitão and Dr. Carolina Frankl-Vilches contributed to the experimental design, collecting of data during fieldwork, data analysis and scientific discussion of **Chapter 3 and 4**. Dr. Carolina Frankl-Vilches also contributed to the development of experimental techniques (molecular sexing and in situ hybridization) in **Chapter 3, 4 and 5**. Rodrigo da Silva Matos, Viviany Costa and Danielson Aleixo contributed to the fieldwork and data collection (**Chapters 3, 4 and 5**). Monika Trappschuh contributed to the hormone analysis of **Chapter 3**. Antje Backer performed the laboratory analysis of samples (molecular sexing and in situ hybridization) in **Chapter 3 and 4** and Nina Sohnius-Wilhelmi (in situ hybridization) in **Chapter 5**. All authors approved the final manuscripts written and published.

# Summary

Although hummingbirds draw attention to their unique hovering flight, as other birds, they strongly rely on sounds to communicate. As songbirds and parrots, hummingbirds convergently evolved the capacity of learning how to produce certain vocalizations early in life in a process similar to speech acquisition, vocal production learning, based on analogous neural substrate. During the past two decades of investigation on vocal production learning in birds, many similarities have been shown among avian vocal learners. However, comparative studies were mainly focused on the North American radiation of hummingbirds which is more than 15 million years apart to the common ancestors originated in the lowlands of South America. Furthermore, investigations on the principles governing vocal production learning in South American hummingbirds meets the lack of information about the basis of their vocal communication. Therefore, in this thesis, I investigate the vocal communication of hummingbirds using a comprehensive approach on an evolutionary perspective by focusing each chapter on a different yet complementary aspect: (*i*) evolution of their vocal behavior, (*ii*) anatomy of their vocal organ, sensibility of their (*iii*) vocal behavior and (*iv*) brain to androgens and (*v*) neural activity during vocalizations in the wild. I found that when investigated comparatively, hummingbirds show a number of dissimilarities concerning their vocal organ, brain and androgen sensibility when compared to other vocal learners and a hitherto overlooked heterogeneity in their vocal communication within the family. Towards the base of the hummingbird phylogeny, both sexes vocalize in similar rates independently of androgen. I propose the black jacobin (*Florisuga fusca*) as a model species here and in future studies to better represent the putative ancestral condition of the family. The inclusion of key yet understudied species, such as the black jacobin, in comparative studies can shed a different light on the understanding of vocal production learning in birds. This thesis paves the way to further

investigations on the evolution of vocal production learning from a more comprehensive perspective.

# General introduction

Living organisms often leave traces of their activities that can be perceived by others and used as a source of information (Bradbury & Vehrencamp, 2011). In aquatic ecosystems, for example, a wide variety of planktonic organisms such as ciliates, algae, rotifers and crustaceans detect the presence of predators by sensing their chemicals diluted in the water and can react consequently (reviewed in Lass and Spaak 2003). One extreme case can take place for example in a tree hole where rainwater accumulates and mosquito larvae develop and feed on a ciliate species. When the ciliate detects chemicals in the water produced by the larvae, their free-living forms start cell division to transform into a parasitic form that is able to infect and eventually kill the larvae (Washburn et al., 1988). Such inadvertent or inevitable by-product of the larvae activity is used by the ciliate as a cue. Cues, like this, can also become signals, conforming the origin of a communication process (Bradbury & Vehrencamp, 2011). A signal can be defined as change in the environment caused by one individual (i.e., the emitter) to convey information to another (i.e., the receiver; Endler et al. 1993). Signals are the centerpiece of communication as they are expected to influence or alter the receiver's behavior and, importantly, have direct fitness consequences either on the emitter, the receiver or both (Bradbury & Vehrencamp, 2011).

There are limited mediums for the transmission of a signal: air, water, or solid substrates with fixed physical properties. However, the strategies of communication are infinite and the way how different organisms develop these strategies is just fascinating. For example, a bean plant infested by the aphid, a small sap-sucking insect, can communicate the danger to the neighbor plants by releasing odorous chemicals into the air, then the neighbors can respond by emitting different chemicals that repel the aphids and attract aphid-hunting wasps (Kost & Heil, 2006). When the infected bean plants were enclosed into plastic bags to prevent their alarm chemicals from spreading into the air, they still could alarm the neighbors, but this time by sending chemicals via mycorrhizal fungi under the soil (Babikova et al., 2013). Either through the air or soil, the plant developed a successful strategy to transmit further the information: “There is something going wrong in here!” Humans could be surrounded by infested bean plants and still not perceive their chemical alarm without the proper tools because this chemical signal is tuned to the plant's morphology, the environment it occupies, and how this environment is sensed.

The evolution of communication signals depend on two factors: the efficacy of the receiver in decoding the message and the signal’s ‘design’ which must contain enough information for receivers to retain their responsiveness (Bradbury & Vehrencamp, 2011; Guilford & Dawkins, 1991). While the signal efficacy is determined by the sender preadaptation, the signal design is determined by the receiver’s (Bradbury & Vehrencamp, 2011). Additionally to sender and receiver preadaptation, the evolution of a signal is shaped by the environment that imposes both physical and biological constraints like attenuation, degradation or even the interaction with other life forms (Endler, 1992; Endler et al., 1993). Here, organisms can evolve different modes of communication like acoustic (also called auditory which is composed of water- or airborne signals), chemical (also called olfactory), electric, seismic (also called vibratory which is composed by substrate-borne signal), tactile and visual (Bradbury & Vehrencamp, 2011).

Among all communication forms, chemical communication constitutes the first one to evolve (reviewed in Tietjen and Rovner 1982). Chemical communication is the most primitive, widespread, and probably the primary mode of communication in the majority of living organisms (reviewed in Wyatt 2014). Although animals seem pre-adapted to detect chemical information in the environment (Wyatt, 2014), after the development of internal cavities (Coelomata), most animal taxa evolved other ways to communicate (Fig. 1). In birds, for example, although some ducks, auklets and petrels use chemical communication (Caro &

Balthazart, 2010), the majority of the species have acoustic and visual as their primary modes of communication (Butcher & Rohwer, 1989; Catchpole & Slater, 1995; Haldane, 1955).

In organisms that disperse in the environment, sender and receiver can be more than a hundred meters apart and the information needs to travel over distance, consequently, some modes of communication such as electric or tactile can be infeasible. Another implication of communication over distance is the presence of obstacles in between, for example, in a dense forest or an ocean, there are many objects or other organisms in between emitter and receiver, thus, the signal must follow a trajectory around trees or a set of corals. Therefore, acoustic communication is used by a wide variety of animals due to the propagation properties of the sound waves in air or water that warrants efficient transmission over distance and obstacles (Bradbury & Vehrencamp, 2011).

The need for structures specialized in the production and perception of sound waves might have been a constraining factor in the early evolution of acoustic communication in Coelomata. Insects, for example, are known for their remarkable ways to produce sounds such as the stridulation in crickets and the loud sounds produced by the contraction of tymbals in cicadas, however, only nine out of 32 orders (~ 28 %) have specializations to perceive sound (Greenfield, 2016). Instead, insects use seismic communication – composed of self-generated vibrations transmitted via a substrate such as the soil or a plant stem (Narins, 1990) – more often because they produce these vibrations by diverse methods independent of morphological specializations and detected them by sensitive receptors present in all six legs (Virant-Doberlet & Cokl, 2004). In contrast, all classes of vertebrates have inner ears structures which seems a synapomorphy that has evolved ~ 400 mya (reviewed in Greenfield 2016).

Acoustic communication is widespread in terrestrial vertebrates (tetrapods, superclass *Tetrapoda*) and prevalent in most amphibians, mammals, crocodilians, and birds except for most lepidosaurs and turtles (Chen & Wiens, 2020). Chen and Wiens (2020) reconstructed the evolution of acoustic communication across tetrapods and found that acoustic communication evolved independently in each of the major tetrapod groups and was preceded by nocturnal activity; the idea is corroborated by the fact that acoustic communication is rare among lepidosaurs but evolved repeatedly within the predominantly nocturnal clade, Gekkota (Chen & Wiens, 2020). In both amphibians and mammals, acoustic communication evolved ~200 mya and has been maintained in many lineages to the present day, however, secondarily losses have happened in several species. Such evolutionary loss was not identified in birds in which acoustic communication was inferred to be present in the most recent ancestor of living birds and the most recent ancestor of living crocodilians, each of these ancestors is ~100 million years old (Chen & Wiens, 2020).

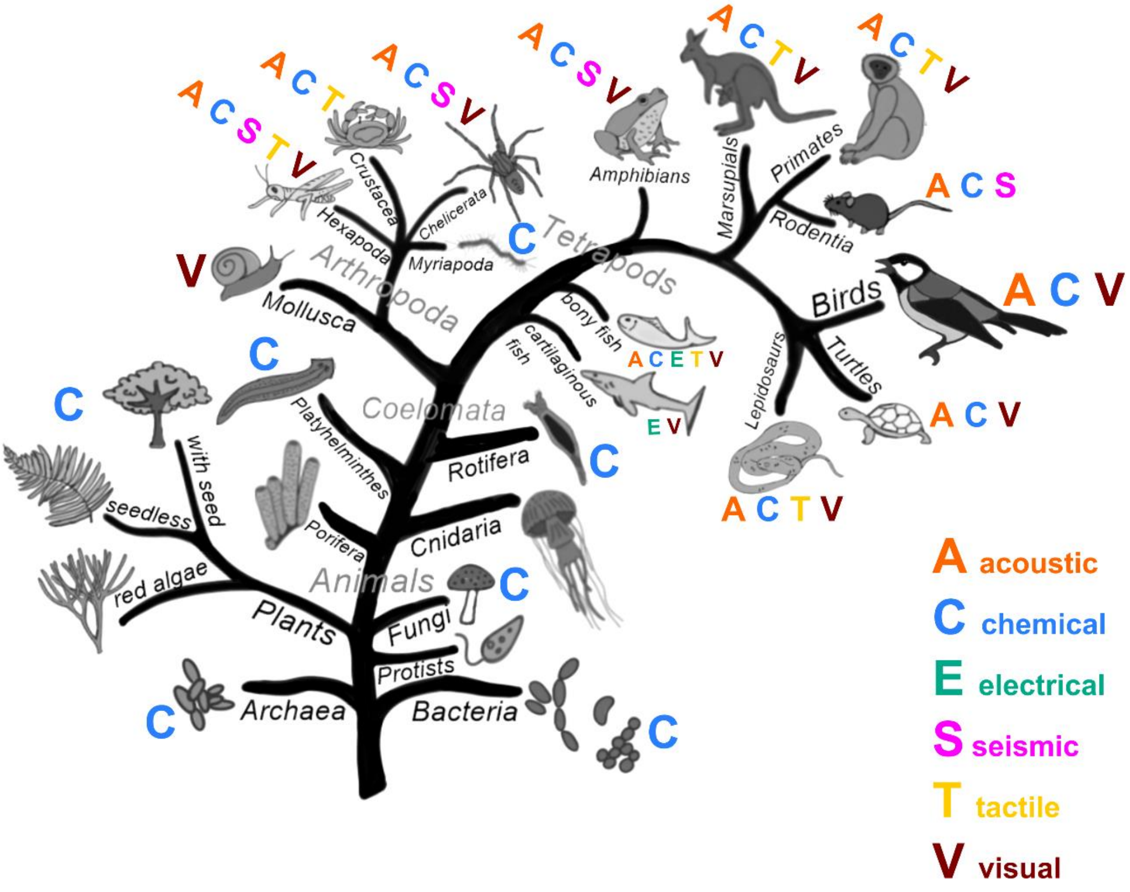

Fig. 1 – The mode of communication is diverse among living organisms. Simplified tree of life redrawn based on an image available in “mammothmemory.net”. Information about phylogenetic relationships is also from the textbooks Ruppert et al. 2004 (invertebrates) and Pough et al. 2004 (vertebrates). To access examples of communication in each taxon, I

performed a keyword search in Google Scholar by entering "communication AND" followed by the Latin taxon name or common taxon name as depicted on the tree, then I inspected the first five results pages. The modes of communication are represented by letters and colors: acoustic (A, orange); chemical (C, light blue); electrical (E, green); seismic (S, magenta); tactile (T, yellow) and visual (V, brown). References: chemical communication in Archae, Bacteria (Keller & Surette, 2006) and Fungi (Cottier & Mühlschlegel, 2011); use of volatile chemicals for communication in plants (Karban et al., 2014); use of pheromones for reproduction in Platyhelminthes (Bone, 1982); pheromones in Rotifera (Snell et al., 1995; Timmermeyer & Stelzer, 2006); visual displays (color-change) in Mollusca (Adamo & Hanlon, 1996; Shashar et al., 2004); acoustic (Greenfield, 2016), chemical (Pelosi et al., 2014), seismic (Virant-Doberlet & Cokl, 2004), tactile (Leonhardt et al., 2016) and visual (dance, Dethier 1957 and bioluminescence, Lloyd 1983) communication in Hexapoda (includes all insects); acoustic (Schmitz, 2002), chemical (Atema & Steinbach, 2007) and tactile (Karplus, 1979) communication in Crustacea; acoustic (Uetz & Stratton, 1982), chemical (Tietjen & Rovner, 1982), seismic (Barth, 1982; Rovner & Barth, 1981) and visual (D. L. Clark et al., 2011; Uetz et al., 2011) communication in Chelicerata (including spiders and scorpions); chemical (Sombke et al., 2011) communication in Myriapoda (centipedes and milipedes); electric (Bratton & Ayers, 1987) and visual (Hart et al., 2006) in cartilaginous fish; acoustic (Bass & McKibben, 2003), chemical (Liley, 1982; Stacey & Sorensen, 1991), electric (Kramer, 1990, 1996), tactile sensed by the mechanosensory lateral line system (Butler & Maruska, 2016) and visual (ultraviolet signaling, Cummings et al. 2003) communication in bony fishes (Teleosts); acoustic (Colafrancesco & Gridi-Papp, 2016), chemical (Madison, 1977), seismic (Narins, 1990) and visual (Hodl & Amézquita, 2001); acoustic, chemical, tactile and visual communication in marsupials (Russell, 1984) and in primates (Peters & Ploog, 1973); acoustic (Okanoya & Screven, 2018), chemical (Johnston, 2003) and seismic (Francescoli & Altuna, 1998) communication in rodents; acoustic (Catchpole & Slater, 1995), chemical (demonstrated for ducks, auklets and petrels, Caro and Balthazart 2010) and visual (Butcher & Rohwer, 1989) in birds; also acoustic (Colafrancesco & Gridi-Papp, 2016), chemical (Madison, 1977) and visual (Brejcha & Kleisner, 2016) communication in turtles (Chelonia) and acoustic (in geckos, Marcellini 1977), chemical (Madison, 1977), tactile and visual (Carpenter, 1977) in Lepidisaurs (lizards, snakes and tatuara).

## Vocal communication in birds

Differently from chemical or visual, acoustic signals can be directly controlled by organs at the disposal of voluntary muscles and produced or interrupted instantly through body movements (Goller & Suthers, 1996; Hartshorne, 1973). Furthermore, sound waves have temporal and spectral parameters that can, to a certain degree, be voluntarily modulated (Hartshorne, 1973; Podos, 1997). Thus, sounds are fitted for signaling rapidly, subtly, and in a variety of ways (Hartshorne, 1973). Probably, the possibility of direct control was one of the factors that contributed to the positive selection for the use of sounds in birds (class Aves) culminating in the evolution of sophisticated systems of acoustic communication (Catchpole, 1982). In fact, acoustic communication was initially proposed as the main driver through the diversification of the most successful radiation of birds, the songbirds (suborder *Passeri*, order *Passeriformes*, ~ 41% of the extant birds). Despite this idea was recently proved wrong, vocal communication is still a well-conserved and fascinating trait within birds (Chen & Wiens, 2020).

Birds produce a wide variety of sounds including a diversity of mechanical sounds that are generated by non-vocal sources such as wings, tails or bills (C. J. Clark & Feo, 2008; Dodenhoff et al., 2001; Gaunt & Nowicki, 2012). Woodpeckers (family *Picidae*), for example, can strike with their bill on a substrate to produce drumming that serves to communicate as this sound can elicit a conspecific response (Dodenhoff et al., 2001). The sounds produced by the vocal organ (vocalizations) have drawn attention particularly because they can be conspicuous, melodic and overlapping with human audible range (Catchpole & Slater, 1995). Moreover, after the 1960s, substantial advances in recording devices and software for sound analysis supported the development of bioacoustics – the study of the sounds of animals (Vielliard & Silva, 2010). Therefore, bird vocalizations, particularly those of songbirds, have been intensively studied for the past six decades being the most studied communication systems after human language (Catchpole & Slater, 1995; Tomasello, 2010). Bioacoustics has a specific terminology to classify birds' vocal communication that is largely but arbitrarily used (Spector, 1994).

### ***Birds' vocal repertoire is divided into song and calls***

Each bird species can produce a set of different vocalizations consistently in certain behavioral contexts, the vocal repertoire, which is arbitrarily classified as calls or song (Catchpole & Slater, 1995; Spector, 1994). The traditional definition is strongly influenced by studies of temperate songbirds that, mostly, follow strict seasonal regimes (Catchpole & Slater, 1995). Thus, the traditional definition states that calls are generally short and simple and are uttered by both sexes at all times of the year each in a distinct context such as alarm or begging, whereas songs are longer, more complex, and generally restricted to the males that emits it during the breeding season for territorial defense and mate attraction (Catchpole, 1982; Catchpole & Slater, 1995). The traditional definition of song is based on multiple criteria (relative complexity of the spectral parameters, sex, seasonality and presumed function), therefore, this definition, which suits most songbirds, suits only partially many further bird species, in particular those from the tropics (Spector, 1994).

Studies focused on tropical species tend to use a broader definition; in these studies, song is the vocalization that, among other functions, primarily serves species-specific recognition (Vielliard 1987, e.g. Mathevon et al. 2008). Thus, each species is supposed to have a unique song that encodes a species signature, whereas calls are all other non-song vocalizations (Vielliard, 1987). Recently, in the attempt to expand the traditional definition of song for southern birds, a broader definition of song was proposed as “a sequence of several vocal elements with species-specific characteristics, performed with a particular rhythmic pattern, which serves for intraspecific communication” (Bonnevie & Craig, 2018).

### *Defining songs in hummingbirds*

The ambiguity in the song definition can be tackled by each study with a simple declaration of the definition used (Spector, 1994). However, studies that compare songs of many species by using information from multiple other studies, as in a systematic review, may come across a homology problem. Given the suitability of the traditional definition, comparative studies of songbirds and maybe other well-studied taxa do not meet such a problem, but this is not the case for other taxa whose song definition is unclear as, for example, hummingbirds (family *Trochilidae*).

In the literature about the vocalization of hummingbirds, I found a few discordances in which of the vocalizations within the vocal repertoire of a species is the song. Authors might have relied on dissimilar song definitions but they do not always disclose a definition in the study. For example, in the case of the long-tailed hermit (*Phaethornis superciliosus*), the males aggregate and vocalize in assemblies during the breeding season where each male perches in its territory to deliver a monotonous vocalization for up to 30 minutes (Stiles & Wolf, 1979). This vocalization was described either as song (Skutch, 1964; Stiles & Wolf, 1979), as call (B. K. Snow, 1973) or reported simply as a "janging noise" (Nicholson 1931). Another example is vocalization of the fork-tailed hummingbird (*Thalurania furcata*) which is also emitted by males in an assembly, Pizo and Silva (2001) classified it as an "advertisement call" whereas Vielliard (1983) classified it as song because "playback experiments elicit strong territorial response" and the response was interpreted as the recognition of a conspecific. Can this lack of consensus be taken as a sign of the unsuitability of the traditional song definition for hummingbirds? This question was addressed in **Chapter 1**.

## Morphological basis of vocal production

In contrast to all other tetrapods which produce vocalizations with their larynx, birds has an unique vocal organ, the syrinx (Ames, 1971; Bass, 1989). The syrinx is located in the caudal end of the Trachea (Fig. 2a), suspended inside of the interclavicular air sac, and composed of vibrating membranes, associated cartilages and muscles (Clarke et al., 2016; King, 1989). In both the larynx and syrinx, sound vibrations are produced by the opening and closing of membranes during upstream airflow (reviewed in Bradbury and Vehrencamp 2011). Birds still have a larynx, but they only use the syrinx to produce sounds because the syrinx offers a biomechanical advantage for sound production over the larynx (Riede et al., 2019). This advantage is given by the location of the syrinx deep in the thorax with a longer vocal tract above the source of sound which improves vocal efficiency by demanding a lower phonation threshold pressure and generating higher sound intensities (Riede et al., 2019).

The anatomy of the syrinx is highly variable across different avian taxa to the extent that it has been used to guide avian phylogenetic classification (Ames, 1971; Suthers & Zollinger, 2008). Up to three pairs of vibrating membranes are present and depending on where these membranes are allocated, the syrinx can be classified into tracheal, tracheobronchial or bronchial (Smolker, 1947). The syrinx musculature can be only partially attached to the syrinx, the extrinsic muscles, or completely attached, the intrinsic muscles (Ames, 1971; Gaunt & Gaunt, 1985). In contrast to the extrinsic, intrinsic muscles are facultative (Gaunt & Gaunt, 1985) and vary largely in number, for example, songbirds have from three up to nine muscle pairs (Ames, 1971; Gaunt, 1983) while parrots (order *Psittaciformes*) have two (Gaunt, 1983; Nottebohm, 1976) (Fig. 2b).

As intrinsic muscles provide finer control over the vibratory membranes, it was hypothesized that a syrinx with intrinsic musculature is a prerequisite for the production of complex vocalizations (Gaunt, 1983). Similar to songbirds and parrots, some hummingbirds species produce complex vocalizations (Ferreira et al., 2006; Olson et al., 2018; Silva & Vielliard, 2006). Hummingbirds have a tracheobronchial syrinx (Müller, 1878; Zusi, 2013) which, in species of a relatively recent taxon, is convergent to the syrinx of songbirds (Riede & Olson, 2020) (Fig 2b). However, it was unknown whether this convergence was also observed in early branched hummingbird species. Therefore, in **Chapter 2**, I investigated the syrinx anatomy of the black jacobin (*Florisuga fusca*), a hummingbird species that is phylogenetically distant to those previously studied and belongs to the sister taxa of all other extent hummingbirds (McGuire et al., 2014). By investigating the black jacobin, I could suggest synapomorphies to the hummingbird family and their implications for the production of complex vocalizations which are potentially learned.

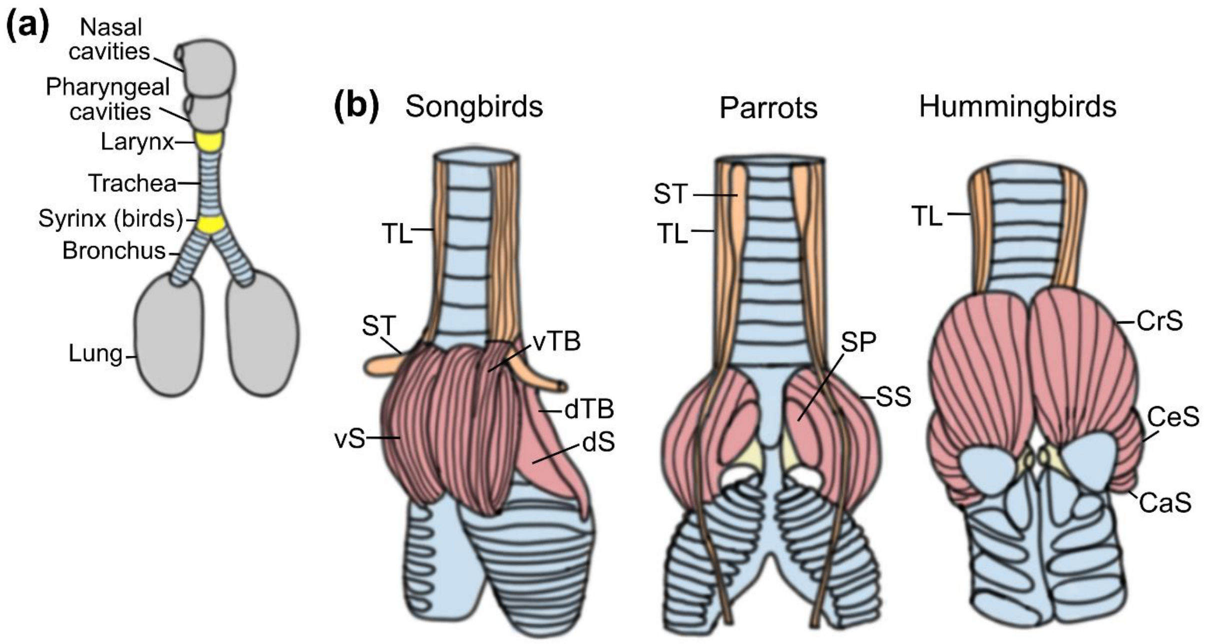

Fig. 2 – The vocal organ of tetrapods is part of their respiratory system and birds evolved a novel vocal organ, the syrinx, which is diverse among avian taxa. (a) Schematic representation of the respiratory system indicating the location of the vocal organ (yellow) that is cranial for the larynx and caudal for the bird syrinx. Redraw from Bradbury and Vehrencamp (2011). (b) General syrinx anatomy of songbirds (ventral view; based on brown thrasher, *Toxostoma rufum*, and cardinal, *Cardinalis cardinalis*), parrots (ventral view; cockatiels, *Nymphicus hollandicus*) and hummingbirds (dorsal view where intrinsic muscles can be better visualized; black jacobin, *Florisuga fusca*). The intrinsic musculature is completely attached (pink) and the extrinsic (orange) is only partially attached to the syrinx. The number of intrinsic muscles varies among taxa but is present in all three of them. The tracheolateral muscle (TL) is present in all of them but the sterno-tracheal muscle (ST), responsible for the stabilization of the syrinx in the interclavicular air sac, is absent in hummingbirds (**Chapter 2**). Trachea and Bronchi are represented in blue. The songbird and parrot syrinxes were redrawn from Larsen and Goller (2002) and the hummingbird from Monte et al. (2020). dS, *musculus syringealis dorsalis*; dTB, *m. tracheobronchialis dorsalis*; SP, *m. syringealis profundus*; SS, *m. syringealis superficialis*; ST, *m. sternotrachealis*; TL, *m. tracheolateralis*; vS, *m. syringealis ventralis*; vTB, *m. tracheobronchialis ventralis*; CrS, *m. syringealis cranialis*; CeS, *m. syringealis centralis*; CaS, *m. syringealis caudalis*.

### *Learned versus innate vocalizations*

In the syrinx, air-induced vibratory movements generates sounds whose acoustic features can be modulated by changes in the geometry of the membranes given by muscle activity (Goller & Larsen, 1997; Larsen & Goller, 1999). Contraction of different syringeal muscles can withdraw or close membranes from the syringeal lumen, causing opening or closing of the syringeal airways (Larsen & Goller, 1999; Rüppell, 1933). Complex sounds such as the song of a canary (*Serinus canaria*) with its high variation in pitch can be reproduced by the simple oscillations in bronchial pressure and vocal fold tension (Gardner et al., 2001). Nevertheless, the production of specific acoustic features such as of the canary song depends on the precise coordination of respiratory and syringeal muscles given by the brain (Alonso et al., 2015). The brain coordinate the production of sounds via a interconected network of nuclei distributed across several regions: brainstem, thalamus and telecephalon that functions in a circular fashion (Alonso et al., 2015). Most of bird vocalizations are produced without the need of auditory preexposure, for example, flycatchers raised in acoustic isolation develop similar songs to wild cospecifics; these vocalizations are innate (Kroodsma, 1984, 1996; Marler, 2004). Whereas some requires a learning process, for example, canaries not exposed to their specific song during their first year developed a abnormal song; these vocalizations are learned (Leitner & Catchpole, 2007). Only learned vocalizations demands a complete brain network including some telencephalic nuclei (reviewed in Nottebohm 2005).

## Vocal production learning

To produce complex vocalizations, some animals need to undergo a learning process that holds striking parallels with speech acquisition in humans, namely vocal production learning or simply vocal learning (reviewed in Marler 1970b and recently in Jarvis 2019). Vocal production learning is the ability to learn how to produce novel sounds based on auditory experiences (Doupe & Kuhl, 1999; Nottebohm, 1970). This ability is rare and evolved independently only in a handful of unrelated mammals and birds (reviewed in Janik and Slater 1997, 2000).

Among mammals, the vocal learners are bats (order *Chiroptera*, reviewed in Knörnschild 2014), cetaceans (infraorder *Cetacea*, including dolphins and whales, reviewed in Janik 2014), some elephants (Poole et al., 2005), pinnipeds (a clade composed by three families of seals, sea lions, and walruses, reviewed in Reichmuth and Casey 2014) as well as humans (reviewed in

Jarvis 2019), and most recently mole rats (Barker et al., 2021). Among birds, the vocal learners are songbirds (suborder *Passeri* or *Oscines*) (Bottjer et al., 1985; Nottebohm et al., 1976), parrots (order *Psittaciformes*) (Jarvis & Mello, 2000) and hummingbirds (family *Trochilidae*) (Baptista & Schuchmann, 1990).

Before reviewing the current knowledge on vocal learning, it is important to first define the difference among usage vocal learning, auditory learning and vocal production learning because only the latter is rare (Jarvis, 2019; Petkov & Jarvis, 2012). Usage vocal learning is the ability to learn through social experience to use vocalizations in a specific context (Petkov & Jarvis, 2012). Meerkats (*Suricata suricatta*), for example, have spectrally different alarm calls that are given to specific predators such as the snake and the avian predator and elicit contrasting conspecific responses (Manser et al., 2002). Both alarm calls are innate, but juvenile meerkats probably need to learn from more experienced ones to match the call to the corresponding predator and which is the appropriate response. Auditory learning is also the ability to associate a sound, vocal or not, with a behavioral reaction but the difference from usage learning is that this association is made with a sound that is heard, for example, the use of different keywords to train a dog (Petkov & Jarvis, 2012). In contrast to usage and auditory learning, vocal production learning is primary a motor rather than associative learning (Feenders et al., 2008).

To be listed as a vocal learner, Kroodsma and Baylis (1982) proposed that a species must show at least one of the following characteristics: (*i*) natural interspecific vocal imitation; (*ii*) intraspecific vocal imitation; (*iii*) experimental vocal imitation of conspecifics, heterospecific or non-avian sounds and; (*iv*) abnormal vocal development under acoustic deprivation. Additionally, the presence of the brain specializations related to vocal production learning is taken as a neurobiological piece of evidence because vocal learners convergently evolved these brain specializations that are absent in non-vocal learners (Gahr, 2000; Jarvis, 2007).

Among the several hypotheses proposed to explain the selective advantages leading to vocal production learning, the sexual selection hypothesis is suggested to have a broader taxonomic application (Jarvis, 2006; Nowicki & Searcy, 2014). The sexual selection hypothesis, also called mate attraction, suggests that vocal production learning enables vocalizations to become more varied as a result of a mating preference for vocal complexity (Nowicki & Searcy, 2014). Nevertheless, in less studied vocal learners such as hummingbirds, it is unclear whether learned vocalizations are subjected to sexual selection in the first place.

Birds are excellent study models to investigate the mechanism of vocal production learning due to their mostly dial activity and conspicuous vocalizations that are relatively accessible and quantifiable (Catchpole & Slater, 1995). During the past five decades, numerous studies focused particularly on a few species of songbirds were conducted and elucidated important neurobiological and physiological aspects underlying the ontogeny of vocal production learning. However, the selective pressures underlying the evolution of high cognitive ability as vocal production learning in such different taxa remains unknown (Jarvis, 2006; Nowicki & Searcy, 2014).

In birds, the ontogeny of vocal production learning can be divided in two phases: sensory and sensorimotor (Doupe & Kuhl, 1999; Nottebohm, 1970). The sensory phase happens when a juvenile gets repeatedly exposed to the sound to be learned and memorizes it, and the sensorimotor phase when the juvenile progressively reproduces a more accurate copy of the memorized model by auditory feedback (Doupe & Kuhl, 1999; Marler & Peters, 1982; Nottebohm, 1970). These phases can be constrained to early life (closed-ended learners) or happen throughout life (open-ended learners) (Nottebohm, 1992). Among songbirds, there are both closed-ended, for example, zebra finches (*Taeniopygia guttata*, Zann 1990) and open-ended learners, for example, canaries (*Serinus canaria*, Nottebohm and Nottebohm 1978); among parrots, all species investigated to date seem open-learners (Bradbury & Balsby, 2016) and among hummingbirds, two distant related species are suggested to be open-learners (Araya-Salas & Wright, 2013; Johnson & Clark, 2020).

Research on vocal communication is primarily associated with songbird species and only to a lesser extent with parrots and hummingbirds. Thus, it is unclear whether the sexual selection hypothesis can explain the origin of vocal production learning in any bird taxa other than songbirds. Comprehensive comparison among bird vocal learners -- songbirds, parrots and hummingbirds -- will benefit from in-depth investigations of hummingbirds' vocal communication. Especially hummingbirds because they have not only the smallest brains capable of vocal production learning but also, they are the vocal learner with the fastest metabolism, in addition to several specializations which makes them unique. Therefore, hummingbirds is a key taxon in comparative studies to investigate which are prerequisites to the evolution of vocal production learning in animals.

### *Vocal production learning in hummingbirds*

Most of the pieces of evidence for vocal production learning in hummingbirds are given by dialects of lekking species (e.g. González and Ornelas 2005, Araya-Salas and Wright 2013, Lara et al. 2015). The first studies suggesting the existence of vocal production learning in hummingbirds date from the end of the 1960s, when intraspecific vocal imitation was described in lekking hermits (subfamily *Phaethornithinae*) (D. W. Snow, 1968). Males of most hermit species aggregate in communal display grounds, the so-called leks, where they perform vocal and complementary visual displays to compete for females (D. W. Snow, 1968; Stiles & Wolf, 1979; Wiley, 1971). These studies found that, in the leks, neighboring males have more similar songs than geographically distant males (D. W. Snow, 1968; Stiles & Wolf, 1979; Wiley, 1971). Such geographical variation of song, the so-called vocal dialect (M. C. Baker & Cunningham, 1985), can be a result of genetic differentiation among populations (M. C. Baker, 1982) rather than vocal imitation, however, it does not seem to be the case in hummingbirds (González & Ornelas, 2014).

In the 1990s, Baptista and Schuchmann (1990) provided the first experimental evidence for vocal production learning in hummingbirds. The authors hand-raised one juvenile of Anna's hummingbirds (*Calypte anna*) acoustically isolated from conspecifics and a group of three juveniles without acoustic exposure to conspecifics other than to each other. They observed that when adults, the bird that was isolated produced a much simpler song than wild conspecifics and those raised in a group resembled each other suggesting intraspecific imitation.

In the 2000s, two independent studies on different species using different methods demonstrated that unrelated species showed brain specializations analogous to those found in songbirds and parrots related to vocal production learning (Gahr, 2000; Jarvis et al., 2000). These brain specializations were shown to be active during song production (Jarvis et al., 2000) in the rufous-breasted hermit (*Glaucis hirsutus*) and sombre hummingbird (*Aphantochroa cirrochloris*), they were present in Anna's hummingbirds and Amazilia hummingbird (*Amazilia amazilia*), but vestigial in ruby-throated hummingbirds (*Archilochus colubris*) and Allen's hummingbirds (*Selasphorus sasin*) (Gahr, 2000).

Meanwhile, other studies demonstrated vocal dialects which is direct evidence for vocal production learning in species other than hermits, for example, in the wedge-tailed sabrewing (*Campylopterus curvipennis*) (González & Ornelas, 2005). As well as indirect evidence for vocal production learning such as high vocal complexity in white-vented violetear (*Colibri serrirostris*) (Silva & Vielliard, 2006) and the blue-throated hummingbird (*Lampornis clemenciae*) (Ficken et al., 2000); intraindividual variation in the long-billed hermit (*Phaethornis longirostris*) which indicated that hummingbirds are open learners (Araya-Salas & Wright, 2013) and convergence in the regulation of specialized genes (SLIT–ROBO) in the brain of Anna's hummingbirds, songbirds and parrots (Wang et al., 2015).

About 20 years later, another study conducted an acoustic isolation experiment and investigated the ontogeny of vocal production learning in the sister species of Anna’s hummingbirds, Costa’s hummingbird (*Calypte costae*) (Johnson & Clark, 2020). This study demonstrated that the simple song of Costa's hummingbird is learned, but juveniles are able to copy adult's songs only after many hours of auditory exposure and if raised in visual contact with a conspecific (Johnson & Clark, 2020). A recent study on bees hummingbirds (tribe *Mellisugini*) showed that producing songs and mechanical sounds with the wing are negatively correlated (C. J. Clark et al., 2018). However, to date, no investigations tackled singing and vocal production learning at the family level, and questions remained open such as whether singing is the ancestral condition in hummingbirds, loss of song is consistently related to evidence against vocal production learning or there are pieces of evidence for losses of vocal production learning in taxa other than bees.

## Endocrinological basis of vocal production

In vertebrates, many processes including vocal production, are coordinated by one or more hormones (reviewed in Baker 2003 and Harding 2008). In particular, steroid hormones such as androgens and estrogens can affect developmental stages and cause unreversible changes (organizational effects) and affect adult stages when changes are reversible (activational effects) (Phoenix et al., 1959).

There are two general types of steroid hormones: adrenocortical and sex (or gonadal) hormones; because both types are synthesized from cholesterol, they have in common a sterol nucleus but differ in organ source and physiological functions (reviewed in Nelson and Cox 2008). Adrenocortical hormones are produced in the cortex of the adrenal gland and can, for example, affect the metabolism of carbohydrates (glucocorticoids) (Olefsky & Kimmerling, 1976) or regulate the concentration of electrolytes in the blood (mineralocorticoids) (reviewed in Connell et al. 2001). Sex hormones (androgens and estrogens) are produced by the gonads, also adrenals to some extent, and the brain (Schlinger & Arnold, 1991, 1992) and promote the development and determination of the reproductive system at the embryonic stage, masculinizing or feminizing the brain at birth, control reproduction, reproductive behavior, and the development of secondary sexual characteristics in adults (reviewed in Harding 2008). Sex differentiation is thought being regulated mainly by higher levels of estrogens in females and androgens in males, although both sexes have both (reviewed in Bear et al. 2007).

Due to their sterol nucleus, steroid hormones are too hydrophobic to dissolve in the blood and need to be transported on specific carrier proteins from their point of release to their target tissues where these hormones can bind to their respective receptors and affect the tissue via two alternative pathways depending on the location of these receptors (reviewed in Wehling 1997, Nelson and Cox 2008 and Harding 2008). In the classic genomic pathway, which may take several hours to days to cause hormonal effects, the hormone passes through the plasma membrane by simple diffusion and can bind to a specific receptor in the nucleus (reviewed in Nelson and Cox 2008 and in Harding 2008). Alternatively, in the non-classical signaling pathway, which happens on the scale of milliseconds to minutes, the steroids are thought to bind to its receptor or a non-specific protein or lipid located at the surface of the plasma membrane that mediates the activation of intracellular signaling cascades (reviewed in Wehling 1997). If there are indeed specific cell surface receptors for androgens and estrogens is a controversial issue, nevertheless, the non-classical signaling may not require the binding to a membrane receptor to play its seemly essential role in full responses of steroid hormones (Cooke & Walker, 2022; Cornil et al., 2006). Interestingly, in the classic genomic pathway, the intracellular steroid receptors act as ligand-regulated transcription factors, binding to DNA response elements and altering the transcription of specific genes and subsequently the production of proteins, including enzymes and other receptors (reviewed in Harding 2008) (Fig. 3a).

### *Testosterone*

Testosterone is the main androgen in mammals and birds and plays an essential role in the anabolic process and androgenic effects in males (reviewed in Cai et al. 2016 for mammals and in Gahr 2020b for birds). Testosterone is synthesized from cholesterol after five enzymatic reactions and released mainly by the testicles (reviewed in Cai et al. 2016). Testosterone can be converted into androgen 5α-dihydrotestosterone (DHT) by the enzyme 5α-reductase and into the estrogen 17β-estradiol (E2), which is the main estrogen released by the ovary, by the enzyme aromatase (reviewed in Frankl-Vilches and Gahr 2018). Testosterone and DHT bind to androgen receptors (AR) and the estrogen to any of both estrogen receptor α (ERα) and estrogen receptor β (ERβ) (reviewed in Frankl-Vilches and Gahr 2018) (Fig. 3b). Due to its greater molarity, DHT has a higher affinity to the AR and a stronger androgenic activity than testosterone (reviewed in Cai et al. 2016). As the enzymes that metabolize testosterone (aromatase and 5α-reductase) are expressed in certain brain regions, active androgens and estrogens can be produced not only in the gonads but also directly in the brain (reviewed in Gahr 2020b). Aromatization of testosterone to E2 in the brain is critical for the activation of innate sexual behavior in rats and birds, quails and zebra finches (Cornil et al., 2006; Vockel et al., 1990). Once secreted, testosterone enters the circulation and is transported to target tissues where it (or its 5a-reduced metabolite 5a-DHT) may bind with AR that then forms protein complexes with coactivators and corepressors to modulate gene transcription. Alternatively, after aromatization, testosterone might bind in form of E2 to the ERa and ERb. AR, respectively ERa and ERb, binds to specific DNA sequences located in the regulatory sequences of genes. However, it is still a matter of debate which genes of a particular genome can be activated by the AR or the ERs or both. Likewise, it is unclear if the pleiotropic effects induced by testosterone AR interaction and by E2 ERs interaction are related to the transcription of a few hub genes or to the parallel AR facilitated transcription of very many genes (Ko et al., in press).

### ***Testosterone dependence of singing***

In birds, often singing behavior is sexually dimorphic and functionally related to reproduction, thus, its development and adult differentiation are sensitive to sex hormones (reviewed in Gahr 2007). Vocalizations related to reproduction was restored in castrated males or induced in juveniles after testosterone implantation in unrelated bird groups such as galliformes (e.g.: domestic chickens - *Gallus gallus* Andrew 1963, Grey Partridge - *Perdix perdix* Fusani et al. 1994, and Japanese quail - *Coturnix japonica* Beani et al. 2000), columbiformes (e.g.: ring doves - *Streptopelia sp.* Adkins-Regan 1981), parrots (e.g.: budgerigars - *Melopsittacus undulatus* Brockway 1968), suboscines passerines (e.g.: golden-collared manakins - *Manacus vitellinus* Chiver and Schlinger 2019) and several songbird species (reviewed in Gahr 2020a).

Specifically, in vocal learners, sex hormones have organizational effects not only on singing behavior but also on the brain (reviewed in Bottjer and Arnold 1986); these effects can be dissimilar among species and result in different degrees of dimorphism ranging from high, for example, in zebra finches only male sing (R. A. Zann, 1996), to low, female canaries rarely sings (Ko et al., 2020). Therefore, some species, as zebra finch, have obligate and others, as canaries, have facultative sex-specific songs (Gahr, 2007). In both vocal learners and vocal non-learners, sex hormones have activational effects in adult singing (reviewed in Harding 2008). In most of the species in which only male sings and follow seasonal reproductive cycles, sex hormones, in this case mainly testosterone, become high in the reproductive season and facilitate singing among other reproductive behaviors (reviewed in Gahr 2020b). As prolonged high levels of testosterone may incur physiological ‘costs’, such as exposure to predators and loss of fat stores (Wingfield et al., 2001), some species with expanded reproductive seasons have reproductive behavior related alternatively to high levels of sex steroid precursors such as dehydroepiandrosterone (DHEA) (Hau et al., 2004).

The effect of testosterone on the singing behavior was experimentally demonstrated in numerous bird species by castration (reviewed in Gahr 2020a). After castration, adult males reduce their singing rate which is recovered after consecutive pharmacological implantation of testosterone (e.g. Arnold 1975). In adult females, such implantations induce masculinized song only in species with facultative sex-specific songs (e.g. Vallet et al. 1996). However, in the female zebra finch, a species with obligate sex-specific songs, the administration of estradiol (17β-estradiol or E2) early in the development induce singing (Gurney & Konishi, 1980). Whereas effects of testosterone in the singing behavior of females are species-specific, in males, testosterone affects at least the singing rate of all species tested to date (Gahr, 2020a). Further temporal and spectral properties of male song are also testosterone sensitive, but such as female singing, this sensitivity is species-specific (Gahr, 2020a). The testosterone dependence of singing in hummingbirds remained completely unknown.

Even in the tropics where seasons are not well marked, hummingbirds reproduce during a specific time of the year (Ruschi, 1964). It is, therefore, expected that males produce certain types of vocalizations only in the breeding season, whose, as in other bird taxa, are probably testosterone sensitive. I investigated the testosterone sensibility of male songs (or any other vocalization) as well as the masculinization of female vocal behavior under high testosterone levels in **Chapter 3**.

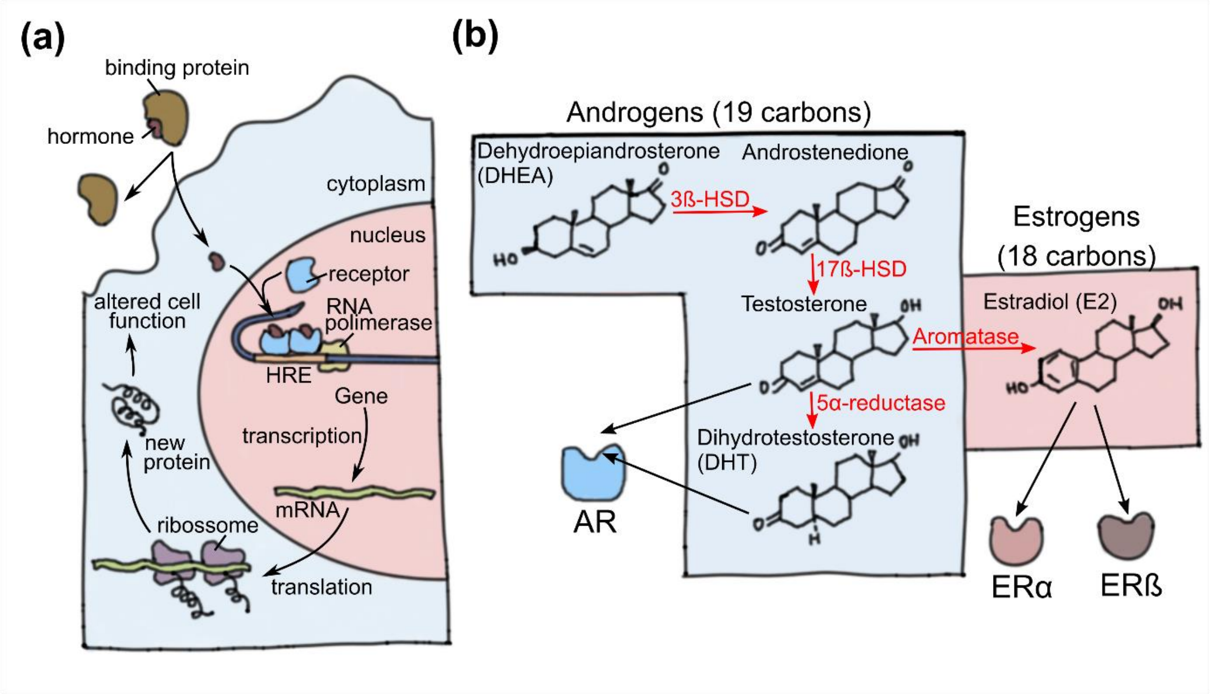

Fig. 3 – General mechanism of action and metabolism of sex steroids. (a) Representation of the main steps of the classic genomic pathway. The hormone is carried to the target tissue on a binding protein across the cytoplasm and binds to its specific receptor. The binding changes the conformation of the receptor which forms a receptor complex that binds to specific regulatory regions called hormone response element (HRE) in the DNA. These HREs are adjacent to specific genes that are regulated by the receptor. The receptor can attract either coactivator or corepressor proteins which increases or decreases, respectively, the mRNA formation rate of the adjacent gene. The mRNA can be translated and produce proteins that coordinate the cellular response to the hormone. Redraw from Nelson and Cox (2008). (b) Simplified diagram showing part of the general pathway for the synthesis of steroids sex hormones. The main androgen (blue box) is testosterone and estrogen (red box) is estradiol. In red are the enzymes and the red arrows indicated their respective metabolite which is represented in molecular form. Black arrows indicate the corresponding receptor which some of the metabolites can bind. Androgen receptor (AR), estrogen receptor α (ERα) and estrogen receptor β (ERβ) are depicted. Based on Häggström and Richfield (2014) and Frankl-Vilches and Gahr (2018).

## Neuronal basis of vocal production

The brain coordinates vocal production by sending motor commands to respiratory, vocal and upper vocal tract muscles (Wild, 1997; Zeigler & Marler, 2008). In birds, distinct brainstem nuclei directly control muscles of the syrinx (tracheosyringeal part of the hypoglossal motor nucleus, nXIIts) and indirectly control muscles of expiration (nucleus retroambigialis, RAm) and inspiration (nucleus parambigualis, PAm) (reviewed in Schmidt and Ashmore 2008). The nXIIts receive projections from a specific steroid-sensitive region of the mesencephalic nucleus intercollicularis (ICo), the dorsomedial subregion (DM) in the mesencephalon, which, in turn, receive afferents from thalamic and hypothalamic nuclei in the diencephalon such as the nucleus pretectalis (PT), ventromedial nucleus (VMN), anterior dorsomedial thalamic region (DMA), occipitomesencephalic tract (OM) and nucleus rotundus (nRt) (Briganti et al., 1996). These mesencephalic and diencephalic nuclei mediate the activation of vocal behavior in all birds including vocal learners and vocal non-learners (Gahr, 2000; Wild, 1997).

Birds perceive sounds through a specialized brain circuit, the auditory system, which shows many similarities to other vertebrates (reviewed in Theunissen et al. 2008; Fig. 4, areas in blue). Briefly, the auditory system is composed of afferents from the cochlea in the ear that projects to the cochlear nucleus in the medulla that converges in the dorsal lateral nucleus of the mesencephalon (MLd). The MLd project to the nucleus ovoidalis (Ov) in the thalamus which, in turn, sends projections to the primary auditory area in the pallium, the Field L (divided into subregions L1, L2a, L2b, L3 and L), more specifically to the subregions L2a and L2b. These subregions make bidirectional connections with the adjacent ones, then L1 and L3, make also bidirectional connections with two secondary areas in the pallium: the nidopallium caudal medial (NCM) and the caudal lateral mesopallium (CLM) which are interconnected via the caudal medial mesopallium (CMM) (reviewed in Theunissen et al. 2008).

### *Vocal control system*

Additionally to the vocal-respiratory and auditory systems, bird vocal learners have a set of anatomically discrete but interconnected nuclei in their forebrain that is involved in the process of vocal production learning, the so-called song control system (reviewed in Nottebohm 2005). This system, present only in vocal learners, is the basic mechanism to control vocal production and receives motor commands from the forebrain (Gahr, 2000; Nottebohm, 1991; Schmidt & Ashmore, 2008). In songbirds, these nuclei and their pathways are as well involved in calling (Ter Maat et al., 2014), thus, they are named broadly the vocal control system. Comparisons among songbirds, parrots, and hummingbirds revealed striking similarities in the neuroanatomy of their vocal control system considering where they occur in the brain (Jarvis and Mello 2000, Jarvis et al. 2000, Gahr 2000; Fig. 4). This similarity is explained by some researchers as convergent evolution of the vocal control system from a pre-existing motor pathway that controls movement which is common to all birds (Feenders et al., 2008). However, this view is highly controversial.

The vocal control system of songbirds includes two nuclei that compose a motor, or posterior, pathway (HVC, proper name and the RA, robust nucleus of the arcopallium) which makes a direct projection from the arcopallium onto the vocal motoneurons in the brainstem and four nuclei that compose an anterior pathway loop which interconnect the nidopallium (HVC and the LMAN, lateral magnocellular nucleus of the anterior neostriatum) with the striatum (Area X) and thalamus (DLM, nucleus of the dorsolateral thalamus) which, in turn, projects back to the nidopallium (reviewed in Brainard and Doupe, 2002). Whereas the motor pathway is necessary for normal song production throughout life, the anterior pathway is important for vocal production learning early in life and song plasticity in adult birds (reviewed in Farries and Perkel, 2008; Fig. 4). HVC is part of both pathways, when bilaterally lesioned, the song is eliminated, whereas when RA is lesioned, song is only partially disrupted (Bottjer & Arnold, 1986). LMAN is the main nucleus of the anterior pathway, when lesioned in deaf birds, the song is not affected, however, lesions in intact birds make the song less variable (Brainard & Doupe, 2000). Thus, the anterior pathway plays a role in the modulation of song even in adult closed-ended learners (reviewed in Brainard and Doupe 2002).

Although convergently evolved, the vocal control nuclei of parrots and hummingbirds differ from that of songbirds in cytoarchitecture and connections, therefore, the nuclei are named differently (Gahr, 2000; Jarvis et al., 2000; Striedter, 1994). In parrots, the vocal control system also includes nuclei that compose a posterior pathway (NLC, central nucleus of the lateral nidopallium and AAC, central nucleus of the anterior arcopallium) and an anterior pathway (MO, oval nucleus of the mesopallium; NAO, oval nucleus of the anterior nidopallium; MMSt, magnocellular nucleus of the anterior striatum and DMm, magnocellular nucleus of the dorsomedial thalamus) (reviewed in Jarvis 2007). However, some aspects seem unique in parrots: additionally to the projection to tracheosyringeal portion (nXIIts), AAc projects to the lingual portion of the hypoglossal nucleus (Striedter, 1994) and all nuclei have an anatomically distinct subdivision in the ventral part with distinct connectivity, the “shell” system, which function is unclear but suggested to be related to parrots’ specific cognitive abilities (Chakraborty et al., 2015). In hummingbirds, the vocal control system also includes a posterior pathway (VLN, vocal nucleus of the lateral nidopallium and VA, vocal nucleus of the arcopallium) which projects to the brainstem and an anterior pathway (VAN, vocal nucleus of the anterior nidopallium and VASt, vocal nucleus of the anterior striatum) (Gahr, 2000; Jarvis et al., 2000). Interestingly in hummingbird, the vocal control system of males in two close-related species seem vestigial when delimited by androgen receptor similar to non-singing females of songbirds (Gahr, 2000). It remains unknown whether this pattern is restricted to this group of hummingbirds, therefore, in Chapter 4 I used androgen receptors to delimitate the vocal control system of phylogenetically distant species and to confirm these delimitations, I used the functional activation of an immediate early gene immediate (EGR-1) of a species that belongs to the radiation evolved in the lowlands of South America in Chapter 5.

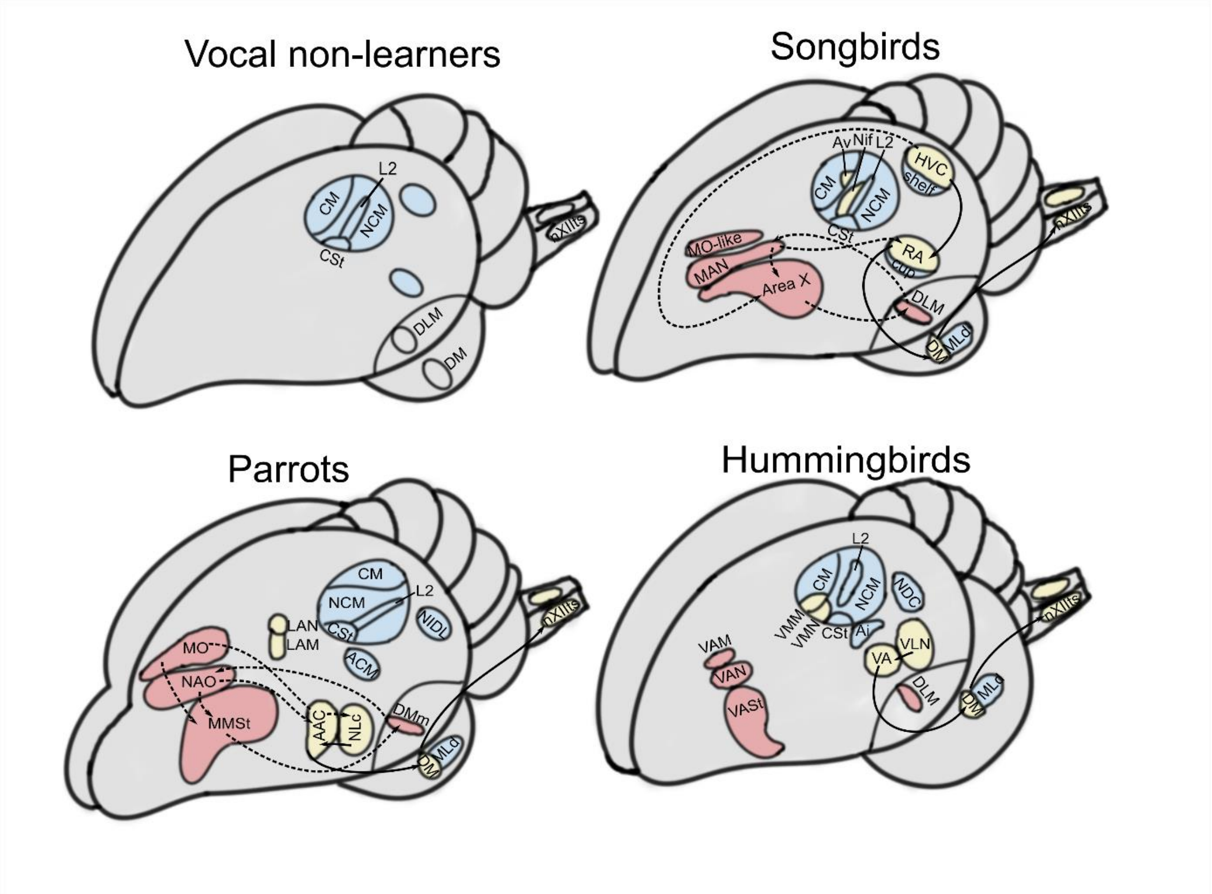

Fig. 4 – Vocal respiratory, auditory and vocal control systems in birds. The vocal control system is an exclusivity of vocal learners (songbirds, parrots and hummingbirds). In blue are the nuclei of the auditory system, in yellow are those of the posterior or motor pathway and in red of the anterior pathway that, together with the former, compose the vocal control system. The full lines indicate connections of the posterior pathway and dashed lines of the anterior pathway. Redraw from Jarvis (2007) and Bruno et al. (2020). AAC, central nucleus of the anterior arcopallium; ACM, caudal medial arcopallium; Ai, intermediate arcopallium; Area X, area X of the striatum; Av, avalanche; CM, caudal mesopallium; CSt, caudal striatum; DLM, medial nucleus of dorsolateral thalamus; DM, dorsal medial midbrain; DMm, magnocellular nucleus of the dorsomedial thalamus; HVC, vocal control area (proper name); HVC shelf, shelf of cells below HVC; L2, field L2; LAN, lateral nucleus of the anterior nidopallium; LAM, lateral nucleus of the anterior mesopallium; MAN, magnocellular nucleus of anterior nidopallium; MLd, mesencephalic lateral dorsal nucleus; MMSt, magnocellular nucleus of the anterior striatum; MO, oval nucleus of the mesopallium; MO-like, similar to the oval nucleus of the mesopallium; NCM, nidopallium, caudal medial; NDC, caudal dorsal nidopallium; NIDL, intermediate dorsal lateral nidopallium; NIf, interfacial nucleus of the nidopallium; NLC, central nucleus of the lateral nidopallium; nXIIts, tracheosyringeal subdivision of the 12th nucleus; RA, robust nucleus of the arcopallium; RA shelf, shelf of cells below RA; VA, vocal nucleus of the arcopallium; VAM, vocal nucleus of the anterior mesopallium; VAN, vocal nucleus of the anterior nidopallium; VASt, vocal nucleus of the anterior striatum; VLN, vocal nucleus of the lateral nidopallium; VMM, vocal nucleus of the medial mesopallium; VMN, vocal nucleus of the medial nidopallium.

### *Hormone sensitivity of the vocal control system*

Despite the overall similarities among species, the vocal control system seems to undergo striking structural species-specific and individual changes during development and adulthood (Frankl-Vilches & Gahr, 2018). These changes are consequence of the organizational (development) and activational (adulthood) effect of sex hormones in the brain (Schlinger & Arnold, 1991). Androgen (AR) and estrogen receptors (ER) are widely distributed in the brain (reviewed in Frankl-Vilches and Gahr 2018). AR is present in nearly all forebrain nuclei of the vocal control system, except for Area-X showing strong inter-individual variation, some nuclei of the auditory system (e.g. NCM), diecephalon (e.g. MLd, nucleus mesencephali lateralis, pars dorsalis) and brainstem (e.g. nXIIts; reviewed in Gahr 2020b). In contrast to AR, ER presence in the vocal and auditory circuits is more restricted. For example, while ERα was found in the NCM of zebra finches, canaries and starlings, ERβ was found only in the NCM of starlings (reviewed in Frankl-Vilches and Gahr 2018 and in Gahr 2020b). AR and ER in the nuclei of the vocal control system in the forebrain are unique to vocal learners (reviewed in Frankl-Vilches and Gahr 2018).

During development, the vocal control system undergoes sex differentiation. The degree of differentiation can vary depending on the species (reviewed in Gahr, 2007). In zebra finches, for example, only males sing and adult females have a vestigial HVC (Bottjer et al., 1985), yet females can develop a masculinized song and HVC if they receive 17ß-estradiol (E2) implantation early in life (Gurney & Konishi, 1980). In canaries, females rarely sing (Ko et al., 2020) and their HVC is strikingly smaller than males (Nottebohm & Arnold, 1976), however, adult females produce masculinized song and HVC after testosterone implantation (Nottebohm, 1980). In blue-capped cordon-bleus, females sing almost as much as males and their HVC is moderately smaller than males (Lobato et al., 2015).

During adulthood, the vocal control system undergoes annual changes in males of some seasonally breeding birds. For example in white-crowned sparrow (*Zonotrichia leucophrys*), HVC, RA and Area X, become larger during the breeding season (longer days) when males sing more than during the non-breeding season (shorter days) when singing declines (Nottebohm 1981; Tramontin et al. 2003). However, this concept of seasonal growth was questioned by detailed analysis of this process in other seasonal species such as the canary (Gahr, 1990, 1997). HVC reorganization during the breeding season can even take place in equatorial songbirds where the difference between the longest and shortest day of the year can be as little as 20 minutes (Quispe et al., 2016). Which organization level may be affected by sex hormones in a particular brain area, e.g. the RA, is likely species-specific but little studied, for example, testosterone affects dendritic morphology in the RA but not HVC of canaries (Vellema et al., 2019) but this has not been studied in other species. Likewise, testosterone induces a generalized restructuration in HVC by affecting, in nearly all cell types, the expression of more than half of the protein-coding genes of the canary genome (Ko et al., 2022) In non-singing female zebra finches, it is the auditory nucleus NCM that undergoes some life stage dependent changes by showing the increased amplitude of neural responses when estrogen levels are high which probably leads to faster replies to their mates during vocal interactions (Adreani et al., 2020).

ARs were found in the vocal control system of all vocal learners investigated to date (reviewed in Frankl-Vilches and Gahr 2018). These constituted at least 19 songbird species (Brenowitz & Kroodsma, 1996), the most studied parrot, the budgerigar (*Melopsittacus undulatus*) (Matsunaga & Okanoya, 2008) and two hummingbird species, Anna's and Amazilia hummingbird (Gahr, 2000). Although the vocal control system shows similarities in morphology and chemical properties across taxa (reviewed in Brenowitz and Kroodsma 1996), these taxa have dissimilar learning phenotypes considering sexual dimorphism, adult song plasticity (Beecher & Brenowitz, 2005) and also in how much of the song is actually learned which is unrelated to phylogeny (Love et al., 2019). It is expected that the hormone sensitivity of the vocal control system is a universal mechanism among vocal learners to modulate the different learning phenotypes, however, the understanding of this mechanism is limited to the studies on a couple of temperate songbirds which may provide a rather limited overview of the possible phenotypes and their modulation. Therefore, the study of hormone sensitivity both in singing behavior and in the vocal control system in non-conventional model systems, in special those with different life stories and physiology such as hummingbirds, has the potential to expand our understanding of sex hormone role on the modulation of multiple learning phenotypes. Thus, the hormone sensitivity of singing (**Chapter 3**) and brain (**Chapter 4**) in an early-branched species such as the black jacobin is the focus of the last chapters of this thesis.

## Natural history of hummingbirds

The hummingbird family (*Trochilidae*) is a radiation (McGuire et al., 2014) composed of 354 species that inhabit the Americas (Winkler et al., 2020). Previously, due to the lack of knowledge about the relationships within the family, the hummingbird family was crudely divided into two subfamilies: *Phaethornithidae*, the hermits with 34 species, and *Trochilinae*, the typical hummingbirds with the remaining species, (Schuchmann, 1999). More recently, McGuire et al. (2014) proposed a time-calibrated phylogeny strongly supported by molecular markers that cohere with geographic events and the distribution of the species. This phylogeny divides the hummingbird family into nine major clades: Topazes (*Topazini*), Hermits (*Phaethornithinae*), Mangoes (*Polytmini*), Coquettes (*Lophoornithini*), Brilliants (*Coeligenini*), Patagona (*Patagonini*), Mountain Gems (*Lampornithini*), Bees (*Mellisugini*) and Emeralds (*Trochilini*) (McGuire et al., 2009, 2014). Six out of nine major clades of hummingbirds (Topazes, Hermits, Mangoes, Coquettes, Brilliants, and Patagona) are nearly entirely composed of species restricted to South America, two clades (Coquettes and Brilliants) of species restricted to the Andes Mountains and one (Bees) to North America (McGuire et al., 2014).

The stem hummingbirds evolved outside of the current geographic range of the family, in Eurasia, where fossils dated from between 34 to 28 mya were found (Mayr, 2004). Then, at about 22.4 million years ago, hummingbirds ocupied the lowlands of South America where they first diversified. More 'recently', about 12 million years ago, the common ancestor of the Bee and Mountain Gem clades ocupied North America where a rapid species accumulation happened during the past five million years ago; at this time the Panamanian uplift was formed and favored multiple independent ocupations by Emeralds, Coquettes, Mangoes, and Hermits and single ocupation by Brilliants and Topazes via the Panamanian land bridge. Also about five million years ago, hummingbirds ocupied the Caribbean (McGuire et al., 2014). Between 10 and 2 million years ago, another uplift, this time that of the Andes Mountains, is suggested to have played a dominant role in the diversification of the South American hummingbirds because in the Andes there are at least 140 species which corresponds to 40% of contemporary hummingbird diversity (McGuire et al. 2014; Fig. 5). The success of the Andean radiation was favored by the lack of natural predators and the independent evolution of hemoglobin with enhanced oxygen-binding properties so these species can thrive in the oxygen-poor environment characteristic of high altitudes (Projecto-Garcia et al., 2013).

The split of the hummingbird family (*Trochilidae*) from their closer sister families swift (*Apodidae*) and treeswift (*Hemiprocnidae*) was suggested to have happened approximately 42 million years ago (McGuire et al., 2014). The phylogenetical proximity of hummingbirds, swifts and treeswifts – traditionally, the order *Apodiformes* – is supported by morphological features, molecular markers, cytogenetics and whole genomic studies (Mayr, 2002). Considering the morphological features, myology and osteology were the most explored. The study of Zusi and Bentz (1984) proposed that swifts and hummingbirds present several similarities in the muscles of the jay, abdomen, wing and especially neck. For example, two morphological synapomorphies of Apodiformes are neck muscle *musculus splenius capites* related to neck movement during the insect capture in fast flights (Zusi, 2013) and a unique quadrate-squamosal articulation in their skull (Cracraft, 1981). More recently, an avian phylogeny based on the whole genome proposed that hummingbirds are sister taxa of the swift family (*Apodidae*) and both are close related to the nightjar family (*Caprimulgidae*) that together compose the order *Caprimulgiformes* embedded in the Neoaves (Jarvis et al., 2014). Another avian phylogeny based on 259 nuclear loci was consistent with the relationship between swift and hummingbird families, but grouped them in the order *Apodiformes* suggested being sister taxa of the nightjar family which together compose the Strisores clade placed at the base of the Neoaves branch (Prum et al., 2015). Recently, an avian phylogeny based on noncoding 3-prime untranslated region (30-UTR) sequences resolved the relationship at a family level and placed the hummingbird family in the Caprimulgiformes order embedded in the Neoaves (Kuhl et al., 2020). Interestingly, although swift and treeswift are close related to hummingbirds, only hummingbirds evolved the brain specializations related to vocal production learning (Gahr, 2000).

Hummingbirds are phylogenetically distant from the songbirds and parrots (Kuhl et al., 2020; Prum et al., 2015) which supports the idea that vocal production learning evolved independently in the group and most likely after the invasion of South America about 22 million years ago. However, it remains unclear whether this evolution happened in the common ancestor of all hummingbirds or independently in a few clades because of the evidence for vocal production learning in Topazes, the sister clade of all other clades, is still lacking. Therefore, the evidence for vocal production learning over a phylogenetic framework is the subject of the first chapter (**Chapter 1**) and morphological (**Chapter 2**), endocrinological (**Chapter 3**) and neuroanatomical (**Chapter 4 and 5**) basis of vocal communication were conducted on model systems from the clades closer to the root of the hummingbird phylogeny. In this thesis, I investigated the fundaments of vocal communication in hummingbirds to pave the way in understanding the intriguing evolution of vocal production learning in such specialized birds in many aspects from diet to metabolism as hummingbirds are. Hummingbirds can potentially unveil overlooked aspects about not only the evolution but the physiological mechanisms underlying vocal production learning.

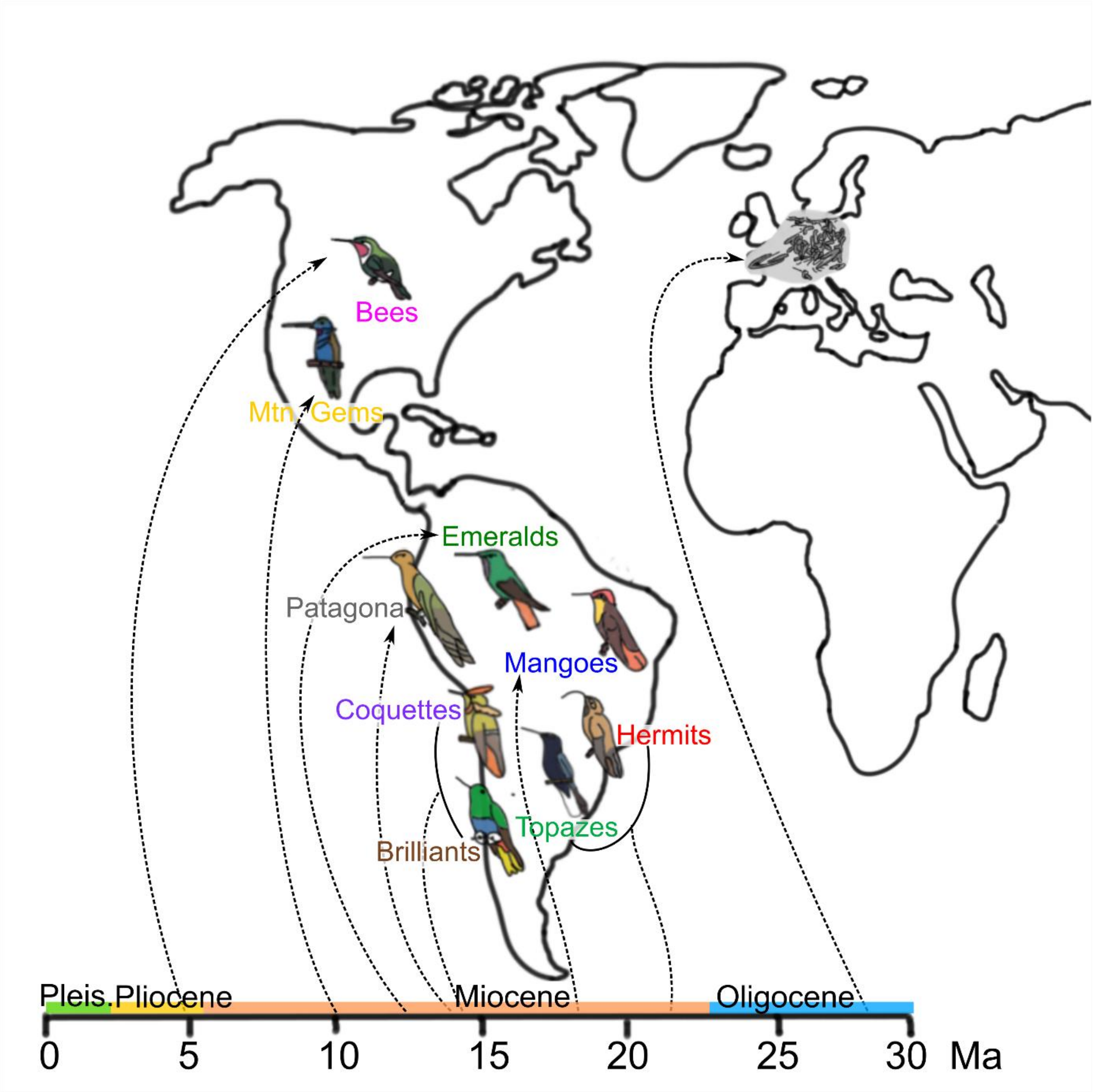

Fig. 5 – Natural history of the Trochilidae family proposed by McGuire et al. (2014). Each of the nine major clades is represented by a drawing. On the bottom, a scale represents the time before the present in million years (Ma) and the respective geological eras (Miocene in orange; Pliocene in yellow and Pleistocene in green). On the top, is a drawing of a partial world map. The arrows indicate when (time scale) and where (world map), according to the phylogeny of McGuire et al. (2014), the clade or the common ancestor of two clades originated. Topazes, Hermits, Mangoes and Emeralds in South America; Coquettes, Brilliants and Patagona in the Andes Mountains, and Mountain Gems (Mtn. Gems) and Bees in Central and North America. A fossil of a stem hummingbird was found in Germany and is dated from between 34 to 28 million years ago (Mayr, 2004). The drawing was modified from McGuire et al. (2014) and the fossil from Mayr (2004).

# Chapter 1

# Absence of song suggests heterogeneity of vocal-production learning in hummingbirds

## Abstract

Hummingbirds have been recognized, along with songbirds and parrots, as capable of learning songs. However, it is still unclear whether singing can be treated as a homologous trait within the family, analogous to songbirds. Therefore, we systematically compared the information about hummingbird vocalizations in the literature in a phylogenetic framework. In general, songs were emitted by perching males in a reproductive context, while calls were mainly in agonistic contexts. Singing was ancestral in most of the nine major hummingbird clades, but has been lost at least once in the mountain gem clade and twice in the bee clade. This evolutionary loss of singing might suggest heterogeneity of vocal-production learning.

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SHORT COMMUNICATION

# Absence of song suggests heterogeneity of vocal-production learning in hummingbirds

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## Abstract

Hummingbirds have been recognized, along with songbirds and parrots, as capable of learning songs. However, it is still unclear whether singing can be treated as a homologous trait within the family, analogous to songbirds. Therefore, we systematically compared the information about hummingbird vocalizations in the literature in a phylogenetic framework. In general, songs were emitted by perching males in a reproductive context, while calls were mainly in agonistic contexts. Singing was ancestral in most of the nine major hummingbird clades, but has been lost at least once in the mountain gem clade and twice in the bee clade. This evolutionary loss of singing might suggest heterogeneity of vocal-production learning.

**Keywords** Evolution · Vocal communication · Vocal learning · Ancestral state estimation · *Trochilidae*

## Zusammenfassung

**Das Fehlen des Gesangs deutet auf Heterogenität beim Gesangslernen von Kolibriarten hin.** Kolibris sind ebenso wie Singvögel und Papageien in der Lage, Gesänge zu lernen. Es ist jedoch unklar, ob das Singen als ein homologes Merkmal innerhalb der Familie der Kolibris zu sehen ist, analog zum Singen der Singvögel. Daher haben wir die in der Literatur vorhandenen Informationen über Kolibri-Gesänge systematisch in einem phylogenetischen Rahmen verglichen. Im Allgemeinen sangen die dabei in der Regel sitzenden Männchen in einem reproduktiven Kontext, während das Rufen hauptsächlich in agonistischen Kontexten stattfand. Der Gesang war in den meisten der neun großen Kolibri-Gruppen evolutiv ursprünglich vorhanden, ist aber mindestens einmal bei Arten der „moutain gem “-Gruppe und zweimal in der „bee “-Gruppe verloren gegangen. Dieser evolutionäre Verlust des Gesangs deutet auf eine Heterogenität beim vokalen Lernen der Kolibris hin.

## Introduction

For communication, birds use a range of species-specific sounds uttered in specific behavioral contexts, arbitrarily divided into two mutually exclusive subgroups: songs and calls (Spector 1994; Catchpole and Slater 1995). In most studies referring to temperate songbirds, the term “song” is used to the elaborate vocalizations emitted by males in the context of reproduction to defend their territory and attract females (Catchpole and Slater 1995). In studies mainly referring to tropical birds, "song" is the vocalization that, among other functions, primarily serves species-specific recognition (Vielliard 1987; Mathevon et al. 2008). In contrast, "call" has always been a broader term, often used for all non-singing vocalizations, such as those used to maintain contact, beg for food, and alarm conspecifics (Catchpole and Slater 1995). The definition of song is of particular interest because there are numerous examples of song learning in songbirds, but very few examples of call learning (Zann 1990). Therefore, song in songbirds is usually considered a learned behavior. Although hummingbirds are less studied, there is compelling evidence of song learning (e.g.: Jarvis et al. 2000; Gahr 2000; Araya-Salas and Wright 2013; Johnson and Clark 2020); however, a direct comparison to songbirds and within hummingbirds may come across a homology

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problem given by the possibility that different authors may have relied on different definitions of song. We investigated this problem in here first, then analyzed the evolution of singing in hummingbirds under the light of the most recent phylogeny (McGuire et al. 2014).

## Methods and results

### Singing in hummingbirds is functionally equivalent to songbirds

First, we gathered information from the literature on the vocalizations of hummingbirds to investigate which are classified as songs or calls and in which context they occur. In our survey, we reviewed 74 publications, from which we extracted 98 vocalizations described as songs and 125 as calls. This comprised 78 species (22% of the 363 species) and three subspecies (Table S1) representing 47 genera (42% of the 112 genera). The hummingbird family (*Trochilidae*) was traditionally subdivided into two subfamilies (*Phaethornithinae* and *Trochilinae*) (Hoyo et al. 1999); however, the most updated phylogeny suggests nine clades (eight tribes and one subfamily) (McGuire et al. 2009). Here, we follow the subdivisions suggested by McGuire et al. (2014) based on a multilocus (six genes and five loci) phylogenetic estimate which recognized nine clades. We sampled eight of these clades: topazes (three species, 75% of the total of 4 species in the clade); hermits (12 species, 30.8% of the total); mangoes (9 species, 32.1%); coquettes (7 species, 10.4%); Patagona (single species clade); mountain gems (3 species, 17.6%); bees (17 species, 48.6%) and emeralds (26 species, 22.6%) (McGuire et al. 2014; Winkler et al. 2020). Then we grouped the references by phylogenetic clades and applied a text mining method using the packages “dplyr” (Wickham et al. 2020), “tidytext” (Silge and Robinson 2016) and “wordcloud” (Fellows 2018) in R (R Core Team 2018). We obtained word clouds for seven of the nine major clades except for the little-studied Patagona and Brilliant clades. Details on the literature survey and word mining are in the Supplementary Material.

The most commonly used terms to describe the behavioral context of songs in all clades were "perch(ed)", "male(s)" and "display" and of calls were "agonistic" and "aggressive", except in hermits, whose calls are emitted mainly in flight ("flight" and "flying", most frequently used words) (Fig. 1, right column). We know from songbirds that perching songs emitted by males are frequently associated with reproductive contexts (Catchpole and Slater 1995), and this seems to be the same for hummingbirds. Although inter-species comparisons of hummingbird show strikingly different levels of temporal–spectral complexity in their songs (Fig. 1, left column), song appears to be functionally equivalent within the family.

### Singing is ancestral, widespread, but not ubiquitous

Since the term "song" was used consistently in the analyzed publications, we used these references to investigate the evolution of singing in the hummingbird family. Seventy-one of the 78 included species and three subspecies were described as singing and 7 species (9%) as non-singing (Fig. 2, green and red circles). The sex of singing species was unknown in 54%, only males sang in 38%, and both sexes sang in 8% (six species, Fig. 2, venus symbol). Singing females are scattered throughout the phylogeny, thus, it is probably overlooked rather than rare. Likewise, non-singing species may be underrepresented since it is not attractive to report negative observations such as “non-singing”. Being aware of these limitations, we, nevertheless, estimated the probabilities of singing occurring in the clades of the phylogeny (McGuire et al. 2014) using the maximum-likelihood method on the equal rate (ER) model and all different rates (ARD) model provided by the R package “ape” (Paradis and Schliep 2019). The ARD model had a better fit to the data compared to the ER model ($LR = 7.12$, $df = 1$, $p < 0.01$). Thus, gains and losses of singing behavior within hummingbirds might have happened at different rates. The ARD model estimated higher transition rates for gains ($0.034 \pm 0.016$) than for losses ($0.003 \pm 0.002$). We obtained the posterior probabilities of the ARD model for singing to estimate the ancestral conditions of the hummingbird family and the clades within the family using the R package “phylotools” (Revell 2012). Support values above 70% are in general thought to be acceptable. Details on ancestral character estimation are provided in the Supplementary Material. In the hummingbird family as a whole, the probability of singing as an ancestral trait (92.6%) is higher than the non-singing (7.4%). Within the family, singing is probably, except for bees (69.1%), the evolutionary ancestral condition in topazes (94.2%), hermits (92.2%), mangoes (92.8%), coquettes (92.1%), mountain gems (90.9%) and emeralds (92.2%). Within bees, the ARD model estimated at least two evolutionary losses of song in the ancestor of the genus *Archilochus* (96.4%) and the common ancestor of the genera *Selasphorus* and *Atthis* (100%). As species in the genus *Atthis* sing, the model estimated a recovery (100%) in ancestors of this genus. Regardless, further loss of song occurred within mountain gems (Fig. 2, pie charts).

The presence of song in hummingbirds appears to correlate with vocal-production learning (Fig. 2, white circles, triangles and squares), as is also the case in songbirds (Kuhl et al. 2020), and evolutionary loss of song with degenerate

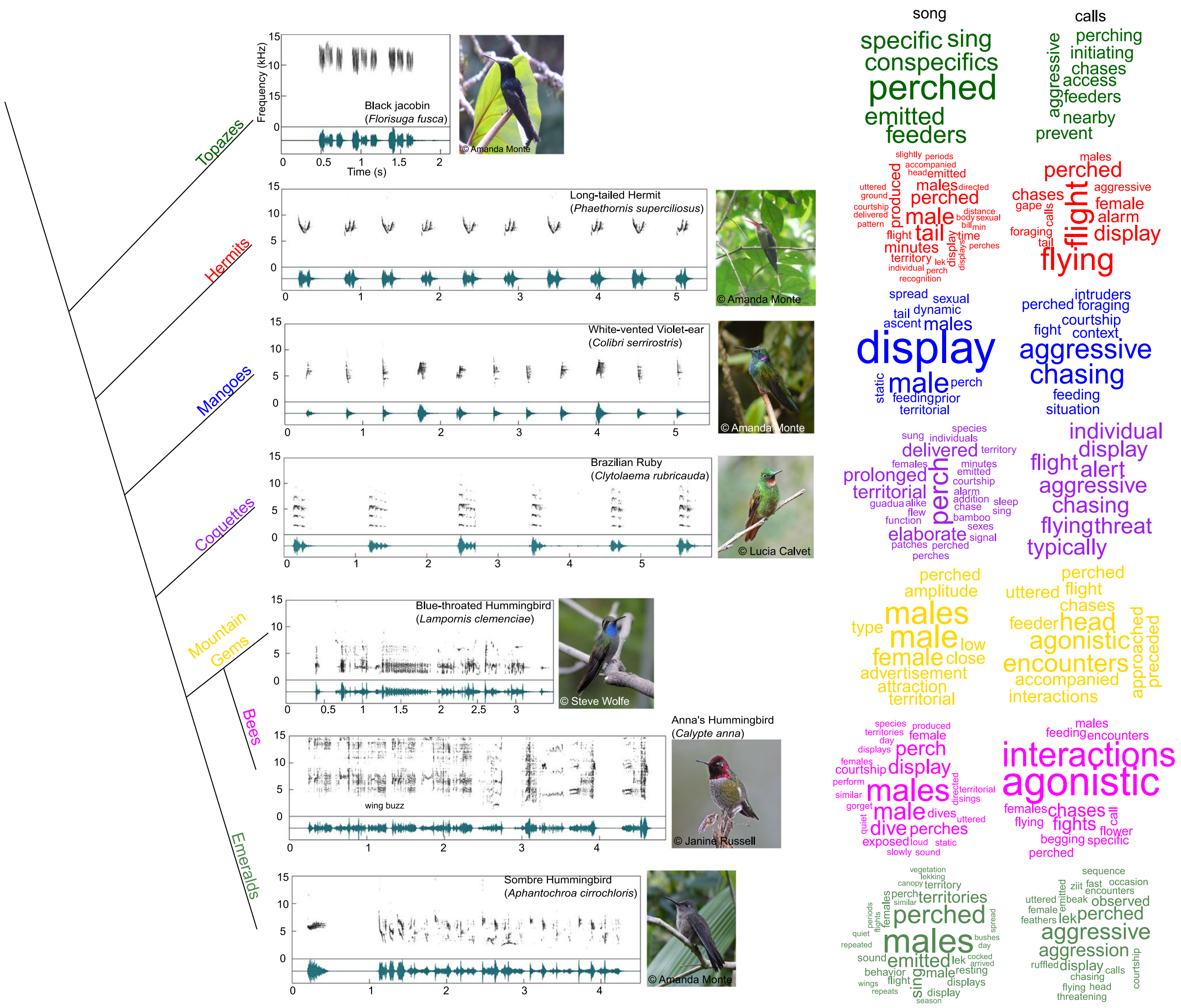

**Fig. 1** Songs in hummingbirds are emitted mainly by perched males and calls mainly in an aggressive context. This result was derived from word cloud analysis containing a maximum of 100 words used at least twice in the literature to describe hummingbirds' songs and calls grouped by clade. The clades are represented in different colors (topazes, dark green; hermits, red; mangoes, blue; coquettes, purple; mountain gems, yellow; bees, pink and emeralds, green). Bigger font sizes in each group represent the word that was repeated the most. Songs of seven of the nine clades are represented by one species and highlighted in different colors. Examples of songs are represented by spectrograms. The recordings were obtained from or deposited in the online sound library xeno-canto (https://www.xeno-canto.org/), except for that of the black jacobin (*Florisuga fusca*), which is available only in our archive. The catalog number of the recordings are as follows: long-tailed hermit (*Phaethornis superciliosus*) (Amanda Monte, XC454960), white-vented violetear (*Colibri serrirostris*) (Luiz C. Silva, XC138158), Brazilian ruby (*Clytolaema rubricauda*) (Jayrson Araujo de Oliveira, XC469392), blue-throated hummingbird (*Lampornis clemenciae*) (Richard E. Webster, XC324498), Anna's hummingbird (*Calypte anna*) (Paul Marvin, XC448778), sombre hummingbird (*Aphantochroa cirrochloris*) (Amanda Monte, XC454963). The xeno-canto recordings are shared under the Creative Commons License (CC BY-NC-SA). The hummingbirds' pictures were reproduced with permission from the rights owners: black jacobin, long-tailed hermit, white-vented violetear, and sombre hummingbird (Amanda Monte); Brazilian ruby (Lucia Calvet); blue-throated hummingbird (Steve Wolfe) and Anna's hummingbird (Janine Russell)

development of the putative vocal control system (Fig. 2, crossed squares), a phenotype similar to that of female songbirds unable to produce learned songs (Gahr 2000) (Table 1).

## Discussion

Hummingbird songs are mainly emitted by males perched on exposed twigs probably because, in several species, males aggregate into singing assemblies (leks) (Stiles

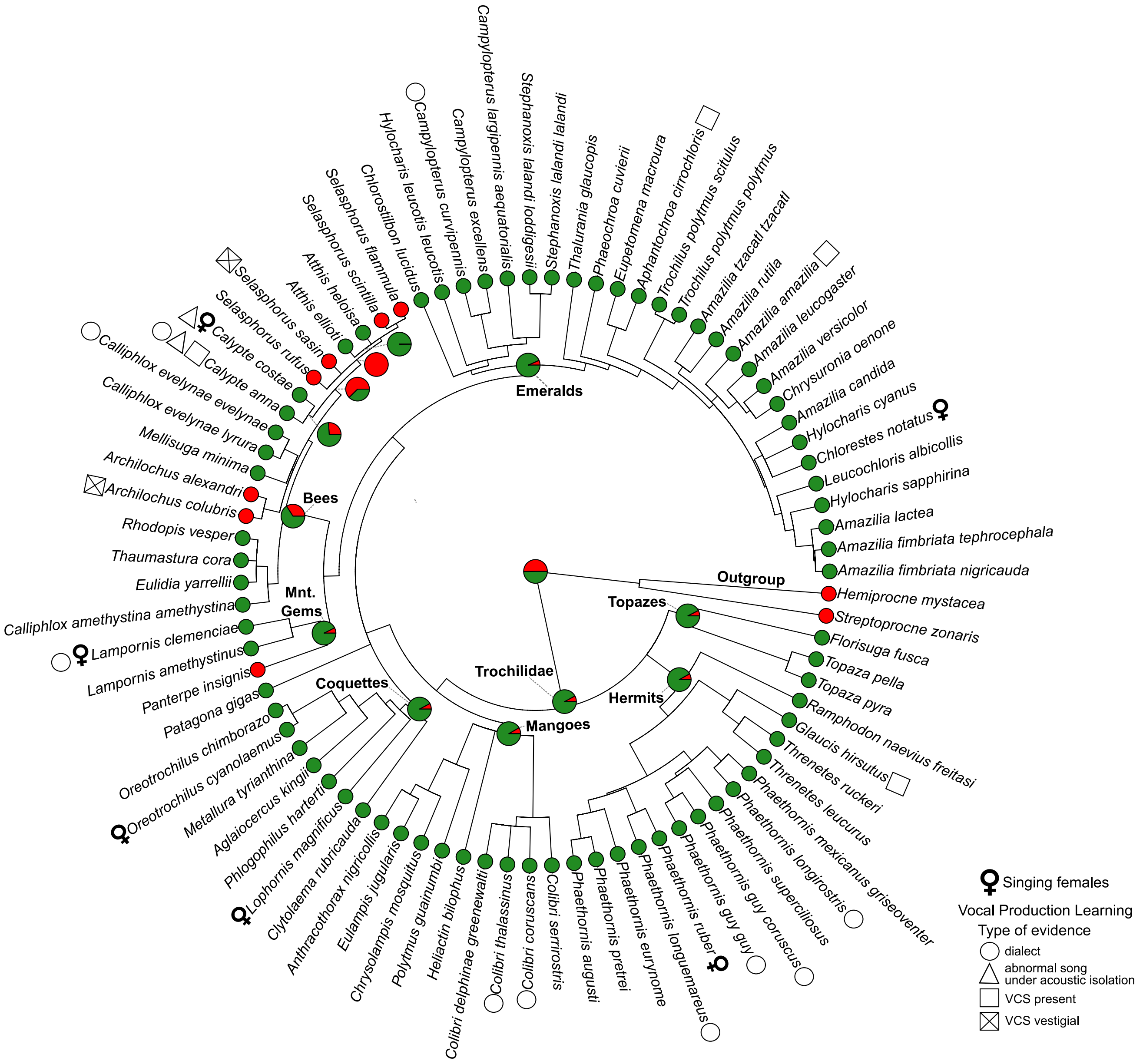

**Fig. 2** Ancestral character estimation of singing behavior in hummingbirds. Evolutionary history of the singing behavior in a sample of 78 hummingbird species (22% of the family) and three subspecies on a phylogenetic tree of the hummingbird family (adapted from McGuire et al. 2014). The outer circle indicates the prevalence of singing. Note that singing behavior is heterogeneously distributed within the hummingbird family. The alternate rates (ARD) model was used to estimate the ancestral character for each node. The area of pie represents the proportion given by the Bayesian posterior probability that the ancestral condition in the node is associated with one of two alternative conditions' presence (green) or absence (red) of singing. Singing behavior was gained, lost, and regained within the hummingbird family. The venus symbol indicates species with singing females. The type of evidence of vocal-production learning (VPL) is also represented: the presence of dialect (circle), the abnormal development of song under experimental acoustic isolation (triangle), the presence of a putative vocal control system (VCS) (square), and vestigial VCS (crossed square). Note that Anna's hummingbird accumulates multiple pieces of evidence for VPL

and Wolf 1979; Martínez-García et al. 2013; Araya-Salas and Wright 2013). Using a broad definition of lek as the mating system in which males gather in communal display grounds for the sole purpose of competing for mates (Payne 1984), lekking behavior is widespread among hummingbirds (reviewed in Martínez-García et al. 2013). Lekking species often have vocal dialects that, in some of them, cannot be explained by genetic differentiation (González and Ornelas 2014, 2019), but rather by cultural transmission. Thus, vocal dialects are also evidence for

**Table 1** Evidence of vocal-production learning (VPL) with respective references

| Clade | Species | Evidence VPL | Reference VPL |
|---|---|---|---|
| Hermits | *Glaucis hirsutus* | VCS present | Jarvis et al. (2000) |
| Hermits | *Phaethornis longuemareus* | Dialect | Snow (1968), Wiley (1971) |
| Hermits | *Phaethornis ruber* | Dialect | Davis (1958) |
| Hermits | *Phaethornis eurynome* | Dialect | Vielliard (1983) |
| Hermits | *Phaethornis longirostris* | Dialect | Araya-Salas and Wright (2013) |
| Hermits | *Phaethornis guy guy* | Dialect | Snow (1977) |
| Hermits | *Phaethornis guy coruscus* | Dialect | Snow (1977) |
| Mangoes | *Colibri coruscans* | Dialect | Gaunt et al. (1994) |
| Mangoes | *Colibri thalassinus* | Dialect | Gaunt et al. (1994)<br>Lara et al. (2015) |
| Mountain Gems | *Lampornis clemenciae* | Dialect | Ficken et al. (2000) |
| Bees | *Archilochus colubris* | VCS vestigial | Gahr (2000) |
| Bees | *Calliphlox evelynae evelynae* | Dialect | Feo et al. (2015) |
| Bees | *Calypte anna* | Dialect<br>VCS present<br>Abnormal song under acoustic isolation | Mirsky (1976), Yang et al. (2007), Gahr (2000), Baptista and Schuchmann (1990) |
| Bees | *Calypte costae* | Abnormal song under acoustic isolation | Johnson and Clark (2020) |
| Bees | *Selasphorus sasin* | VCS vestigial | Gahr (2000) |
| Emeralds | *Campylopterus curvipennis* | Dialect | González and Ornelas (2005, 2009, 2014) |
| Emeralds | *Aphantochroa cirrochloris* | VCS present | Jarvis et al. (2000) |
| Emeralds | *Amazilia amazilia* | VCS present | Gahr (2000) |

Pieces of evidence of VPL are the occurrence of similarities in the song of a certain population that is attributed to natural intraspecific vocal imitation ("Dialect"); the abnormal vocal development under acoustic deprivation observed under experimental conditions ("Abnormal song under acoustic isolation"), and the presence of brain nuclei related to VPL, the vocal control system (VCS) ("VCS present"), which is vestigial in two species ("VCS vestigial")

vocal-production learning (Kroodsma and Baylis 1982; Araya-Salas and Wright 2013) (Table 1).

It is, therefore, possible that males of a lek use dialects to acoustically distinguish neighbors, which are most likely to hold singing territories with stable boundaries within the leks, from newcomers, which are probably seeking a singing territory. Thus, territorial males can use this information to modulate their aggression during territorial responses, according to the risk of the threat. This hypothesis predicts two selective benefits: (1) males can increase their detectability and consequently their mating success and (2) decrease the costs of a fight, thus increasing their survival odds. The reduced aggression toward familiar individuals is called the "dear enemy effect" and has been shown in songbirds (Briefer et al. 2008), but this effect is awaiting experimental confirmation in hummingbirds.

In bees, the evolution of singing is negatively correlated to the production of sounds with the wing during dive displays (wing trills) as they seem functionally equivalent (Clark et al. 2018). Dive display is ancestral (Clark and Feo 2010; Clark et al. 2018), whereas dive or homologous behaviors seem rare outside the bees (Clark et al. 2018). Furthermore, their relatively small body sizes may lower the energetic requirements for dives and modified wings may facilitate maneuverability (Payne 1984; Clark et al. 2011). Interestingly, bumblebee hummingbird (*Atthis heloisa*) and wine-throated hummingbird (*Atthis ellioti*) which inhabit forests, pine-oak woodlands, and neighboring shrubby areas (Arizmendi et al. 2020; Thurber et al. 2020) are non-diving and regained singing (Clark et al. 2018). Overall, the expansion of bees to North America and consequent occupation of open habitats combined with their anatomical specializations might have favored the enhancement of visual displays and associated mechanical sounds.

Most of the bees species that lost the song occupy open habitats, for example, Allen's hummingbird (*Selasphorus sasin*) is a riparian breeder that often perches conspicuously on leafless branches (Clark and Mitchell 2020). The predation of adults has been, nevertheless, seldom observed and predation does not seem a significant risk to hummingbirds

(Miller and Gass 1985). It is, therefore, possible that some bees avoided perched song because, particularly in open habitats, learned songs that are, in general, more variable and less susceptible to habituation, made them more conspicuous to predators. A relaxed predatory pressure seems to precede the evolution of vocal-production learning because predators, likely, habituate more easily to the constancy of innate calls than to the variation of learned songs (Jarvis 2006; Nowicki and Searcy 2014). Predatory risks must be investigated comparatively between singing and non-singing bees to elucidate this possibility.

## Conclusion

In summary, singing is ancestral in most of the hummingbird clades and commonly associated with lekking behavior whereas evolutionary losses happened especially in bees, a clade that also evolved sophisticated visual displays such as the dive. We especulate that gains, losses and regains of singing and maybe vocal-production learning seem to have happened under distinct evolutionary pressures. It is important to bear in mind that such a literature survey can be biased toward singing species. Particularly species of clades in which singing is ancestral need to be investigated to confirm our findings. Although recent studies have shed light on the ontogeny of vocal-production learning in some hummingbirds (Johnson and Clark 2020, 2022), experimental studies that tackle vocal-production learning are still scarce and the related neurobiological correlates need confirmation; the brain areas suggested by connectivity, histology and gene expression to control song of hummingbirds (Jarvis et al. 2000; Gahr 2000) have not yet been confirmed using electrophysiological or lesion approaches. Nevertheless, the diversity in the vocal communication of hummingbirds including songs of different complexity levels which can be combined with a variety of visual displays or predominately visual offers a unique opportunity to investigate the mechanistic convergence, and perhaps the phylogenetic constraints, of vocal-production learning. Furthermore, comparative studies involving little-studied songbird and parrot taxa would reveal whether a similar heterogeneity exists in the other two vocal learner bird groups.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s10336-023-02057-9.

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**Author contributions** AM conceived the idea for the paper, and collected and analyzed the data; MLS and MG supervised the research; AM, MLS, and MG wrote and/or substantially edited the paper; and MG secured funding for this work.

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**Data availability** All datasets, scripts, and supplementary information are available at: https://osf.io/u7z4s/

## Declarations

**Conflict of interest** The authors report no conflict of interest.

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# Supplementary Material

# Absence of song suggests heterogeneity of vocal-production learning in hummingbirds

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## Supplemental Methods

### *Literature survey*

We compiled data on hummingbirds' vocalizations by conducting an informative rather than comprehensive literature survey. We collected descriptions of vocalizations that were classified by the author as song or calls and provided information further than onomatopoeic representations used for species identification in the field. We aimed for publications which focus was describing the vocal repertoire of the species. To include as many species as possible with a replicable method, reliable and detailed information, we searched for peer-reviewed articles in electronic databases. As exceptions, we included peer-reviewed conference articles if these had methods and results sections, and doctoral theses if these were available in an online repository. We are aware that this is far for being a comprehensive survey, nevertheless, for our goal to investigate the evolution of song within the family, it was important that we verified whether different authors had comparable song definitions before considering singing as a homologous trait.

#### Search strategy

We conducted the electronic search in January 2018 and carried out an updated version in April 2021. The search was divided into three steps: first, we used the web search engine "Web of Science" (https://apps.webofknowledge.com); second, the web search "Google Scholar" (https://scholar.google.com/); and third, we inspected the database of the journal "Boletim do

Museu de Biologia Mello Leitão" (http://boletim.sambio.org.br/index.html) because their articles were not fully covered by the web search. The first search in the "Web of Science" included "All databases" in the basic research tool. The timespan selected was all years from 1864 to date. In the field "topic," we used the keywords "hummingbird AND vocalization." Then, we refined the output by setting the document type selection for "article," "meeting," "review" and "other." We inspected the abstract of all 101 results back in 2018 and additional 10 results obtained in the last update. In the second search in the "Google Scholar," we included only the box "articles," enabled with the same keywords, "hummingbird AND vocalization." Then, we refined the output by disabling the options "patents" and "citations." The output, sorted by relevance, was 5,110 results back in 2018 and 7,820 in the last update. We inspected the abstract of all results on a page (10 results); then, if at least one of the results on the current page had new information about hummingbirds' vocalization, we inspected the following page. We inspected in total 20 pages (200 results) until no further information was found. The third search was conducted in the database of the journal "Boletim do Museu de Biologia Mello Leitão". This journal was funded by the naturalist Augusto Ruschi, who successfully bred several species of South American hummingbirds and documented their biology. This journal also includes a sonographic catalog of the hummingbirds' vocalizations conducted by Jacques Vielliard, who founded the largest sound library in South America. These articles were not included in the web search because the text is in Portuguese. We inspected the abstracts of all articles in the series "Biology" (http://boletim.sambio.org.br/biologia.html), "Zoology" (http://boletim.sambio.org.br/zoologia.html) and "Divulgation" (http://boletim.sambio.org.br/divulgacao.html). Additionally, we inspected the reference lists of the articles that provided information about hummingbirds' vocalizations and included cited articles in our survey.

## Inclusion criteria

Initially, we sorted out publications which title indicated that the main subject of the study is not birds (e.g.: "Sensory ecology of the frog-eating bat, *Trachops cirrhosus*, from DNA metabarcoding and behavior"). Subsequently, we inspected the abstracts of the remaining publications and accepted publications for viewing at the full text if it appeared that they may contain information about hummingbirds' vocalizations or if the abstract was ambiguous about their content. The criteria which studies had to meet for inclusion into the final stage of the survey were: (*i*) the main subject of the study was hummingbirds; (*ii*) the author mentioned a vocalization emitted by a valid species or subspecies of hummingbirds – a species or subspecies was considered valid if listed in "Birds of the World" online database and (*iii*) the author specified either clearly or implicitly (e.g., intermittent use of the term vocalization and song in any part of the text) whether the vocalization is either a song or a call. Field guides were not included because they aim to provide information used for species identification in the field, thus vocalizations are often described as onomatopoeic representations.

From all inspected results, 74 publications ranging from 1930 to 2020 met the inclusion criteria. A complete list of publications is in the Table S1.

## Data extraction

From these publications, we collected data about the prevalence of songs. Species which song was described were assigned as singing and those reported to lack a song were assigned as non-singing, when the term song was not mentioned or ambiguous, we considered as "unknown." In the case of an existing conflict between authors, the song was defined as present if at least one of the authors described a song for the species. We generated a dataset containing information about at least one trait for 78 hummingbird species and three subspecies from eight of the major hummingbird clades proposed by McGuire et al. (2009, 2014). We additionally inspected the entries for each of these species in the "Birds of the World" (BotW) online database (https://birdsoftheworld.org/) and added missing information on the prevalence of song. Except for field guides and books unavailable online, nearly all articles cited in BotW were already covered by our survey (Table S1).

Additional to song, we also obtained information on the pieces of evidence for vocal production learning demonstrated for hummingbird species. As behavioral evidence, we listed the occurrence of similarities in the song of a certain population that is attributed to natural *intra*specific vocal imitation, or dialect (Kroodsma and Baylis, 1982); as experimental evidence, the abnormal vocal development under acoustic deprivation observed under experimental conditions (Kroodsma and Baylis, 1982) and as neurobiological evidence, the prevalence of a set of brain nuclei analogous to the vocal control system (VCS) present in songbirds (Gahr, 2000; Jarvis et al., 2000).

### *Word cloud*

We obtained data about the behavior of the emitter during vocal production by selecting the corresponding quote from the publications with the descriptions of the spectral and temporal characteristics of the song or call ("song or call description") and the emitter's behavior during the production ("song or call context"). When the publication was not in English, we translated it.

We used the data about the behavior of the emitter during the production of songs and calls to access the probable function of the hummingbirds' vocalizations. We sorted the quotes with the description of the songs' or calls' behavioral context by clade and applied the text-mining method. The text-mining method consists of highlighting the most frequent word in a text. For text mining, we used the package "dplyr" (Wickham et al., 2020) and "tidytext" (Silge and

Robinson, 2016) in R (R Core Team, 2018). We excluded from the descriptions all prepositions (e.g., ‘for’ and ‘to’), determiners (e.g., ‘the’ and ‘a’), numbers, and words we listed as noninformative: song(s), singing, vocalization(s), note(s), syllable(s), phrase(s), kHz, hummingbird(s), bird(s) and unknown. We represented the most common words separately for songs and calls with a word cloud. The word cloud is a visual representation of the frequency of words in a text as represented by their font size: the most frequent words have the biggest font size. For the word cloud, we used the package “wordcloud” (Fellows, 2018) in R (R Core Team, 2018). In the word clouds, we represented a maximum of 100 words used at least twice, except when there were less than 40 words; then, we kept words used only once.

### *Ancestral character estimation*

#### Phylogeny

We reproduced a phylogenic tree for the 78 hummingbirds species and the three subspecies by subsetting the complete tree published by McGuire et al. (2014). To create the subsetted tree, we used the Tree Snatcher Plus software (Laubach and von Haeseler, 2007). In the software, we draw the tree tips and respective nodes over the phylogenetic tree available in the supplementary material of McGuire et al. (2014). For five species and three subspecies present in our dataset but absent in McGuire’s phylogeny, we assigned the node of the closest species of the same genus. TreeSnatcher Plus detected the original tree structure from the image, recreated the phylogenetic tree with the nodes given and produced the Newick representation of the subsetted tree with the estimated branch lengths. The representation of the phylogenetic tree was generated in R (R Core Team, 2018) with the packages “ape” (Paradis and Schliep, 2019) and “phytools” (Revell, 2012).

Estimation of the ancestral trait

We investigated the evolution of singing in hummingbirds by estimating the probabilities that this trait occurs across the phylogeny. Thus, we used discrete databases with presence ("yes") and absence ("no") of singing and the subsetted hummingbirds' phylogeny (see subsection Phylogeny) to run an ancestral state reconstruction. For the reconstruction, we excluded species in which data were unknown both from the datasets and the phylogeny. We ran the reconstruction with 78 species and three subspecies by using the function "ace" set to maximum likelihood (ML) in the R package "ape" (Paradis and Schliep, 2019). The ML method reconstructs the ancestral character states that are most likely to predict the contemporary state (Pagel, 1999). We fitted two alternative evolutionary models: in the first, the gains and losses were supposed to have happened at the same rate (equal rate, – ER model), and in the second, the gains and losses were supposed to have happened at different rates (all rates different, – ARD model) (Felsenstein, 2003). Because the trait is dichotomic ("yes" or "no"), a symmetrical model (– SYM model) would give results identical to the ER model; thus, a SYM model was not necessary. Then, to verify whether one of the models presented a better fit to the data, we compared the likehoods of the models using a likelihood ratio test (LR) as in Pagel (1994). For the LR test, the degrees of freedom (df's) were given by the difference in the number of parameters required to estimate each likelihood: one parameter for the ER model and two parameters for the ARD model. Then, we used the ARD model to estimate the probabilities of song being ancestral in each of the main clades of the phylogeny only if this model fits the data better than the ER model.

We used the function "make.simmap" in the package "phylotools" (Revell, 2012) to simulate stochastic character maps on each subsetted phylogenetic tree. We ran 1000 simulations to estimate the posterior probabilities of each node. We then integrated information across this set of stochastic maps into a Bayesian posterior probability for each branch in the tree using the function “describe.sinmap” in the package “phylotools” (Revell 2012). For each node, we plotted a pie chart showing the relative Bayesian posterior probability of the character state. We selected the nodes that aggregate all species of each of the major clades in the subsetted phylogeny as a representation of the ancestral condition for the respective clade.

## Recordings and pictures information

The recordings are taken from or deposited in the online sound library xeno-canto (https://www.xeno-canto.org/), except for the recordings of the black jacobin (*Florisuga fusca*), which is available on request. The xeno-canto recordings are shared under the Creative Commons License (CC BY-NC-SA). The catalog number of the xeno-canto recordings are as follows: XC454960 (long-tailed hermit), XC138158 (white-vented violetear), XC469392 (Brazilian ruby), XC608771 (blue-throated hummingbird), XC501895 (Anna’s hummingbird) and XC454963 (sombre hummingbird). The photographers of the pictures are: Amanda Monte (black jacobin, long-tailed hermit, white-vented violetear and sombre hummingbird); Lucia Calvet (Brazilian ruby); Steve Wolfe (blue-throated hummingbird) and Janine Russell (Anna's hummingbird).

## Supplemental Tables

Table S1 – List of hummingbird species with respective publications where was obtained the information about the presence (“yes”) and absence (“no”) of song. Species in which only calls were reported in the literature without information about song were assigned as “unknown” and not included in the ancestral reconstruction neither in this list. The sex of the songs’ emitter is depicted as provided in the song descriptions (“male” or “both” for male and female). When the sex of the emitter was not mentioned in the literature, we assigned as “unknown”. In the case of an existing conflict between authors, the song was defined as present if at least one of the authors described a song for the species. “NA”s are inserted where information does not apply

| Clade | Species | Song | Emitter’s sex | Reference song |
|---|---|---|---|---|
| Topazes | *Florisuga fusca* | yes | Unknown | Ruschi 1973<br>Vielliard 1983<br>Olson et al. 2018 |
| Topazes | *Topaza pella* | yes | Male | Davis 1958 |
| Topazes | *Topaza pyra* | yes | Unknown | Hu et al. 2000 |
| Hermits | *Ramphodon naevius freitasi* | yes | Unknown | Ruschi 1978 |
| Hermits | *Threnetes ruckeri* | yes | Male | Skutch 1964 |
| Hermits | *Threnetes leucurus* | yes | Male | Snow 1973<br>Vielliard 1983 |
| Hermits | *Glaucis hirsutus* | yes | Unknown | Vielliard 1983<br>Ferreira et al. 2006 |
| Hermits | *Phaethornis mexicanus griseoventer* | yes | Unknown | del Hoyo et al. 2020a |
| Hermits | *Phaethornis longuemareus* | yes | Unknown | Nicholson 1931<br>Snow 1968<br>Wiley 1971 |
| Hermits | *Phaethornis ruber* | yes | Both | Nicholson, 1931<br>Snow 1973<br>Vielliard 1983<br>Felton et al. 2008 |
| Hermits | *Phaethornis eurynome* | yes | Unknown | Vielliard 1983 |
| Hermits | *Phaethornis augusti* | yes | Male | Ramjohn et al. 2003 |
| Hermits | *Phaethornis petrei* | yes | Unknown | Vielliard 1983 |
| Hermits | *Phaethornis supercilious* | yes | Male | Skutch 1964<br>Stiles and Wolf 1979<br>Snow 1973 |
| Hermits | *Phaethornis longirostris* | yes | Male | Araya-Salas and Wright 2013 |
| Hermits | *Phaethornis guy guy* | yes | Unknown | Nicholson, 1931<br>Snow 1977 |
| Hermits | *Phaethornis guy coruscus* | yes | Unknown | Skutch 1964<br>Snow 1977<br>Macdougall-Shackleton and Harbison 1998 |
| Mangoes | *Colibri serrirostris* | yes | Unknown | Vielliard 1983<br>Silva and Vielliard 2006 |
| Mangoes | *Colibri coruscans* | yes | Male | Gaunt et al. 1994 |
| Mangoes | *Colibri delphinae greenewalti* | yes | Unknown | Vielliard 1983 |
| Mangoes | *Colibri thalassinus* | yes | male | Gaunt et al. 1994<br>Barrantes et al. 2008 |

|  |  |  |  |  |
|---|---|---|---|---|
|  |  |  |  | Lara et al. 2015 |
| Mangoes | *Heliactin bilophus* | yes | male | Vielliard 1983 |
| Mangoes | *Polytmus guainumbi* | yes | unknown | Vielliard 1983 |
| Mangoes | *Chrysolampis mosquitus* | yes | unknown | Vielliard 1983 |
| Mangoes | *Anthracothorax nigricollis* | yes | unknown | Vielliard 1983<br>Araujo-Silva and Bessa 2010 |
| Mangoes | *Eulampis jugularis* | yes | male | Schuchmann and Schuchmann-Wegert 1984 |
| Brilliants | *Clytolaema rubricauda* | yes | unknown | Vielliard 1983 |
| Coquettes | *Lophornis magnificus* | yes | both | Ruschi 1973b |
| Coquettes | *Phlogophilus harterti* | yes | unknown | David et al. 2018 |
| Coquettes | *Aglaiocercus kingii* | yes | unknown | Stiles and Cortés-Herrera 2015 |
| Coquettes | *Oreotrochilus chimborazo* | yes | male | Duque et al. 2020 |
| Coquettes | *Oreotrochilus cyanolaemus* | yes | both | Sornoza-Molina et al. 2018 |
| Coquettes | *Metallura tyrianthina* | yes | unknown | Stiles and Cortés-Herrera 2015 |
| Patagona | *Patagona gigas* | yes | unknown | Heynen et al., 2020 |
| Mountain Gems | *Panterpe insignis* | no | NA | Wolf 1969<br>Wolf and Stiles 1970 |
| Mountain Gems | *Lampornis clemenciae* | yes | both | Ficken et al. 2000, 2002 |
| Mountain Gems | *Lampornis amethystinus* | yes | male | Ornelas et al. 2002 |
| Bees | *Calliphlox amethystina amethystina* | yes | male | Ruschi 1973a |
| Bees | *Rhodopis vesper* | yes | male | Clark et al. 2012 |
| Bees | *Thaumastura cora* | yes | male | Clark et al. 2013 |
| Bees | *Eulidia yarrellii* | yes | male | Clark et al. 2013 |
| Bees | *Archilochus alexandri* | no | NA | Rusch et al. 1996 |
| Bees | *Archilochus colubris* | no | NA | Clark et al. 2018 |
| Bees | *Mellisuga minima* | yes | unknown | Clark 2006 |
| Bees | *Calliphlox evelynae evelynae* | yes | unknown | Feo et al. 2015 |
| Bees | *Calliphlox evelynae lyrura* | yes | male | Feo et al. 2015 |
| Bees | *Calypte anna* | yes | male | Mirsky 1976<br>Wells et al. 1978<br>Baptista and Matsui 1979<br>Stiles 1982<br>Baptista and Schuchmann 1990<br>Zyskowski et al. 1998 |
| Bees | *Calypte costae* | yes | both | Wells et al. 1978<br>Williams and Houtman 2008 |

| | | | | |
|---|---|---|---|---|
| | | | | Clark and Feo 2010 |
| Bees | *Atthis heloisa* | yes | male | Zyskowski et al. 1998 |
| Bees | *Atthis ellioti* | yes | male | Bent 1940<br>Zyskowski et al. 1998 |
| Bees | *Selasphorus scintilla* | no | NA | Clark et al. 2011 |
| Bees | *Selasphorus flammula* | no | NA | Clark et al. 2011 |
| Bees | *Selasphorus sasin* | no | NA | Wells and Baptista 1979 |
| Bees | *Selasphorus rufus* | no | NA | Hammersley 1928<br>Clark and Feo 2010 |
| Emeralds | *Campylopterus excellens* | yes | male | Winker et al. 1992 |
| Emeralds | *Campylopterus curvipennis* | yes | male | González and Ornelas 2005, 2009 |
| Emeralds | *Stephanoxis lalandi lalandi* | yes | male | Vielliard 1983 |
| Emeralds | *Stephanoxis lalandi loddigesii* | yes | male | Pizo 2012 |
| Emeralds | *Campylopterus largipennis aequatorialis* | yes | unknown | Ruschi 1973c |
| Emeralds | *Chlorostilbon lucidus* | yes | unknown | Bündgen et al. 2020 |
| Emeralds | *Hylocharis leucotis leucotis* | yes | male | Bent 1940 |
| Emeralds | *Thalurania glaucopis* | yes | unknown | Ruschi 1973e<br>Vielliard 1983 |
| Emeralds | *Phaeochroa cuvierii* | yes | unknown | Skutch 1964a<br>Stiles and Wolf 1970 |
| Emeralds | *Aphantochroa cirrochloris* | yes | unknown | Vielliard 1983<br>Ferreira et al. 2006 |
| Emeralds | *Eupetomena macroura* | yes | unknown | Vielliard 1983 |
| Emeralds | *Trochilus polytmus polytmus* | yes | male | Schuchmann 1978 |
| Emeralds | *Trochilus polytmus scitulus* | yes | male | Schuchmann 1978 |
| Emeralds | *Amazilia rutila* | yes | unknown | Arizmendi et al. 2020b |
| Emeralds | *Amazilia tzacatl tzacatl* | yes | male | Bent 1940 |
| Emeralds | *Amazilia amazilia* | yes | unknown | Weller 2000 |
| Emeralds | *Chrysuronia oenone* | yes | unknown | Vielliard 1983 |
| Emeralds | *Amazilia versicolor* | yes | unknown | Vielliard 1983 |
| Emeralds | *Amazilia leucogaster* | yes | unknown | Vielliard 1983 |
| Emeralds | *Amazilia candida* | yes | unknown | Atwood et al. 1991 |
| Emeralds | *Hylocharis cyanus* | yes | unknown | Vielliard 1983 |
| Emeralds | *Chlorestes notatus* | yes | both | Carvalho 1958 |
| Emeralds | *Leucochloris albicollis* | yes | unknown | Vielliard 1983 |
| Emeralds | *Hylocharis sapphirina* | yes | unknown | Vielliard 1983 |
| Emeralds | *Amazilia lactea* | yes | unknown | Vielliard 1983 |
| Emeralds | *Amazilia fimbriata tephrocephala* | yes | unknown | Vielliard 1983 |

| | | | | |
|---|---|---|---|---|
| Emeralds | *Amazilia fimbriata nigricauda* | yes | unknown | Vielliard 1983 |

## Data availability

All datasets, scripts and supplementary information is available at https://osf.io/u7z4s/

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# Chapter 2

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

## Abstract

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. 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. 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.

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

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## 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

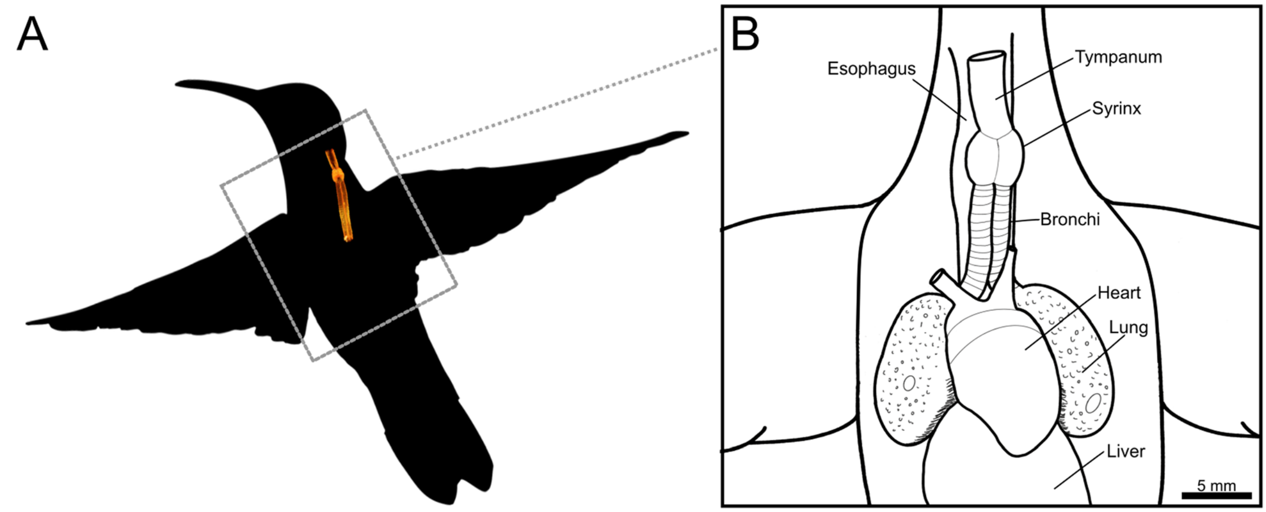

**Fig. 1** Extra-thoracic location of the black jacobin's syrinx. **a**, schematic drawing of a flying hummingbird showing the relative position of the dorsally located syrinx. The syrinx is dorsally located. We generated the schematic by overlaying a surface rendering of the black jacobin's syrinx on 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 craniocaudal 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.

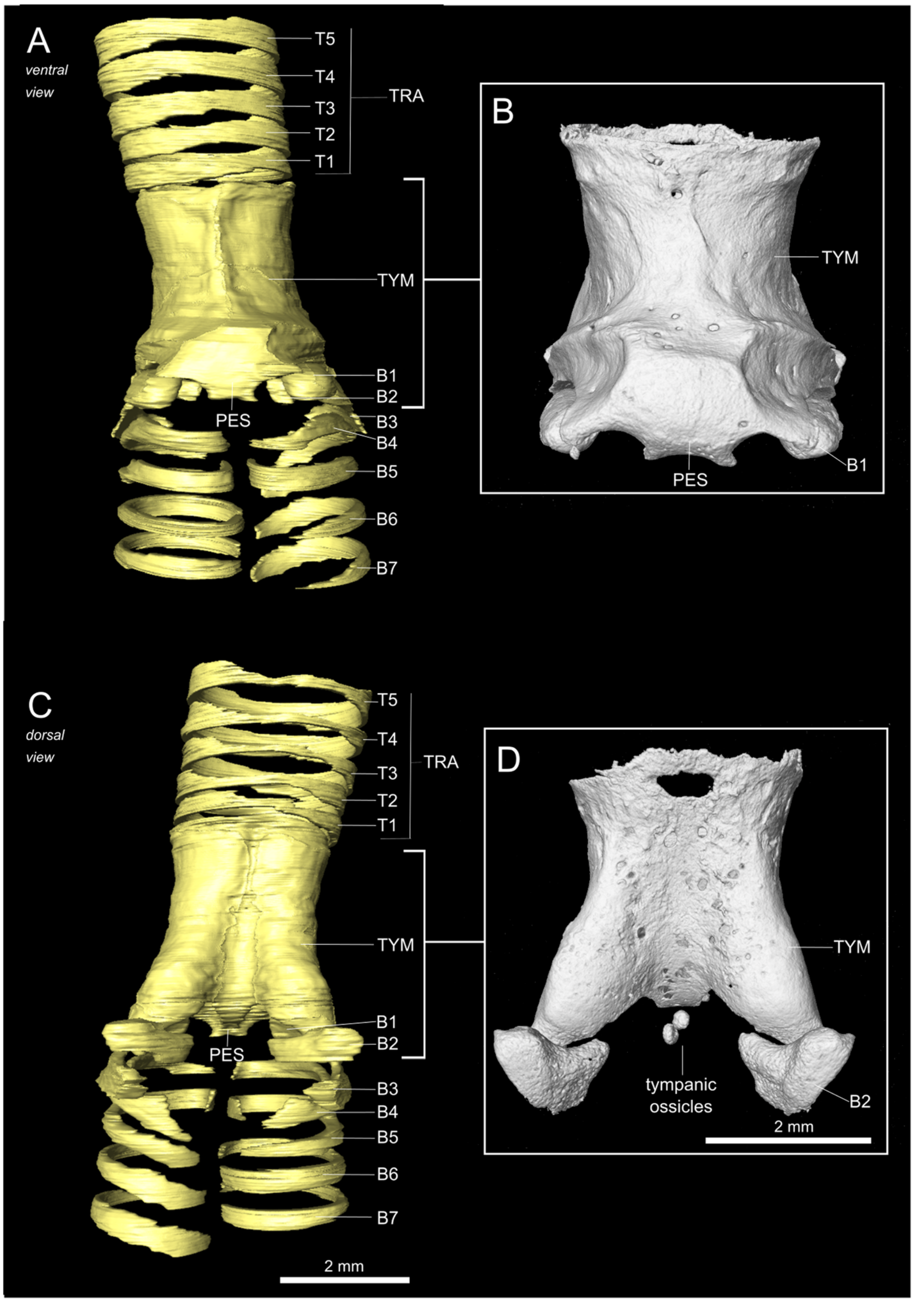

**Fig. 2** The syringeal osseous and cartilaginous elements of the black jacobin. 3D visualizations of µCT data. **a** and **c**, surface renderings of the contrasted sample; **b** and **d**, volume renderings of the non-contrasted sample. **a** and **b**, ventral view; **c** and **d**, dorsal view. 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

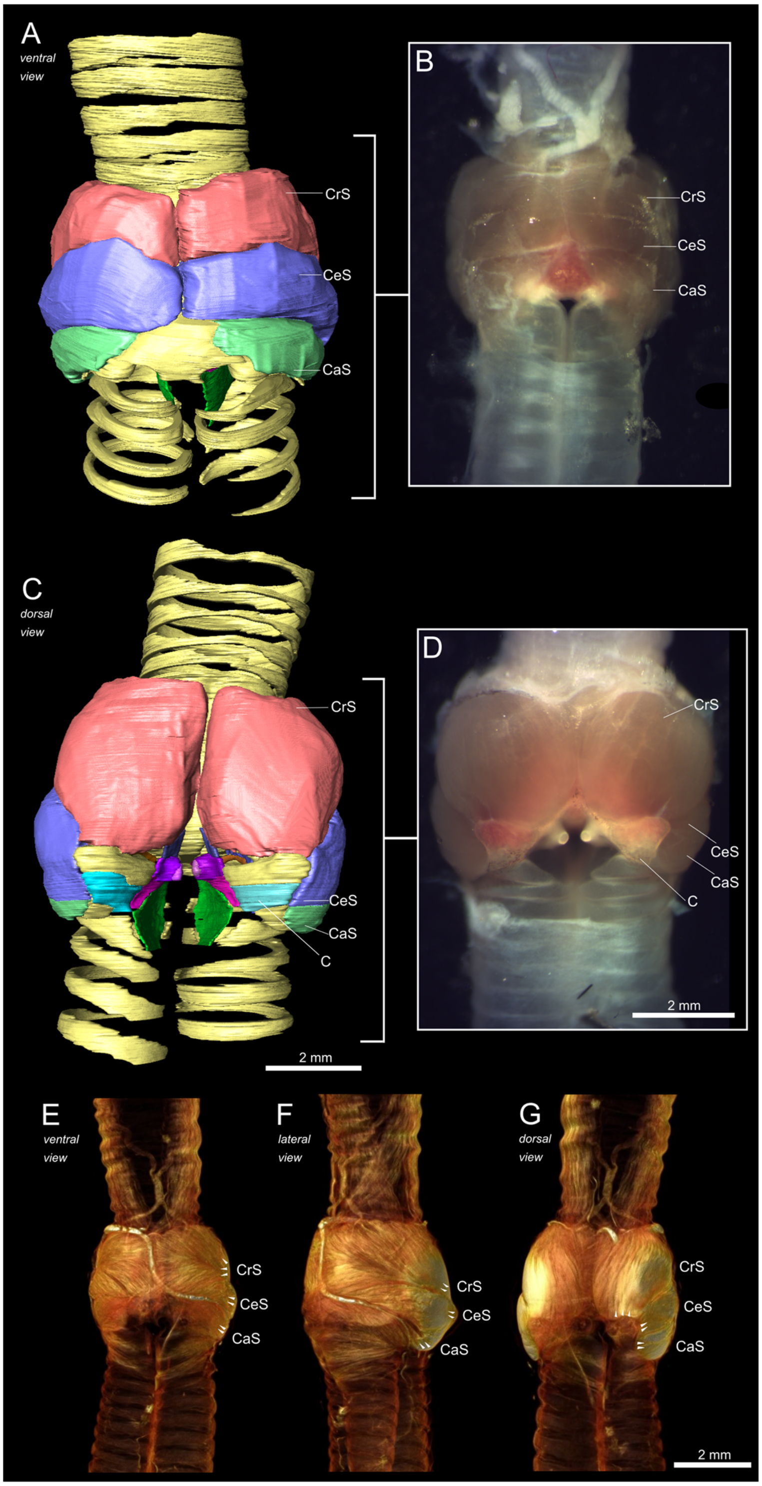

**Fig. 3** Musculature of the black jacobin's syrinx. **a** and **c**, surface renderings; **b** and **d**, dissected syrinx and **e**, **f** and **g**, volume renderings. **a**, **b** and **e**, ventral view; **c**, **d** and **g**, dorsal view and **f**, lateral view. The arrows indicate the orientation 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.<br>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.<br>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 (± 0.5, $n$ = 66 syllables); click-like chirps with an F0 average of 7.9 kHz (± 1, $n$ = 148 syllables); and high-pitched vibratos with an F0 average of 11.8 kHz (± 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 ± 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 ± 0.5 kHz, $n$ = 401 crest-trough pairs, Fig. 6b) with a periodicity of around 2.4 ms (± 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 (± 37.5, $n$ = 18 syllables). For example, Whitney Houston produced an average vibrato rate of 5.1 Hz (± 0.6, $n$ = 12 vibratos) with a vibrato extent of 0.05 kHz (± 0.01) (Fig. 6c). The human vibrato rate is similar between different music

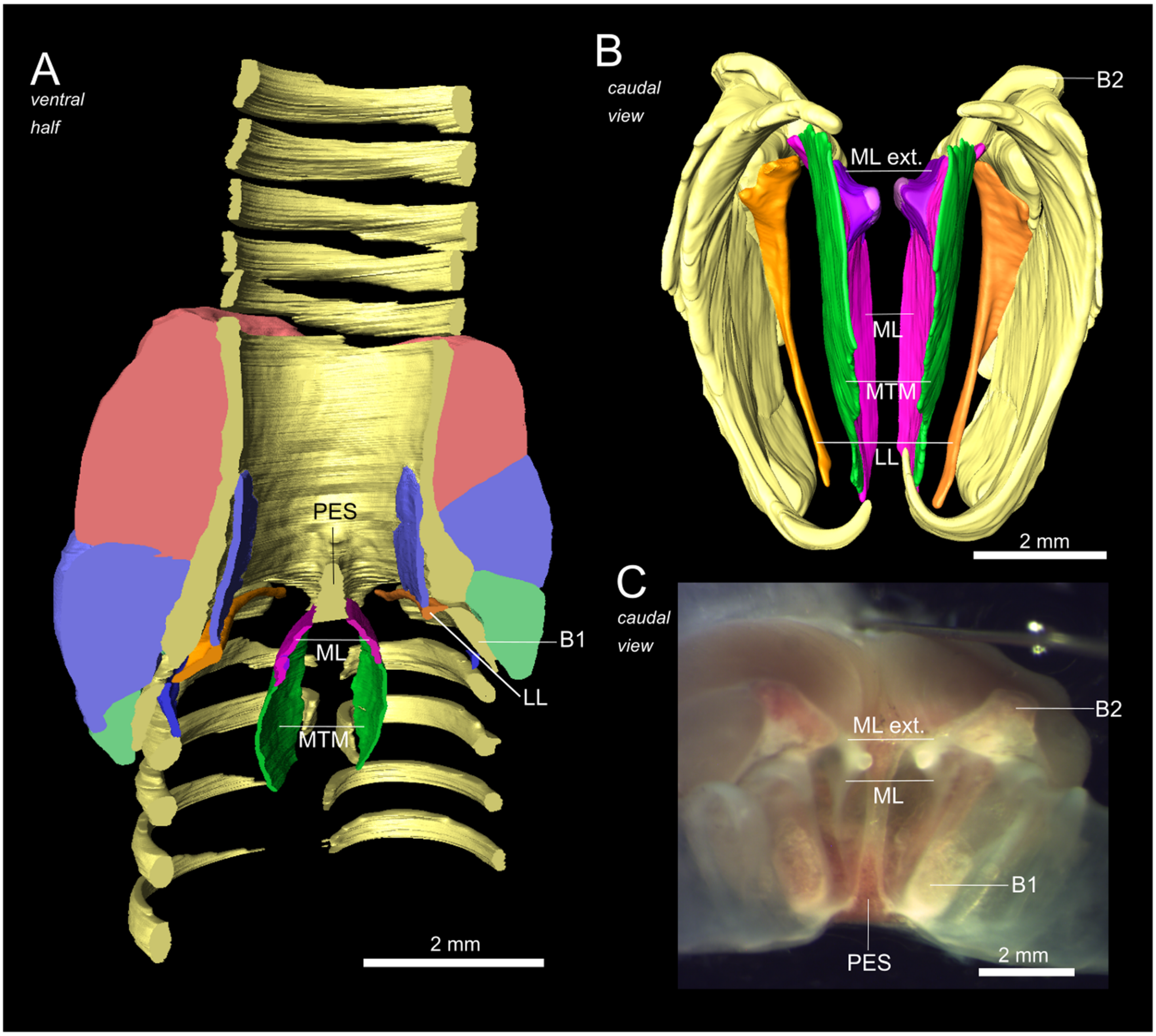

**Fig. 4** Vibratory membranes of the black jacobin's syrinx. **a** and **b**, surface renderings and (**c**), dissected syrinx. **a**, ventral view and (**b**) and (**c**), caudal view. In **b**, the first three pairs of bronchial half-rings and all pairs of vibratory membranes: LL (orange), MTM (green), 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

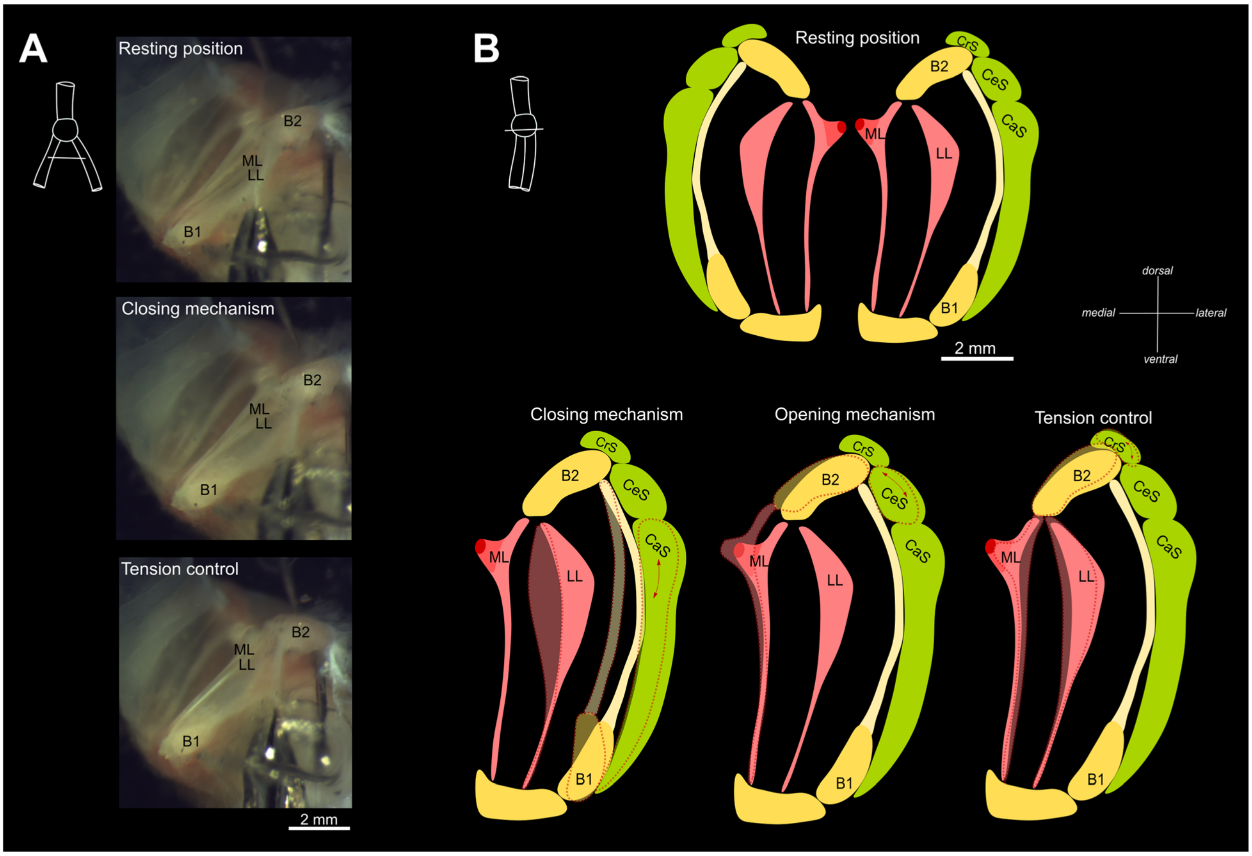

**Fig. 5** Hypothetical biomechanics of the black jacobin's syrinx. In **a**, caudal view of a fresh dissection. Upper panel: resting position; middle panel: inwards movement of the LL, the closing mechanism; lower panel: stiffening of the ML, tension control. In **b**, schematic representation of the 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,

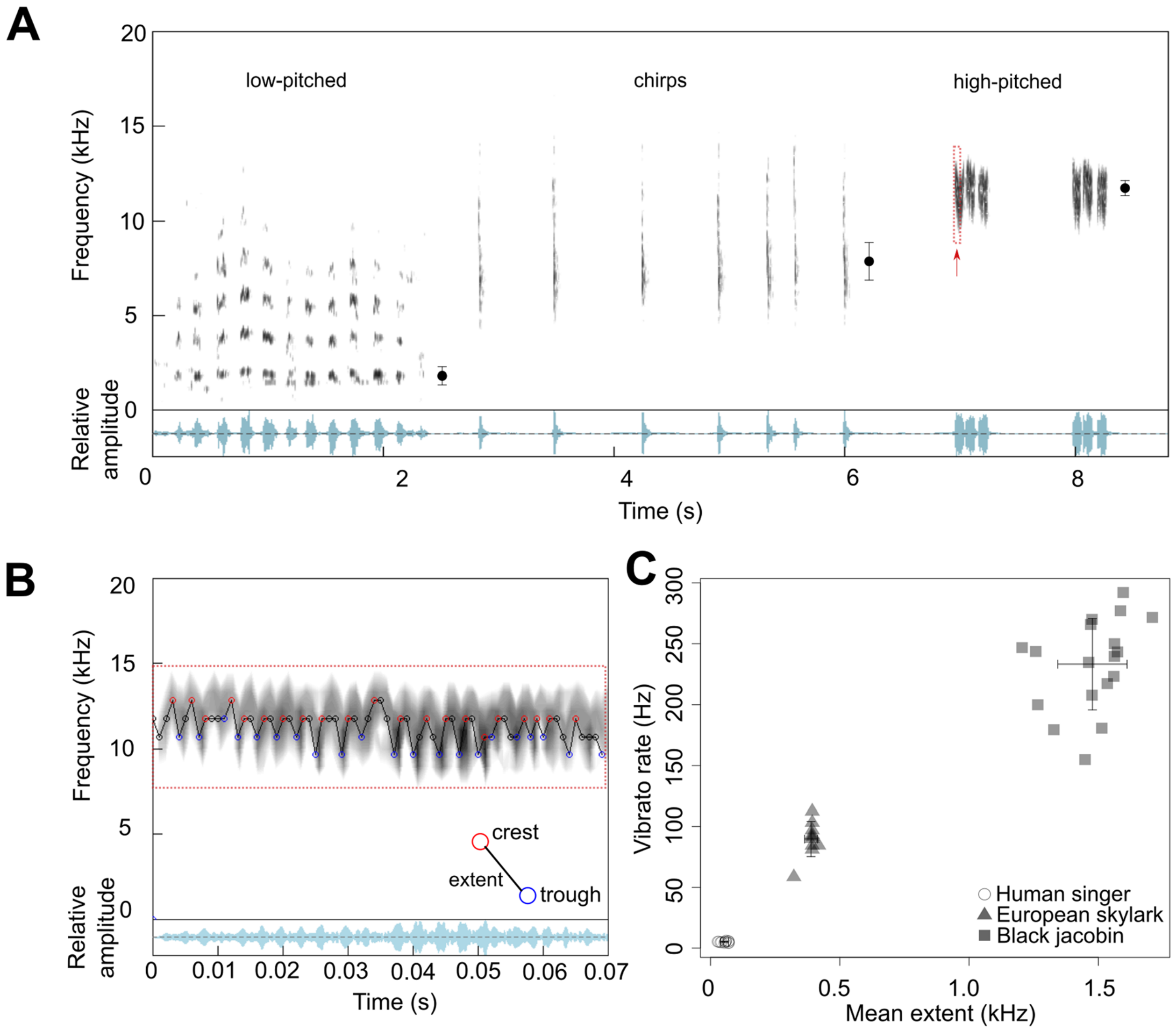

**Fig. 6** Black jacobin vocalizes in a wide frequency range with rapidly modulated vibratos. In **a**, spectrogram (frequency) and oscillogram (relative amplitude) of the three vocalization types produced by the black jacobin in the wild: low-pitched, chirps and high-pitched. The dots represent the 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, 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).

## Abbreviations

µCT: Micro-computed tomography; ST: *Musculus sternotrachealis* or sternotracheal muscle; T1 to Tn: First to the last cartilaginous rings; B1 to Bn: First to the last bronchial half-rings; TL: *Musculus tracheolateralis* or tracheolateral muscle; CrS: *Musculus syringealis cranialis* or cranial syringeal muscle; CeS: *Musculus syringealis centralis* or central syringeal muscle; CaS: *Musculus syringealis caudalis* or caudal syringeal muscle; LL: Lateral labia; ML: Medial labia; MTM: Medial tympaniform membrane; MVM: Medial vibratory mass; F0: Fundamental frequency; PTP: Phonation threshold pressure

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## Authors' contributions

AM and DD conceived the study. AM, AC, BR, and DD contributed to data acquisition. DD, AC, BR, and MG contributed reagents, materials, and analytical tools. All authors contributed to data analysis. AM wrote the initial draft of the manuscript. All authors contributed to manuscript revision and approved the final version.

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## Availability of data and materials

The µCT scans and derived 3D surfaces of the black jacobin's syrinx generated during the current study are available in the Morphobank repository under the project number 3691, http://morphobank.org/permalink/?P3691

The customized R script generated during the current study to detect crest-trough pairs in vibrato is available in the GitHub repository under the file name "vibrato_scan.R", https://github.com/amandamonte/black_jacobin.git

The sound recordings and respective datasets analysed during the current study are available from the corresponding author on reasonable request.

### 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

# Chapter 3

# Testosterone treatment unveils testosterone-insensitive song in an early-branched hummingbird

## Abstract

Birdsong often plays a crucial role in sexual behavior, including territorial defense and mate attraction. This behavior is particularly well studied in species in which only males sing and respond to gonadal testosterone depending on the breeding season. Although hummingbirds are long known to sing in a breeding context, the sensitivity of these vocalizations to testosterone in both sexes remains unknown. Therefore, we investigated (*i*) which vocalizations are emitted during their breeding season, (*ii*) the testosterone levels in free-ranging birds, and (*iii*) the effect of testosterone on these vocalizations in a semi-natural environment in both sexes of the black jacobin (*Florisuga fusca*), an early-branched hummingbird. Females and males of black jacobin produced the same set of vocalizations (high-pitched song and chirp call) at a similar rate during the breeding season, although testosterone levels in males were significantly higher. Testosterone treatment did not affect the vocal activity or acoustic parameters of their song in both sexes. However, testosterone maintained high levels of aggression in treated birds of both sexes and hyper-developed the oviduct of females. In contrast to almost all well-studied bird species studied so far, the vocal behavior of the black jacobins in their breeding season seems to be insensitive to high testosterone levels.

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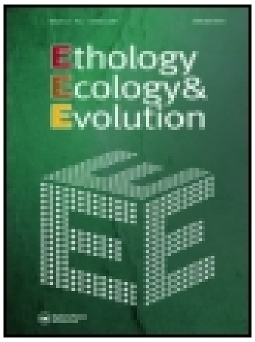

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# Testosterone treatment unveils testosterone-insensitive song in an early-branched hummingbird

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Birdsong often plays a crucial role in sexual behavior, including territorial defense and mate attraction. This behavior is particularly well studied in species in which only males sing and respond to gonadal testosterone depending on the breeding season. Although hummingbirds are long known to sing in a breeding context, the sensitivity of these vocalizations to testosterone in both sexes remains unknown. Therefore, we investigated (i) which vocalizations are emitted during their breeding season, (ii) the testosterone levels in free-ranging birds, and (iii) the effect of testosterone on these vocalizations in a semi-natural environment in both sexes of the black jacobin (*Florisuga fusca*), an early-branched hummingbird. Females and males of black jacobin produced the same set of vocalizations (high-pitched song and chirp call) at a similar rate during the breeding season, although testosterone levels in males were significantly higher. Testosterone treatment did not affect the vocal activity or acoustic parameters of their song in both sexes. However, testosterone maintained high levels of aggression in treated birds of both sexes and hyper-developed the oviduct of females. In contrast to almost all well-studied bird species studied so far, the vocal behavior of the black jacobins in their breeding season seems to be insensitive to high testosterone levels.

KEY WORDS: vocal behavior, sexual trait, breeding season, Trochilidae, black jacobin

## INTRODUCTION

Sexual male-typical traits, such as song in Northern Hemisphere songbirds are known to be activated during the reproductive periods with high levels of testosterone (reviewed in Wingfield & Farner 1978; Gahr 2014). These traits are important for

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courtship (mate attraction) and agonistic encounters (territorial defense) (Catchpole & Slater 1995). Evidence of testosterone-dependent vocal performance is nevertheless not exclusive to songbirds (oscine passerines) since it has also been found in other taxa, as in suboscine passerines (Kroodsma 1984) and parrots (Brockway 1968; Nespor et al. 1996). Crowing of roosters in domestic chicken (*Gallus gallus*) as well as of other galliform species, such as the Japanese quail (*Coturnix japonica*) and the grey partridge (*Perdix perdix*), is another typical vocal trait known to be testosterone dependent (Hamilton 1938; Marler et al. 1962; Andrew 1963; Beani et al. 2000). A particular vocalization type, the nest-coo used in the courtship of male ring doves (*Streptopelia risoria*) was induced by brain implantation of testosterone (Cohen & Cheng 1982).

In all well-studied bird species, changes in vocalization were found in association with naturally elevated testosterone plasma levels of the breeding season or after testosterone implantation (Gahr 2020), for example, increase of vocal output (song or/and call rate), acoustic features such as frequency range or stereotypy (Arnold 1975; Heid et al. 1985; Fusani et al. 1994), and utterance of testosterone-specific vocalizations such as trills (Pröve 1974; Fusani et al. 1994; Voigt & Leitner 2013). Further, a masculinized song can be induced in females (that do not sing otherwise) by testosterone implants, not only in songbirds such as canaries, chaffinches, white-crowned sparrows and white-browed sparrow weavers (Kern & King 1972; Nottebohm 1980; Voigt & Leitner 2013; Chiver & Schlinger 2019; Vellema et al. 2019) but also in non-songbirds such as golden-collared manakins (*Manacus vitellinus*) (Chiver & Schlinger 2019), budgerigars (*Melopsittacus undulatus*) (Lahaye et al. 2012) and Japanese quail (Balthazart et al. 1983).

High levels of testosterone appear to be necessary to activate sexually relevant vocal behaviors in Northern Hemisphere songbirds during a short breeding season (Wingfield & Farner 1978; Gahr 2020), whereas, in tropical songbirds such as the silver-beaked tanager (*Ramphocelus carbo*), during a long breeding season (between 6 and 7 months), the testosterone levels are only high at the end of the season coinciding with higher rates of dawn-song, but this species show a higher brain sensitivity to testosterone in the beginning of the season which indicates testosterone sensitivity of their dawn-song (Quispe et al. 2016). However, testosterone sensitivity of these vocal behaviors are less clear in tropical and sub-tropical non-songbird species with protracted breeding periods or year-round territoriality (Goymann et al. 2004). Such a bird family are the hummingbirds in which males and in some species females are known to sing (Baptista & Schuchmann 1990; Ficken et al. 2000; da Silva & Vielliard 2006; Ferreira et al. 2006).

Hummingbirds have neural song control areas analog to songbirds which are active during song production (Jarvis et al. 2000) and express androgen receptors (binding site of testosterone and its converted product 5α-dihydrotestosterone, DHT) (Gahr 2000). Androgen receptors were also found in hummingbird syrinx (A. Monte unpublished data), the sound-producing organ of birds. Nevertheless, it is unknown whether song is a male-typical trait or whether any vocalization is testosterone sensitive in hummingbirds, hence our motivation to study these aspects in the black jacobin (*Florisuga fusca*). It is a species of the Topazes clade (tribe Topazini) which is a sister taxon of all other extant hummingbirds according to the most recent evolutionary tree (McGuire et al. 2009, 2014). Therefore, the study of the black jacobin may give insights into the ancestral condition of testosterone-sensitive vocalizations in the hummingbird family.

The black jacobin occurs in the Atlantic forest on the extent of the Brazilian coast until Uruguay and Argentina (Sick 1997; Schuchmann & Bonnan 2019). Black jacobins produce a high-pitched song composed of very fast frequency oscillations (Olson et al. 2018; Monte et al. 2020) and uttered in the breeding season (from September until March; Ruschi 1964); however, it is unknown whether this high-pitched or other vocalizations produced in the breeding season are the exclusivities of males and whether they are testosterone sensitive. To investigate the sensitivity of their vocalizations to testosterone, firstly, we measured testosterone plasma levels and body weight of free-living black jacobins from both sexes in the middle of the breeding season (December). Secondly, we carried out an experiment with wild-caught birds placed in semi-natural aviaries. We recorded the vocalizations and associated behaviors before and after testosterone implants. We established their vocal repertoire and quantified their vocal output (song and call rate), as well as the acoustic parameters of the song. Additionally, we investigated the number of attacks as a proxy for aggressiveness and their feeding behavior. We found higher circulating testosterone levels and body weight in males than females, but no sex differences in their vocalizations. The testosterone treatment induced the hypertrophy of the oviduct but did not affect the testes weight. Both sexes maintained their aggressive behavior before and after testosterone implantation whereas the control group implanted with placebos reduced aggressiveness during the study period. Unexpectedly, we could not show any changes in their vocal behaviors following testosterone implantation. These results indicate that in contrast to songbirds and most other birds studied to date, the vocalizations of hummingbirds in their breeding season seem insensitive to testosterone.

## METHODS

### *Animals*

This study was carried out in the park of the Museum of Biology Prof. Mello Leitão in Santa Teresa, Espírito Santo, Brazil in the middle of the breeding season that lasts from September until March (Ruschi 1964). Free-living black jacobins (*Florisuga fusca*) were captured between 6:00–8:00 and 17:00–18:00 during their regular visit to feeder bottles filled with sugary water (solution of water with 20% sugar), hanging inside a trap called “Ruschi net trap” developed and optimized to capture hummingbirds (Ruschi 2009).

Black jacobin’s plumage is not sexually dimorphic (Ruschi 1973), the birds captured for the testosterone experiment ($n = 32$) were individually marked with plastic-colored tags attached to their back (Kapoor 2012) and kept randomly in outdoor aviaries until molecular sexing. Upon sexing results, we kept 14 males and 10 females that were distributed in four adjacent outdoor aviaries (2.35 m long × 2.70 m high × 3.20 m deep; A1 to A4) under natural conditions of daylight, rain, temperature, and humidity. We placed six birds per aviary. Three males and three females in aviaries A1 and A4 and four males and two females in aviaries A2 and A3. The unequal number of females and males was due to two mistakes in the molecular sexing results that were only noticed at the end of the experiment when we checked the gonads. Additionally, we caught and released 206 wild birds for blood sampling (50 females and 153 males, 3 died) and captured and sacrificed further 14 birds (seven females and seven males) for future tissue analyses.

The aviaries contained vegetation to offer shadow, branches of different thicknesses to be used as perches, two hanging pots with flowers and thin perches, three feeder bottles with watery sugar, a wooden box containing fermented fruits to attract fruit flies (part of the diet of the black jacobins), and an irrigation sprinkler activated only between observations when the temperature was above 35 °C to spread water in half of the aviary to be used for bathing. The feeder bottles were refilled once a day before the morning observations. At the end of the aviary experiment, the birds were killed by decapitation, as for the 14 extra caught birds, organs were immediately frozen in dry ice and stored at − 80 °C until use.

All procedures of this study were conducted in conformity with the instruction no. 03/2014 of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and with the permission of the Brazilian Institute of Environment and Renewable Natural (IBAMA) under the Biodiversity Information and Authorization System (SISBio) licenses numbers 30319–1, 41794–2 and 49097–1. The bird handling and housing procedures were conducted in accordance with Law no. 11.794/2008, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA) and was approved by the Ethics Committee on Animal Use of the Federal University of Para (CEUA/UFPA) under the protocol number 5937130718 (ID 000953).

### *Experimental procedure and testosterone implants*

The first 6 days of captivity were used as a time for the housed birds ($n = 24$) to acclimate to the aviaries (no recorded observations), and then we started monitoring observations for 1 week (days − 6 to − 1) before the testosterone (T) or empty implant treatment for baseline activity ("before treatment"). On the 7th day (day 0), a total of seven females (aviaries A1, A3 and A4 with two each; A2 with one) and five males (aviaries A1, A3 and A4 with one each; A2 with two) were implanted with testosterone ("T-treated group") and the others with placebo empty implants ("control group") and monitored for the next 2 weeks (days 1 to 12, "after treatment", Fig. S1 in Supplemental Data). Each bird was observed for 20 min twice a day, in the morning (between 5:30, after sunrise, and 12:00) and the afternoon (between 12:00 and 18:30, before sunset; except days 8 and 12, in which birds were monitored only in the morning due to logistic problems). All the birds within an aviary were observed consecutively in a randomized order. Each of the two observation sessions per bird was conducted by a different observer (A. Monte or R. da Silva Matos) to minimize the observer bias. In total, we sampled 96 hr of observations before and 176 hr after T-treatment.

The implants were self-made with a 3 mm silastic tube (Dow Corning, USA; 1.47 mm inner diameter, and 1.96 mm outer diameter) filled with ca 0.9 mg of testosterone (Sigma T1500, Sigma-Aldrich GmbH, Germany) (T implant) or left empty (placebo implant) and the endings sealed with liquid silicone sealant (ASC 40064, USA). The implant size was estimated in proportion to the body size as it was reported in other species studied (Quispe et al. 2015; Prior et al. 2016). The implants were subcutaneously inserted at the lower back of the birds after local anesthesia with lidocaine hydrochloride (Lidogel 2%, Neoquimica).

### *Sound recording and analyses of vocalizations*

In each session we monitored the vocal behavior, feeding behavior and aggressiveness in the four aviaries following a focal-bird sampling technique (Altmann 1974; Martin & Bateson 2007).

For sound recording, we used a digital recorder Marantz PMD 671 (USA) mounted with a shotgun directional microphone (Sennheiser MKH 70 P48, Germany; Rode NTG2, Australia). The recordings were obtained in the mono channel, with frequency sample of 48 kHz and 32 bits. The audio recordings were screened manually in the software Audacity (version 2.0.3, Audacity Team 2013), and the descriptions were transcribed into audio labels that were exported as text files. The labels were quantified by a customized algorithm written on R version 3.0.3 (R Core Team 2014).

We identified three vocal types based on spectral parameters as previously described for the species (Olson et al. 2018; Monte et al. 2020): high-pitched (most common, 68.1% of emission in the entire monitoring), low-pitched (0.6%) and chirps (31.3%) (Fig. S2 in Supplemental Data).

The high-pitched vocalization has harmonics up to 80 kHz with a fundamental frequency between 10 and 14 kHz. The fundamental frequency was fast-modulated and had periodic oscillation called vibrato (Monte et al. 2020). It is the most spectrally complex of black jacobin's vocalizations and is suggested as the song of the species (Olson et al. 2018). The song is composed of one or several units (or syllable) emitted in sequence with each unit composed of three (triplet), two (double) or one (single) subunit (or notes) (Fig. S3 in Supplemental Data). Chirps can be emitted alone or followed by a few repetitions with variable silent interval (single chirp or s-chirp) or followed by many repetitions with progressively longer silence intervals (multiple chirps or m-chirp) (Fig. S2 in Supplemental Data) as previously described for rufous-breasted hermit (*Glaucis hirsutus*) (Ferreira et al. 2006). Given that m-chirp was rare, we quantified the number of chirps in the m- and s-chirps and presented them together in the results. The low pitch was also a very rare event and was not analyzed.

We quantified the number of songs and chirps emitted per bird in each 20 min observation session (songs or chirps/20 min). We subsampled the longest song bouts with a good signal-to-noise ratio and selected ~ 200 songs per bird (range: 5–1000 song bouts per bird, per session). We labeled the triplets, doubles and singles manually in the software Audacity (version 2.0.3, Audacity Team 2013) and saved the labels as text files. We quantified their repeatability (R) which represents how much of the total variance in the number of triplets, doubles and singles is attributed among birds and was calculated with the function "rpt" implemented by the package "rptR" (Stoffel et al. 2017) and using a customized algorithm written in R version 3.0.3 (R Core Team 2014). For the analysis of the spectral and temporal acoustic parameters (in total 26, Table S1 in Supplemental Data), we used the function "specam" and package "warbleR" (Araya-Salas & Smith-Vidaurre 2017). We analyzed in total 3660 songs. We calculated the averages of each parameter per bird before, 1 and 2 weeks after T-treatment followed by a Principal Component Analysis (PCA) to reduce the number of parameters and maintain those with higher variance (see Statistical analyses).

### *Quantification of other associate behavioral observations*

We quantified the number of attacks per focal bird per session (a proxy for aggressiveness), that is when the focal bird chased another bird away or pecked the bird with the bill (attacks/20 min). We also quantified the feeding events per session as the number of times the focal bird drank sugary water from the feeder or caught a fruit fly (a proxy for metabolism). These feeding counts were also a proxy for the well-being of the birds as lethargy and anorexia are signs of sickness (Hart 1988). Those two behaviors have been previously documented to be influenced by testosterone in other birds, especially in males (Wingfield et al. 2001; Soma 2006).

### *Blood sampling*

For sex determination, a droplet of blood was collected from each bird with a heparinized capillary tube after the section of the hallux's nail as described by Ruschi (1950) diluted in Queens Lysis Buffer (0.01 M EDTA; 0.01 M Tris; 0.01 M NaCl; 1% n-Lauroylsarcosine and pH 8.0) and stored in the fridge (~ 5 °C). For the measurement of the testosterone plasma levels, blood (mean µL, 30–80 µL) was collected from the jugular vein as described by Hoysak and Weatherhead (1991) otherwise from the trunk after decapitation. A droplet of blood was diluted in Queens Buffer for later sexing and for the rest of the blood, the plasma was separated by centrifugation (10 min at 6000 rpm) and stored in dry ice (~ – 80 °C).

## *Sexing protocol*

The blood diluted in Queens Lysis Buffer was used for genomic DNA extraction with the NucleoSpin Blood QuickPure DNA Extraction Kit (Macherey-Nagel 740569.250, Germany). We specifically designed primers pair for the black jacobins: floFus3-gold-fw (5'-TGCTCATGGCTAGAGGGACT-3') and floFus3-gold-rv (5'-TAATCCCCCTCTCCTCCCTA-3') that allowed successful sex determination with 92% of efficiency (calculated as the percentage of correct sexing result for those birds which gonads could be inspected, $n = 24$). The primers were designed based on the sequence information of a female *Florisuga fusca* genome assembly available at the Department for Behavioral Neurobiology of the Max Planck Institute for Biological Intelligence (former Max Planck Institute for Ornithology) in Seewiesen, Germany (manuscript in preparation). For the PCR reaction, we used 2 uL primer mix (10 pmol/µL each primer), 2 µL 10× Buffer B1 (Hot FirePol, Solis BioDyne, 01-02-00500, Estonia), 1.6 µL $MgCl^2$ 25 mM (Solis BioDyne, 01-02-00500, Estonia), 0.4 µL dNTPs 10 mM each nucleotide (Solis BioDyne, 02-31-00020, Estonia), 0.5 µL Taq Polymerase 5 u/µL (Hot FirePol, Solis BioDyne, 01-02-00500, Estonia) and 1 µL DNA template. All were diluted in 12.5 µL nuclease-free distilled water for a total volume of 20 µL. The PCR protocol for all samples was run in a T 3000 Thermocycler 48 (050–723, Biometra, Germany) and consisted of a first 15 min at 95 °C, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 45 sec and 72 °C for 50 sec with the last extension step at 72 °C for 2 min. The PCR products were separated by electrophoresis at 100 V between 1.5 and 2 hr in a 2% agarose gel stained with ethidium bromide.

## *Testosterone level measurements*

In total, we measured the plasma testosterone levels of 241 black jacobins. The samples for this profile were obtained within 10 consecutive days in December, during the middle of the breeding season (Ruschi 1964) for 57 females (52 adults and five juveniles) and 160 males (145 adults and 15 juveniles). First-year juveniles could be identified by differential plumage (Ruschi 1973). Since there was no significant difference between 1st-year juveniles and adults (Table S2 in Supplemental Data), they were pooled together.

The testosterone plasma levels were measured by radioimmunoassay as described by Goymann et al. (2006). In total, three assays were realized. The samples from the testosterone-treated birds were assayed 3 times, and the final testosterone value was obtained by the simple mean. These triplicates were conducted in different batches with consistent outcomes; thus, it is unlikely that the values obtained for testosterone-treated birds are an artifact. The lower detection limits were between 0.33 and 0.4 pg/mL with all samples above the detection limit. The extraction recovery of testosterone per assay was respectively 89, 90 and 87%, and the intra-assay coefficients of variation 7, 5 and 12.2%. The inter-assay coefficient of variation was 3.2%.

## *Statistical analyses*

The statistical analyses were performed using R version 3.0.3 (R Core Team 2014). All data and code are available in the online repository and accessible in this link (https://osf.io/9fs5w/).

The testosterone plasma levels (T-levels) were $\log_{10}$ transformed. For wild black jacobins (57 females and 160 males), to explore the effect of sex, body weight, age and day on T-levels, and effect of sex, T-levels, age and day on body weight, we used linear models. First, we fitted models including age (1st-year juveniles × adults) which had no effect both on T-levels (estimate = 0.22 ± 0.20, $t = 1.13$, $P = 0.262$, Table S2 in Supplemental Data) and body weight (estimate = 0.18 ± 0.11, $t = 1.60$, $P = 0.112$, Table S2 in Supplemental Data). Then we fitted models without age, and we plotted 1st-year juveniles and adults together. For captive black jacobins (10 females and 14 males), to compare T-levels among sex and experimental groups (control and T-treated), we used

Wilcoxon rank sum exact test (non-parametric). To compare control to wild birds, we randomly subsampled 10 T-levels values of each sex from the wild birds.

To compare gonadal and brain weight, we used Kruskal–Wallis rank sum test (non-parametric) to compare wild, control and T-treated. We used non-parametric statistics in all cases in which at least one of the groups (T-treated females) had only three data points. In these cases, we reported the median interquartile range (IQR) [25%, 75% quartiles]. Following a significant Kruskal–Wallis test, we performed Dunn's Kruskal–Wallis multiple comparisons as post hoc analysis with the function "dunnTest" implemented by the package "FSA" (Ogle et al. 2020). To compare body weight, we weighed the aviaries birds every time they were handled: when they were housed (before T), on the day of the implantation (T) and at the end of the experiment (after T). Then, we used repeated measures one-way ANOVA (parametric because all the assumptions were met). In the results, we reported the mean (± SD). Following a significant ANOVA, we performed Tukey multiple comparisons of means as post hoc analysis. In both post hoc analyses, we adjusted the *P*-values with the Bonferroni method. Given that the body weight of males had a wide variance, we further performed the F test to compare two variances (control and T-treated) with the "var.test" function. The significance level for all tests was $\alpha = 0.01$.

To test whether the testosterone treatment (T-treatment) affected the number of songs and chirps emitted by the black jacobins, their feeding behavior and aggressiveness in the aviaries, we used generalized linear mixed models (GLMMs). In all models, to account for the non-independence of the samples, we included "bird id" as a random effect. To determine the best fit model for each response variable (song, chirp, feeds or attack counts), we fitted models with all possible combinations of the predictors that were biologically meaningful for the respective response variable. Then, we compared the models using the differential Akaike's information criterion (dAIC) obtained by the function "AICtab" implemented by the "bbmle" package (Bolker & R Development Core Team 2020) and selected the most parsimonious models based on the lowest AIC (Burnham & Anderson 2016). We accessed the statistical significance of the best-fitting models' predictors via asymptotic Wald's z-tests ($P < 0.01$). In the results, we reported the estimated mean 95% CI [lower, upper confidence interval] for song and chirp counts models and the means of the simulated values from the posterior distributions and the predicted 95% credible intervals (CrI) [lower, upper credible interval] for feeding and attack counts models. Details about the fitting of the GLMMs the assessment of their assumptions and post-hoc analysis are provided in the Supplemental Data.

We performed a Principal Component Analysis to reduce song parameters using the function "prcomp" implemented by the package "car" (Fox & Weisberg 2018). We plotted the first two Principal Components (PC1 and PC2) using a customized version of the function "ggbiplot" implemented by the package "ggbiplot" (Vu 2011). We inspected visually the PC1 and PC2 plot to identify whether the birds were grouped by treatment and/or sex at the different time points (before, 1 and 2 weeks after T-treatment). We further investigated one of the variables that were heavily loaded within the PC1 vector (mean dominant frequency of the song) and one within the PC2 vector (song duration). We compared the averages of the dominant frequency and song duration for each of these four groups: control females, T-treated females, control males and T-treated males at the time points: before, 1 and 2 weeks after T-treatment using the Friedman Rank Sum Test which is an ideal statistic to use for a repeated measures type of experiment to determine if a particular factor has an effect (Scheff 2016). We also calculated the repeatability (R) using the function "rpt" implemented by the package "rptR" (Stoffel et al. 2017).

## RESULTS

### *Testosterone plasma levels and body weight differ between sexes in free-living birds during the breeding season as well as in captivity*

Adult males had higher testosterone levels [estimated mean 95% CrI (lower, upper credible interval); $n = 160$ birds, 102.19 pg/mL (90.24, 117.18)] than adult females [$n = 57$, 54.39 pg/mL (44.12; 6838)] (estimate = 0.52 ± 0.14, $t_{213} = 3.82$, $P < 0.001$; Fig. 1A, left panel) and had a higher body weight [7.85 g (7.74, 7.96)] (estimate = 0.26 ± 0.08, $t_{213}$ = 3.33, $P = 0.001$; Fig. 1A, right panel) than adult females [7.47 g (7.33, 7.60)] (Table 1). The model for T-levels indicated a strong effect of body weight (estimate = 0.34 ± 0.12, $t_{213}$ = 2.90, $P = 0.004$; Table 1). Thus, to verify whether the sex difference found in T-levels is not a side effect of the differences in body weight, we selected males ($n = 115$), which fall into the weight range of the females (female weight mean ± SD) and compared T-values again. The difference remained and females still had lower T-levels than males ($t = -3.40$, df = 142.27, $P = 0.001$). The model for T-levels also indicated a strong effect of sampling time (estimate = − 0.29 ± 0.02, $t_{213} = -13.79$, $P < 0.001$; Table 1). This temporal correlation may reflect a decline in the baseline testosterone in the second half of their breeding season. Furthermore, nearly 7% of the males ($n = 11$) showed T-levels above 1000 pg/mL and 30% of the females ($n = 17$) showed T-levels above 100 pg/mL which is expected for birds in breeding condition in the tropics (e.g. Goymann et al. 2004; Goymann & Wingfield 2014).

T-levels of captive (aviaries) control birds were inside the range of the free-living birds in both females (W = 26, $P = 0.077$) and males (W = 44, $P = 0.968$); therefore, captivity did not affect T-levels. T-levels of T-treated birds were higher than controls in both females (W = 0, $P < 0.017$) and males (W = 0, $P = 0.001$): 2.5k-fold above wild average for females and 300-fold for males (Table 2, Fig. 1B).

### *Captivity, but not testosterone treatment decreased body weight only in females*

Captivity decreased the weight of females, but not that of males. The body weight of females changed with time [F (2,18) = 6.47, $P = 0.008$], but not between treatment groups (W = 16, $P = 0.267$). Before T application, females' weight did not differ between the control (mean ± SD, 7.1 g ± 0.3) and T-treated (7.2 g ± 0.3) groups. At the T-treatment day, weight of all females increased slightly, but not significantly compared to before ($P = 0.723$) (control, 7.3 g ± 0.1; T-treated, 7.3 g ± 0.4). Two weeks after the testosterone treatment (after T) weight decreased significantly compared to baseline ($P = 0.041$) and to T day ($P = 0.008$) (control, 7 g ± 0.4; T-treated 6.8 g ± 0.4) (Fig. 1C, upper panel). This effect was not observed in males, as their weight did not change in time [F (2,26) = 1.36, $P = 0.276$] and not between groups [F (8,4) = 0.21, $P = 0.058$]: Before T: control group 7.7 ± 0.4 g and T-treated group 7.6 ± 0.4 g. On the T-treatment day: control group 7.9 ± 0.4 g and T-treated group 8 g ± 0.6; after T: control group 7.9 ± 0.5 g and T-treated group 7.7 ± 1 g (Fig. 1C, lower panel).

### *Testosterone treatment increased oviduct weight but did not affect testes weight*

The oviduct weight differed between groups (H = 6.56, df = 2, $P = 0.038$): wild ($n = 6$), captivity control ($n = 3$), and captivity T-treated ($n = 7$) (see Table 3 for medians). Ovary weight was lower in control captive birds than in the wild ($P = 0.021$) and T-treated females ($P = 0.017$), but this was not significant after Bonferroni correction ($P$ adj. = 0.063 and 0.051, respectively) (Fig. 2A, left panel). However, the oviduct weight differed between groups (H = 11.21, df = 2, $P = 0.004$) with heavier oviducts in T-treated (by five and six-fold, respectively) compared to the wild ($P$ adj. = 0.011) and to control ($P$ adj. = 0.025) (Fig. 2A, right panel; Table 3). The testis weight was similar between groups [F (2,18) = 2.14, $P = 0.147$] (Fig. 2C, Table 3).

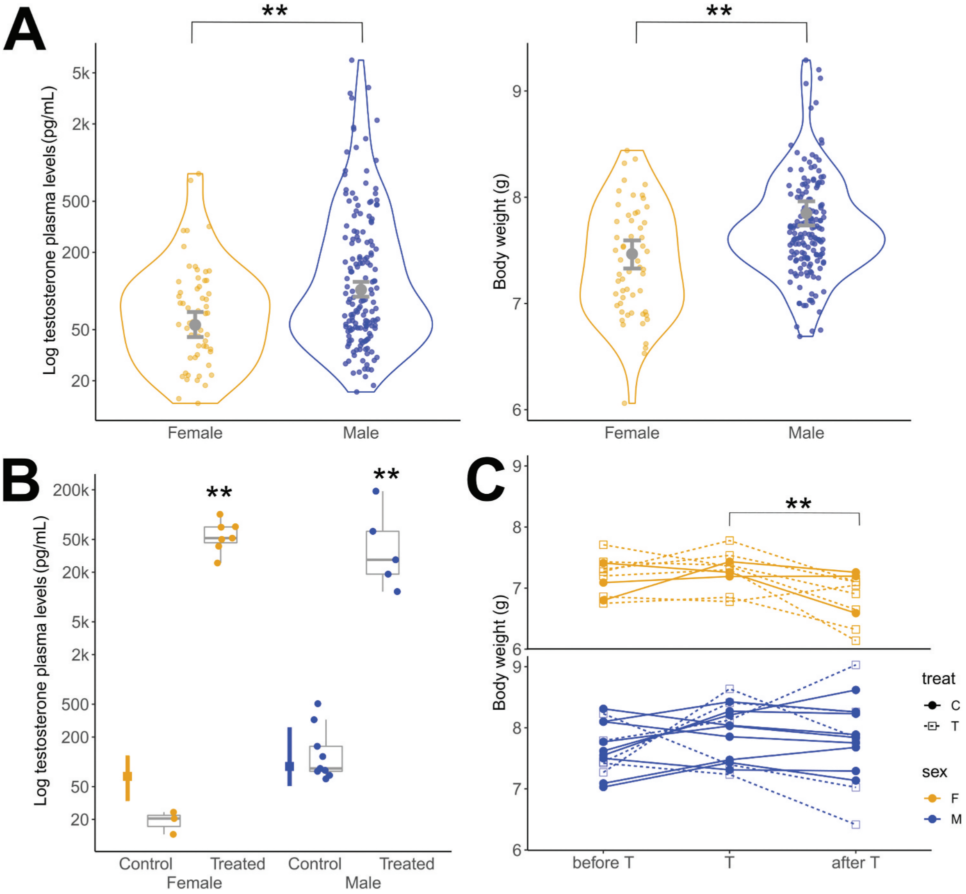

Fig. 1. — Circulating levels of plasma testosterone (T plasma levels) and body weight of wild and captive black jacobins. All T plasma levels are represented on a logarithmic scale (Log10). (A) Wild black jacobin is sexually dimorphic in the T plasma levels (left panel) and body weight (right panel). The outlines of the violin plot represent the proportion of the data located in each value given by kernel probability density. The data points represent individual measurements of females ($n = 57$, lighter points) and males ($n = 160$, darker points). The error bars in gray represent the fitted mean and the 95% credible interval given by the linear models. (B) Captive black jacobins under T implantation had T plasma levels increased 2000-fold for females and 300-fold for males in relation to those of the same sex that received placebo implants (controls). The error bars on the left indicate the median and interquartile range (25 to 75%) of the wild black jacobins as a reference to the physiological range. The box plots indicate the median and interquartile range (25 to 75%) of the captive black jacobins. The data points represent individual measurements of females (control, $n = 3$ and T-treated, $n = 7$; lighter points) and of males (control, $n = 9$ and T-treated, $n = 5$; darker points). (C) Females lost weight in captivity independently of T-treatment. The body weight of the captive black jacobins was obtained at three time points: on the day there were placed in the aviaries (before T), on the day of the T implantation (T), and at the end of the experiment (after T). Although T-treated black jacobins presented T levels above the physiological range, neither females nor males had their body weight affected by the treatment, instead, females were affected by the captive condition.

Table 1.

Results of linear models explaining variation in the circulating testosterone plasma levels (T plasma levels) and body weight of wild black jacobins. In the wild, T plasma levels were affected by sex, body weight, and time, whereas body weight was affected by sex and T plasma levels. In bold are the significant predictors ($P < 0.01$).

| | Estimate | SE | t value | *P*-value |
|---|---|---|---|---|
| T plasma levels | | | | |
| Intercept | 5.793 | 0.925 | 6.260 | **< 0.001** |
| Sex (ref. female) | 0.518 | 0.136 | 3.823 | **< 0.001** |
| Body weight | 0.344 | 0.119 | 2.898 | **0.004** |
| Time (days) | – 0.289 | 0.021 | – 13.790 | **< 0.001** |
| Body weight | | | | |
| Intercept | 6.404 | 0.365 | 17.537 | **< 0.001** |
| Sex (ref. female) | 0.258 | 0.077 | 3.330 | **0.001** |
| Log T plasma | 0.110 | 0.038 | 2.898 | **0.004** |
| Time (day) | – 0.290 | 0.016 | 2.258 | 0.025 |

Table 2.

Median and quartiles of the testosterone plasma levels for wild and captive black jacobins grouped by sex and treatment: birds treated with placebo (control) and birds treated with testosterone implants (T-treated).

| | Sex | | n | Testosterone plasma levels (pg/mL) | | |
|---|---|---|---|---|---|---|
| | | | | Median | First quartile (25%) | Third quartile (75%) |
| Wild | F | Adult | 52 | 67.08 | 33.22 | 110.67 |
| | | Juvenile | 5 | 37.40 | 37.33 | 146.41 |
| Control | | | 3 | 20.51 | 16.84 | 22.51 |
| T-treated | | | 7 | 51845.30 | 45697.95 | 70807.90 |
| Wild | M | Adult | 145 | 89.29 | 51.58 | 264.05 |
| | | Juvenile | 15 | 73.02 | 41.17 | 165.64 |
| Control | | | 9 | 83.09 | 76.73 | 154.33 |
| T-treated | | | 5 | 28302.50 | 18927.90 | 62721.50 |

No difference in brain weight was found between groups in either females and males [females, median (25, 75) quartiles: wild 0.235 g (0.224, 0.242), control 0.229 g (0.219, 0.232) and T-treated 0.214 g (0.212, 0.223), ($H = 1.79$, $df = 2$, $P = 0.409$); males, wild 0.241 g (0.229, 0.245), control 0.221 g (0.213, 0.238) and T-treated 0.230 g (0.227, 0.237); $F(2,18) = 2.98$, $P = 0.076$; Fig. 2B].

Table 3.

Median and quartiles of testes, ovaries, and oviduct weights for wild and captive black jacobins grouped by treatment: birds treated with placebo (control) and birds treated with testosterone implants (T-treated).

| Treatment | n | Median | 2.5% | 97.5% |
|---|---|---|---|---|
| Ovaries weight (mg) | | | | |
| Wild | 6 | 24 | 14.75 | 35.5 |
| Control | 3 | 4 | 2.5 | 7.5 |
| T-treated | 7 | 27 | 15 | 35.5 |
| Oviduct weight (mg) | | | | |
| Wild | 6 | 46 | 28.75 | 70.75 |
| Control | 3 | 38 | 27.5 | 54 |
| T-treated | 7 | 215 | 197.5 | 226 |
| Testes weight (mg) | | | | |
| Wild | 7 | 24 | 23 | 26.5 |
| Control | 9 | 21 | 21 | 24 |
| T-treated | 5 | 21 | 19 | 22 |

### *Testosterone treatment did not affect song or chirp emissions of males and females*

Males tended to sing more than females (% of male plus female songs emitted in a time window) before T-treatment (males: 79%, females: 21%), 1 week after T-treatment (males: 76%, females: 24%) and 2 weeks after T-treatment (males: 57%, females: 43%); however, this difference was not significant (fixed effects estimate = 0.46 ± 0.54, z = 0.84, $P = 0.398$, Table 4). The likelihood ratio test showed that T-treatment did not affect the emission of song in males and females ($\chi^2 = 6.12$, df = 4, $P = 0.191$). Disregarding T-treatment or sex, black jacobins sang less in the afternoon than in the morning in the aviaries (linear: fixed effects estimate = – 20.46 ± 2.81, z = – 7.29, $P <$ 0.001 and quadratic term: fixed effects estimate = – 8.13 ± 1.73, z = – 4.71, $P < 0.001$, Table 4, Fig. S4 in Supplemental Data). This singing pattern is similar to the daily variation in singing usually found in birds in the wild (McNamara et al. 1987; A. Monte personal observation). Disregarding T-treatment, 2 weeks after T-treatment, males sang less than before (fixed effects estimate = – 0.91 ± 0.27, z = – 3.39, $P = 0.001$, Table 4). Males emitted fewer songs [predicted means 95% CI (lower, upper confidence interval)] the longer they were in the aviaries [before T-treatment: control 26.2 songs/20 min (14.8, 46.7) and T-treated 14.8 (6.3, 35), 1 week after T-treatment: control 17.5 songs/20 min (9.2, 33.4) and T-treated 10 (4.5, 22) and 2 weeks after T-treatment: control 15.8 songs/20 min (8.2, 30.2) and T-treated 6.1 (2.6, 14)] and females, in special those from the control group, sang more [before T-treatment: control 16.6 (6.5, 42.5) and T-treated 12.8 (6.4, 25.4), 1 week after T: control 18.7 (7.1, 49.5) and T-treated 14.5 (7.4, 28.5) and 2 weeks after T: control 24.8 songs/20 min (9.9, 62) and T-treated 13 (6.8, 24.9), Fig. 3B, Table S4 in Supplemental Data]. Dispersion of the data tended to be greater in males in

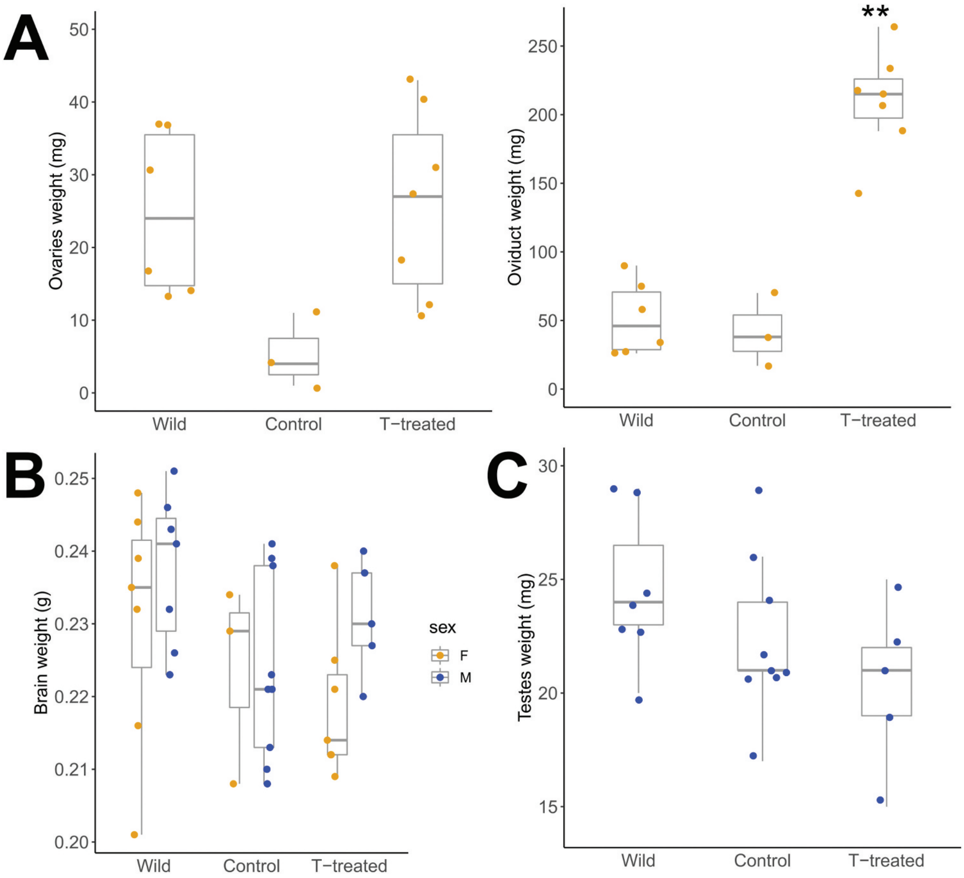

Fig. 2. — Although both sexes of black jacobin have androgen receptors in their gonads, the testosterone treatment (T-treatment) affected only the gonads of the females. (A) The ovary weight (left panel) of wild and T-treated females does not differ, although the ovary of control females is slighter lighter. The oviduct weight (right panel) of T-treated females is significantly heavier than wild and control females. The data points represent individual measurements of females (wild, $n = 6$; control, $n = 3$ and T-treated, $n = 7$). (B) The brain weight of both sexes wild, control and T-treated black jacobins did not differ. The data points represent individual measurements of females (wild, $n = 7$; control, $n = 3$ and T-treated, $n = 7$; lighter dots) and males (wild, $n = 7$; control, $n = 9$ and T-treated, $n = 5$; darker dots). (C) The testes weight of wild, control and T-treated males also did not differ (wild, $n = 7$; control, $n = 9$ and T-treated, $n = 5$). All box plots indicate the median and interquartile range (25 to 75%).

comparison to female (dispersion effects, estimate = 0.54 ± 0.22, z = 2.48, $P = 0.013$, Table 4).

Males and females emitted similar number of chirps (Fig. 3C) (% of total male plus female chirps per time window; before T-treatment: males: 56% and females 44%; 1 week after T: males 58%, females 42%; 2 weeks after T: males 49%; females 51%, fixed effects estimate = 0.57 ± 0.67, z = 0.85, $P = 0.395$, Table 4). The likelihood ratio test indicated that T-treatment also did not affect the emission of chirps in both sexes ($\chi^2 = 12.75$, df = 8, $P = 0.121$). Disregarding T-treatment or sex, black jacobins

Table 4.

Results of the generalized linear mixed model explaining variation in the number of songs emitted per observation session (20 min) of captive black jacobins. For a better fit, the hour of the day was transformed into linear, quadric and cubic terms. The birds tend to emit fewer songs throughout the day and males sing less than females 2 weeks after the testosterone treatment. In bold are the significant predictors ($P < 0.01$).

| | Estimate | SE | z-value | *P*-value |
|---|---|---|---|---|
| Conditional fixed effects | | | | |
| (Intercept) | 4.801 | 1.844 | 2.604 | 0.009 |
| T-treatment (ref. control) | – 0.265 | 0.564 | – 0.471 | 0.638 |
| Time after 1 week (ref. before T-treatment) | 0.120 | 0.285 | 0.419 | 0.675 |
| Time after 2 weeks (ref. before T-treatment) | 0.402 | 0.242 | 1.661 | 0.097 |
| Sex male (ref. female) | 0.457 | 0.541 | 0.844 | 0.398 |
| Temperature | – 0.005 | 0.041 | – 0.125 | 0.900 |
| Humidity | – 0.022 | 0.016 | – 1.341 | 0.180 |
| **Hour 1** (linear term) | **– 20.456** | **2.807** | **– 7.287** | **< 0.001** |
| **Hour 2** (quadratic term) | **– 8.133** | **1.725** | **– 4.714** | **< 0.001** |
| Hour 3 (cubic term) | – 3.005 | 1.683 | – 1.785 | 0.074 |
| T-treatment: sex male | – 0.306 | 0.709 | – 0.432 | 0.666 |
| Time after 1 week: sex male | – 0.523 | 0.289 | – 1.807 | 0.071 |
| **Time after 2 weeks: sex male** | **– 0.910** | **0.268** | **– 3.391** | **0.001** |
| T-treatment: time after 1 week | 0.011 | 0.276 | 0.038 | 0.969 |
| T-treatment: time after 2 weeks | – 0.384 | 0.265 | – 1.448 | 0.148 |
| Conditional zero-inflation effects | | | | |
| (Intercept) | – 0.661 | 0.955 | – 0.692 | 0.489 |
| T-treatment | 0.049 | 0.758 | 0.065 | 0.949 |
| Time after 1 week | 1.002 | 0.446 | 2.246 | 0.025 |
| Time after 2 weeks | – 0.191 | 0.428 | – 0.447 | 0.655 |
| Sex male | – 0.431 | 0.843 | – 0.511 | 0.610 |
| Hour | – 0.007 | 0.076 | – 0.090 | 0.929 |
| T-treatment: sex male | – 0.977 | 1.727 | – 0.565 | 0.572 |
| Time after 1 week: sex male | – 1.423 | 0.792 | – 1.797 | 0.072 |
| Time after 2 weeks: sex male | – 2.230 | 4.001 | – 0.557 | 0.577 |
| Conditional dispersion effects | | | | |
| (Intercept) | 3.279 | 0.247 | 13.269 | 0.000 |
| **Sex male** (ref. female) | **0.541** | **0.218** | **2.484** | **0.013** |
| Aviary A2 (ref. aviary A1) | 0.202 | 0.281 | 0.721 | 0.471 |

(*Continued*)

Table 4.

(*Continued*)

| | Estimate | SE | z-value | *P*-value |
|---|---|---|---|---|
| Aviary A3 (ref. aviary A1) | 0.402 | 0.251 | 1.601 | 0.109 |
| Aviary A4 (ref. aviary A1) | − 0.541 | 0.306 | − 1.768 | 0.077 |
| Random effect | Variance | SD | | |
| Conditional random effects | | | | |
| Bird id (intercept) | 0.509 | 0.714 | | |
| Zero-inflation random effects | | | | |
| Bird id (intercept) | 0.650 | 0.806 | | |

emitted fewer chirps one (fixed effects, estimate = − 1.30 ± 0.38, z = − 3.39, $P < 0.01$) and 2 weeks after T-treatment (fixed effects, estimate = − 0.96 ± 0.35, z = − 2.77, $P < 0.01$) (Table 5, Fig. 3D). When the birds were not emitting chirps, they were feeding in the feeders (zero-inflated effects, estimate = − 1.14 ± 0.34, z = − 3.34, $P < 0.01$). T-treatment and sex jointly had an effect closer to a significance for T-treated males in relation to controls and females (fixed effects interaction between T-treatment and sex, estimate = − 2.12 ± 0.87, z = − 2.44, $P = 0.015$). The means estimated from the best-fitting model for chirp counts show that T-treated females produced 15.67 chirps (7.26, 33.81) [estimated mean (lower, upper 95% CI)] before, 7.19 chirps (3.25, 15.9) 1 week after T and 12.73 chirps (6.02, 26.93) 2 weeks after T (Fig. 3D, Table S5 in Supplemental Data). The dispersion of the data explained by the model was greater in aviary A1 in relation to aviary A2 (dispersion effects, estimate = − 1.48 ± 0.25, z = − 5.84, $P < 0.01$), A3 (dispersion effects, estimate = − 0.68 ± 0.25, z = − 2.69, $P < 0.01$) and A4 (dispersion effects, estimate = − 1.29 ± 0.25, z = − 5.13, $P < 0.01$).

### *Testosterone treatment did not affect the acoustic parameters of the song of males and females*

Black jacobin song can be composed of one (single), two (double) or three (triplets) subunits (Fig. S3A in Supplemental Data). The proportion of singles, doubles and triplets was stable before, 1 week and 2 weeks after T-treatment, because these proportions showed high repeatability within birds ($R = 0.46 \pm 0.11$, $P < 0.01$). Singles were rare, most of the birds emitted triplets and three males emitted mostly only doubles (Fig. S3B in Supplemental Data).

Concerning the acoustic parameters of the song, a Principal Components Analysis (PCA) reduction revealed the first three eigenvalues to contain ~ 75% of the overall variation. PC1 (~ 36% of the variation) described mainly spectral parameters (such as frequency mean, frequency standard deviation, median, lower (25%) and upper (75%) frequency quantiles, spectral entropy, spectral flatness, dominant frequency, minimum dominant frequency, dominant frequency range, start dominant frequency, as well as peak frequency mean); PC2 (~ 23% of the variation) described

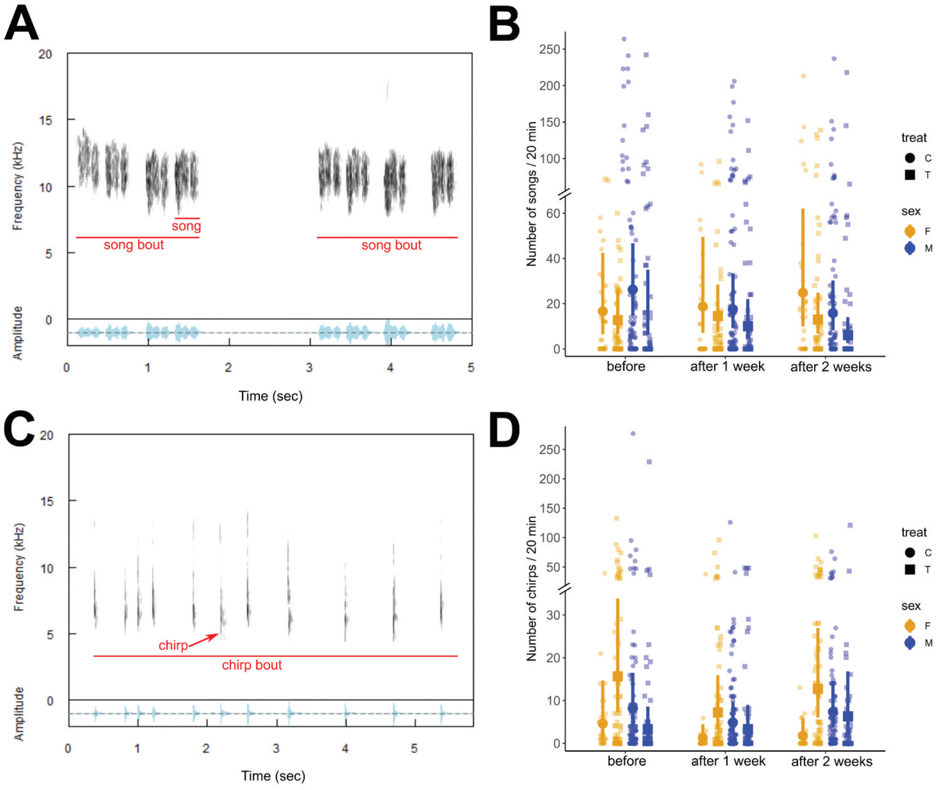

Fig. 3. — Testosterone treatment (T-treatment) did not affect the number of songs and chirps emitted by both sexes in black jacobin. (A) Spectrogram (frequency with time) and oscillogram (relative amplitude with time) of the black jacobin's song and (C) chirp. The song is composed of a single high-pitched syllable (vocal unit) that can be repeated multiple times in a song bout. The chirp is characterized by a wide frequency bandwidth in a fast duration that can also be repeated consecutively in a chirp bout. (B) The number of songs produced by both sexes in control and T-treated groups did not differ before, 1 or 2 weeks after T-treatment. (D) Although T-treated females tended to consistently produce more chirps, the number of chirps produced by both sexes in control and T-treated groups also did not differ significantly before, 1 or 2 weeks after T-treatment. The data points represent song or chirp counts obtained per bird in 20 min of observation. The error bars represent the fitted mean and the 95% confidence interval given by the generalized linear mixed models.

temporal parameters [duration, median time, upper (75%) quantile and time interquartile range as well as time entropy] and PC3 (~ 16% of the variation) described skewness and kurtosis (Tables S6-S7 in Supplemental Data). PC1 and PC2 did not separate control from T-treated birds neither in females nor in males, although showing a slight separation between sexes (Fig. 4A).

We further investigated the parameters with the highest eigenvalue in PC1, the mean dominant frequency of the song, and in PC2, the song duration. The mean dominant frequency of the male and female songs was similar in control and T-treated groups across time ($F_r = 9$, df = 3, $P = 0.029$). The mean dominant frequencies

Table 5.

Results of the generalized linear mixed model explaining variation in the number of chirps emitted per observation session (20 min) of captive black jacobins. The birds tend to emit fewer chirps across time. In bold are the significant predictors ($P < 0.01$).

| | Estimate | SE | z-value | *P*-value |
|---|---|---|---|---|
| Conditional fixed effects | | | | |
| (Intercept) | 1.359 | 0.606 | 2.243 | 0.025 |
| T-treatment (ref. control) | 1.223 | 0.694 | 1.763 | 0.078 |
| **Time after 1 week** (ref. before T-treatment) | **– 1.300** | **0.384** | **– 3.388** | **0.001** |
| **Time after 2 weeks** (ref. before T-treatment) | **– 0.960** | **0.347** | **– 2.766** | **0.006** |
| Sex (ref. female) | 0.569 | 0.669 | 0.850 | 0.395 |
| Feeding | 0.085 | 0.048 | 1.754 | 0.079 |
| T-treatment: sex male | – 2.125 | 0.869 | – 2.444 | 0.015 |
| Time after 1 week: sex male | 0.789 | 0.379 | 2.085 | 0.037 |
| Time after 2 weeks: sex male | 0.856 | 0.359 | 2.383 | 0.017 |
| T-treatment: time after 1 week | 0.521 | 0.352 | 1.482 | 0.138 |
| T-treatment: time after 2 weeks | 0.752 | 0.342 | 2.195 | 0.028 |
| Conditional zero-inflation effects | | | | |
| (Intercept) | – 0.262 | 1.255 | – 0.209 | 0.835 |
| T-treatment | 0.265 | 1.412 | 0.187 | 0.851 |
| Time after 1 week | – 0.052 | 0.691 | – 0.075 | 0.940 |
| Time after 2 weeks | – 3.322 | 5.508 | – 0.603 | 0.546 |
| Sex male | 0.521 | 1.208 | 0.431 | 0.666 |
| **Feeding** | **– 1.143** | **0.342** | **– 3.342** | **0.001** |
| T-treatment: sex male | – 0.812 | 1.595 | – 0.509 | 0.610 |
| Time after 1 week: sex male | – 0.279 | 0.950 | – 0.293 | 0.769 |
| Time after 2 weeks: sex male | 2.775 | 5.586 | 0.497 | 0.619 |
| Conditional dispersion effects | | | | |
| (Intercept) | 0.227 | 0.241 | 0.940 | 0.348 |
| **Aviary A2** (ref. aviary A1) | **– 1.475** | **0.253** | **– 5.836** | **< 0.001** |
| **Aviary A3** (ref. aviary A1) | **– 0.675** | **0.251** | **– 2.687** | **0.007** |
| **Aviary A4** (ref. aviary A1) | **– 1.291** | **0.252** | **– 5.130** | **< 0.001** |
| Random effect | Variance | SD | | |
| Conditional random effects | | | | |
| Bird id (intercept) | 0.797 | 0.893 | | |
| Session (intercept) | 0.194 | 0.440 | | |
| Zero-inflation random effects | | | | |
| Bird id (intercept) | 0.000 | 0.000 | | |

[data are: median and (25, 75) quartiles] of control females were 10.9 kHz (10.5, 11.3) before T ($n = 178$ songs), 10.8 kHz (10.3, 11.1) ($n = 107$ songs) 1 week after T and 10.9 kHz (10.6, 11.1) ($n = 146$) 2 weeks after T-treatment. For the T-treated females, the mean dominant frequencies were 11 kHz (10.5, 11.3) before T ($n = 342$ songs), 10.9 kHz (10.5, 11.2) 1 week after T ($n = 201$ songs) and 11 kHz (10.7, 11.2) 2 weeks after T ($n = 370$ songs). For control males, mean dominant frequencies were 11.1 kHz (10.8, 11.4) before T ($n = 624$ songs), 11.1 kHz (10.8, 11.4) 1 week after T ($n = 545$ songs) and again 11.1 kHz (10.8, 11.4) 2 weeks after T ($n = 466$ songs). For T-treated males, the mean dominant frequencies were 11.3 kHz (11, 11.5) before T ($n = 268$ songs), 11.2 kHz (10.9, 11.4) 1 week after T ($n = 228$ songs) and 11.1 kHz ± (10.8, 11.3) 2 weeks after T treatment ($n = 185$ songs) (Fig. 4B, left panel).

The same consistency was observed in the medians of the song duration. Control females had nearly the same median before, 1 and 2 weeks after T-treatment [0.32 sec median interquartile range (0.30, 0.33)]. T-treated females had little variation in their medians before [0.30 sec (0.28, 0.31)], 1 [0.30 sec (0.29, 0.32)] and 2 weeks after T-treatment [0.30 sec (0.29, 0.31)]. Control males had also nearly the same median before, 1 and 2 weeks after T-treatment [0.29 sec (0.25, 0.31), except for the median 1 week after T-treatment which was 0.30 sec]. T-treated males tended to sing slighter shorter songs before [0.29 sec (0.24, 0.31)] in relation to 1 [0.30 sec (0.28, 0.32)] and 2 weeks after T-treatment [0.31 sec (0.27, 0.32)]. Although male's songs were around 10 msec shorter than those of the females, the averages of the control females, control males, T-treated females and T-treated males were similar before, 1 and 2 weeks after the T-treatment ($F_r = 7$, df = 3, $P = 0.072$) (Fig. 4B, right panel). Both the dominant frequency and duration of the song were highly repeatable within birds (dominant frequency: $R = 0.40 \pm 0.07$, $P < 0.001$; duration: $R = 0.37 \pm 0.07$, $P < 0.001$).

### *Testosterone treatment maintained high aggressiveness in males in comparison with a decrease in placebo birds*

We counted the number of attacks (chasing other birds or beak pecking on another bird) as a proxy for aggressiveness. In all observations and regardless of sexes, the observed birds attacked another bird twice or more in 55% of the observations, once in 37% and did not attack in 8%, which indicates the intrinsic high aggressiveness of the black jacobins. The number of attacks was weakly associated with song counts ($r = 0.16$, $P < 0.001$).

The number of attacks before T compared to 2 weeks after T was influenced by time (estimate = $-0.67 \pm 0.19$, z = $-3.58$, $P < 0.001$), by temperature (estimate = $-0.20 \pm 0.05$, z = $-3.76$, $P < 0.001$) and by the feeding counts to the feeders (estimate = $0.25 \pm 0.03$, z = 7.21, $P < 0.001$) (Table 6). A post-hoc analysis of interactions indicated that the control birds of both sexes attacked less as longer they spent in captivity (females $\chi^2 = 12.79$, df = 2, $P = 0.007$ and males $\chi^2 = 23.37$, df = 2, $P < 0.001$), whereas T-treated birds of both sexes kept the number of attacks similar to baseline values (females $\chi^2 = 3.56$, df = 2, $P = 0.674$ and males $\chi^2 = 3.68$, df = 2, $P = 0.635$). The number of attacks of the T-treated females was relatively low before T-treatment by chance [1.84 attacks 95% CrI (1.36, 2.46)], but these values remained similar 1 [1.59 attacks (1.18, 2.13)] and 2 weeks after the T-treatment [1.37 attacks (1.03, 1.89)]. The number of attacks of the T-treated males attacked was similar before [2.65 attacks (1.93, 3.70)], 1 week [2.68 attacks (1.97, 3.76)] and 2 weeks after the T-treatment [2.03 attacks (1.46, 2.87)] (Fig. 5).

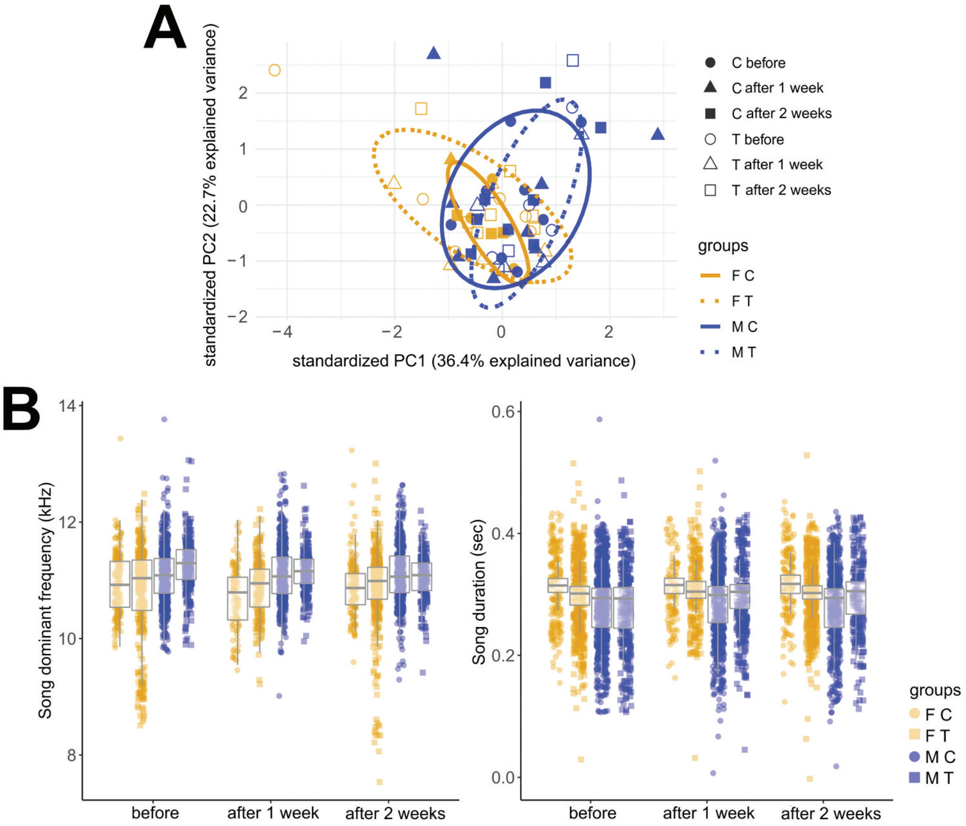

Fig. 4. — Testosterone treatment (T-treatment) did not affect the acoustic parameters of the song in both sexes of black jacobin. (A) Biplot including the first two components (PC1 versus PC2) of a Principal Components Analysis including 26 acoustic parameters of black jacobin's song (see Table S1 in Supplemental Data for a list of the parameters). The first component PC1 explained 36% of the overall variation and described mainly spectral parameters such as dominant frequency. The second component PC2 explained 23% of the variation and described temporal parameters such as duration. Each data point represents the mean of the PCA loads per bird in three time points: before, 1, and 2 weeks after T-treatment. If T-treatment had affected and differentiated the acoustic parameters of the song, we expected to see a clear separation of the PCA points of T-treated birds (dashed circle) after the T-treatment in comparison to the controls (solid circle). Instead, the biplot shows a complete overlap between T-treated and controls in both sexes indicating that there are no clear sex or treatment differences in the acoustic parameters of the song before, 1 or 2 weeks after the T-treatment. Nevertheless, a small separation between sexes can be observed, because some males had consistently more positive loadings along PC1 axis and some females more negative reflecting a trend of male's song toward higher pitch independent of treatment or time. (B) The dominant frequency (left panel) and the duration (right panel) of black jacobin's song in both sexes of control and T-treated groups did not differ before, 1 or 2 weeks after T-treatment. Males consistently tended to sing higher-pitched songs than females, whereas females consistently tended to sing longer songs than males, but the differences were not significant. All box plots indicate the median and interquartile range (25 to 75%). Each data point indicates an individual song measurement (see Table 3 for details on sample size). FC, female control; FT, female T-treated; MC male control and MT, male T-treated.

Table 6.

Results of the generalized linear mixed model explaining variation in the number of attacks per observation session (20 min) of captive black jacobins. For a better fit, all continuous predictors were mean-centered (z-scored). The number of attacks was affected by time 2 weeks in relation to before treatment, temperature and number of feeds on the feeder. In bold are the significant predictors ($P < 0.01$).

| | Estimate | SE | z-value | *P*-value |
|---|---|---|---|---|
| (Intercept) | 1.079 | 0.195 | 5.540 | **< 0.001** |
| T-treatment (ref. control) | – 0.456 | 0.210 | – 2.174 | 0.030 |
| Time after 1 week | – 0.318 | 0.178 | – 1.789 | 0.074 |
| **Time after 2 weeks** | – 0.668 | 0.187 | – 3.575 | **< 0.001** |
| Sex (ref. female) | – 0.043 | 0.204 | – 0.212 | 0.832 |
| Hour (z-scored) | 0.054 | 0.046 | 1.170 | 0.242 |
| **Temperature** (z-scored) | – 0.202 | 0.054 | – 3.765 | **< 0.001** |
| **Feeding** (z-scored) | 0.251 | 0.035 | 7.210 | **< 0.001** |
| T-treatment: time after 1 week | 0.171 | 0.163 | 1.047 | 0.295 |
| T-treatment: time after 2 weeks | 0.372 | 0.177 | 2.094 | 0.036 |
| T-treatment: sex male | 0.407 | 0.246 | 1.656 | 0.098 |
| Time after 1 week: sex male | 0.157 | 0.168 | 0.934 | 0.350 |
| Time after 2 weeks: sex male | 0.031 | 0.182 | 0.173 | 0.862 |

The number of times the birds feed on the feeders in the aviary was not affected by T-treatment (estimate = – 0.06 ± 0.20, z = – 0.30, $P = 0.761$), nor by the time they spent in captivity 1 (estimate = – 0.27 ± 0.14, z = – 1.93, $P = 0.054$) or 2 weeks after T-treatment (estimate = 0.14 ± 0.14, z = 1.06, $P = 0.290$) in relation to the baseline before T (Fig. S5, Table S3 in Supplemental Data). However, T-treated males and females tended to catch more fruit flies 2 weeks after T-treatment. The best-fitting model for fruit-fly captures indicated that the number of captures was affected by the interaction of T-treatment and the time they spent in captivity after 2 weeks (estimate = 1.57 ± 0.43, z = 3.68, $P < 0.001$) and also by the number of feeding from the feeders (estimate = 0.27 ± 0.05, z = 5.39, $P < 0.001$, Table S3 in Supplemental Data). A post hoc analysis of interactions indicated that the control birds of both sexes captured the same number of fruit flies across time (females $\chi^2 = 7.34$, df = 2, $P = 0.102$ and males $\chi^2 = 4.86$, df = 2, $P = 0.353$), whereas T-treated birds tended to capture more fruit-flies as longer they spent in captivity, but this difference was non-significative (females $\chi^2 = 9.86$, df = 2, $P = 0.029$ and males $\chi^2 = 9.71$, df = 2, $P = 0.031$) (Fig. S5 in Supplemental Data).

## DISCUSSION

This study presents the first profile of testosterone plasma levels (T-levels) and the effects of testosterone treatment (T-treatment) on the vocal behavior in females and males of a hummingbird species, the black jacobin. Although T-levels of black

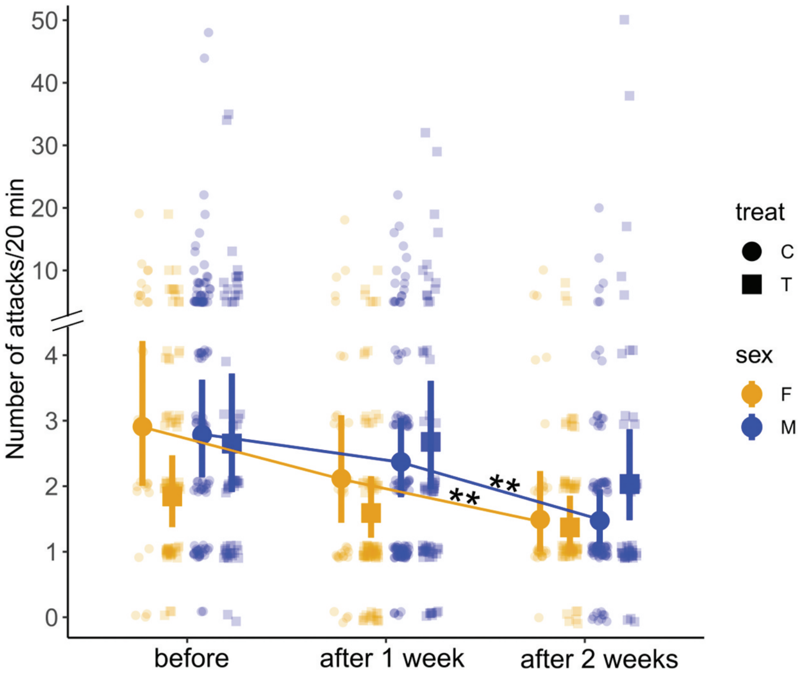

Fig. 5. — Testosterone treatment (T-treatment) maintained aggressiveness of T-treated birds towards initial values (post hoc analysis of the mixed-effect model; female $\chi^2_2 = 3.56$, $P = 0.67$; male, $\chi^2_2 = 3.68$, $P = 0.63$), whereas controls become less aggressive with time (female, $\chi^2_2 = 12.79$, $P = 0.007$; male, $\chi^2_2 = 23.37$, $P < 0.001$). The data points represent attack counts obtained per bird in 20 min of observation as a proxy for aggressiveness. The error bars represent the estimated mean and the 95% credible interval given by the generalized linear mixed model.

jacobins in the wild were higher in males, vocal behaviors of control and testosterone treated males and females did not differ. Therefore, the vocal behavior of these hummingbirds seems insensitive to testosterone in contrast to species of other avian families in which testosterone either affects vocal output or vocal pattern or both (Gahr 2014, 2020). Note that T-levels tended to get lower in both sexes towards the end of the breeding season (March compared to December), indicating that they can change during the breeding season or/and throughout the year. In relation, 7% of the males had T-levels comparable to reproductively active males of other species in the tropics (Goymann et al. 2004), which, too, indicates that T-levels vary during the breeding season. The large individual differences of plasma T-levels are likely due to the long breeding period of tropical species, which leads to de-synchronized reproductive activities within populations.

The T-levels of wild populations of hummingbirds were so far documented in only a few species given the methodological constraints to obtaining blood samples of such small birds. Previously, T-levels of hummingbirds were estimated from measurements of testosterone concentrations in the cloacal fluid of three species, all of the clade Bees, during the breeding season. In the black-chinned hummingbird (*Archilochus alexandri*), males had higher T-levels than females, whereas in Anna's hummingbird (*Calypte anna*) and the rufous hummingbird (*Selasphorus rufus*) males and females had similar T-levels (González-Gómez et al. 2014). Surprisingly, although these species weigh about half of black jacobin's body weight, their average T-levels were about 90-fold higher than that of black jacobins, their T-levels ranged between ~ 8 and ~ 23 ng/mL (González-Gómez et al. 2014), whereas the T-levels of wild black jacobins ranged (25 to 75% quantiles) from ~ 0.03 to 0.1 in females and ~ 0.05 to 0.2 ng/mL in males (Table 2). However, a fraction of males and females had testosterone levels typical of reproductively active male and female birds of the temperate zones with T-levels above 1 ng/mL plasma for males and 100 pg/mL plasma for females. This difference may reflect (i) the distant relationship of black jacobins (clade Topazes) and the Bees in the hummingbird family; (ii) a sampling methodology difference as T-levels quantified from the cloacal fluid can be distorted due to individual differences caused by the metabolism of hormones by gut bacteria and by matrix effects (Goymann 2012) or, more likely, (iii) methodological problems in measuring testosterone levels from cloacal fluid; such high T-levels are only found in birds after testosterone treatment (Goymann & Wingfield 2014; this study).

The T-levels of the wild black jacobin were collected in the middle of their 7 months long breeding season (Ruschi 1964), although there was no information about the exact life stage such as incubating or having off-springs of the sampled birds, the high T-levels of 7% of wild males indicate their reproductive status. Interestingly, if we calculate the median of the plasma T-levels by considering only wild males with values above 1 ng/mL ($n = 11$), the outcome is 1.8 ng/mL (1.4, 3.3) which is rather low when compared to average plasma T-levels of northern temperate birds in the wild during the breeding season (2.8 ± 0.4 ng/mL), but on the range of tropical birds (1.3 ± 0.2 ng/mL) (Goymann et al. 2004). For tropical birds, it is suggested that their maxima T-levels is mainly determined by the length of the breeding season and the altitude they occur: species with longer breeding seasons (above 4 months) tends to show relatively lower T-levels and species inhabiting higher altitudes, higher values (Goymann et al. 2004). Therefore, the black jacobin as a species with long breeding season that occupy relatively lower altitudes is expected to have the observed T maxima.

The T-values of the T-treated black jacobins at the end of the experiment was about 300 times higher than control in males but 33 times higher than wild males with highest T-levels (> 1 ng/mL, $n = 11$) and 2,500 times higher than control in females but 350 times higher than wild females with highest T-levels (> 0.1 ng/mL, $n = 17$). In relation, T-values of intact breeding males of zebra finches after T implantation that are ~ 4 times higher than controls has been shown to induce singing and reduce time spent in close proximity to their partner (Prior et al. 2016). In castrated males of canaries, T-values 2 days after implantation can be ~ 10 times higher than controls and induce higher singing rates (Shevchouk et al. 2019). In intact and photostimulated males of house sparrow (*Passer domestics*), a dose-dependent effect was observed: after low doses, T-values were up to 2 times higher than control and induced testes atrophy, whereas after high doses, T-values ranged from 2 to 10 times higher while spermatogenic activity was maintained (Turek et al. 1976). Despite the much higher circulating T-levels in both sexes of T-treated black jacobins, neither gonadal, body and brain weights nor vocal behavior were affected; testosterone only affected oviduct growth in females and the maintenance of high aggressiveness in both sexes.

It is important to keep in mind that, in some species, T-treatment induce singing in castrated or intact birds in non-breeding condition but has no effect when administered in intact birds in breeding condition because T-levels reached a certain physiological threshold (reviewed in Fusani 2008; Goymann & Wingfield 2014). In ring doves (*Streptopelia risoria*), for example, castrated males vocalize more after T-treatment (Cohen & Cheng 1982); however, in intact males in breeding conditions both low and high dosage T-implants did not affect any element of their courtship activity including the sexually relevant vocalizations (Fusani & Hutchison 2003). Although T-treatment was conducted in intact and potentially breeding birds, this is unlikely the case for the black jacobin because T-treatment modulated aggressiveness and induced oviduct atrophy indicating that there was not a generalized lack of effect due to the supraphysiological T-levels in treated birds. Furthermore, a sex difference was observed in the T-levels of wild birds but not on vocal rates which reinforce the hypothesis that none of these vocalizations are a T-sensitive trait. T-implantation in castrated black jacobins would require developing surgical procedures and is, therefore, difficult to perform. Nevertheless, implants with anti-androgens in birds whose life-stages are known may help to further confirm our findings. The quantification of testosterone metabolites DHT (binds to androgen receptors) and estradiol (binds to estrogen receptors) also remains to be seen. In chickens, testosterone and estrogen act synergically on the preparation of the magnum – part of the oviduct – for normal secretion of albumen into the egg; when administered alone, testosterone increased magnum weight, but did not induce the formation of albumin secreting cells (Yu & Marquardt 1973). In black jacobin females, the presence of androgen receptors in the part of the oviduct, that is histologically similar to the magnum, may explain the testosterone-induced oviduct growth (A. Monte unpublished data). Testosterone treatment did not affect the weight of the testis of male black jacobins. This result is in line with some other species in which high T-levels did not decrease testis weight (Turek et al. 1976; Desjardins & Turek 1977) while in other species elevated T-levels regressed the testis (Chu 1940).

Hummingbirds are aggressive year-round, especially while foraging (Greenewalt 1990; Schuchmann 1999). Aggressiveness levels (number of attacks) of T-treated black jacobins were maintained during the whole experiment compared to a reduction in control birds over time in captivity. In seasonal species, aggressiveness is correlated with T-levels (Wingfield et al. 1987; reviewed in Soma 2006). To cope with the costs of high T-levels, these birds raise T-levels, particularly in periods of social instability during territorial challenges. Conversely, when their status or territory boundaries are maintained (social stability), T-levels are kept low (Wingfield et al. 1990). In the aviaries, the reduction in the number of attacks of control black jacobins across time may be explained by social stability; there was one feeder for every two birds, thus some birds might have alternated instead of disputed the same feeder. A similar use of the feeders was also observed in wild black jacobins, they awaited a free feeder instead of competing for feeders (A. Leitão personal observation).

We observed no effects of the T-treatment on the vocal rates and the acoustic parameters of the song, in contrast with other bird species investigated to date (reviewed in Gahr 2014, 2020). In the brain, testosterone can act on the medial preoptic nucleus (POM) or directly in the vocal control system (reviewed in Gahr 2014; Shevchouk et al. 2019). For example, direct testosterone implantation in POM of castrated male canaries induced high vocal rates but increased either vocal rate and song stereotypy when administered concomitantly in POM and HVC, an important nucleus of the vocal control system (reviewed in Alward et al. 2017). In the POM and song control regions of songbirds, there are androgen receptors (AR) and estrogen receptors that mediate the activity of testosterone and its androgenic and estrogenic metabolites on the cellular level (reviewed in Ball & Balthazart 2007). Similar, AR is expressed in the putative song control region of two hummingbird species, Anna's hummingbird (*Calypte anna*) and amazilia hummingbird (*Amazilia amazilia*) (Gahr 2000). AR and estrogen receptors in the POM are a general feature of all birds (reviewed in Gahr 2020). Although the reason for life-stage dependent effects of testosterone is unknown, we can speculate that the number of AR (Fusani et al. 2000; Quispe et al. 2016) or of testosterone metabolizing enzymes such as 5ß-reductase varies seasonally; birds reduce testosterone to 5ß-dhihydrotestosterone that cannot activate the AR (Floch et al. 1985). Likewise, we could assume that AR is expressed at low levels or 5ß-reductase is expressed at high levels in the putative song control brain regions of the black jacobin so that testosterone cannot affect song rate and pattern. Further, although we quantified AR in the syrinx of the black jacobin, these AR were not in muscles but in skeletal parts of the syrinx (A. Monte unpublished data), which makes an activating effect of testosterone on the syrinx and song unlikely. The only finding that indicates hormonal regulation of the song of the black jacobin is a previous report suggesting a seasonal change in the entropy of the song (Olson et al. 2018). However, these birds were not ringed and were recorded in the wild at the beginning (September) and end of the breeding season (March) (Olson et al. 2018). Thus, this seasonal change could be due to the fact that more juvenile birds were recorded at the end of the breeding season rather than an effect of testosterone. Entropy measurements of the song of our birds were not affected by testosterone ($F_r = 5$, $df = 3$, $P = 0.172$, Fig. S6 in Supplemental Data).

The insensitivity of vocalization rates and song features to testosterone in both sexes combined with our observations of the behavior of the birds indicates that none of these vocalizations are used exclusively in a sexual context. Chirps were mainly produced in flight shortly before feeding, whereas song was mainly produced when perched or sometimes in flight when chasing another bird (Fig. S7 in Supplemental Data). Black jacobins were observed to use their songs outside of the breeding season for defending food resources (Olson et al. 2018; A. Monte personal observation). Hence, it could be conceivably hypothesized that year-round song types that are also used in non-sexual contexts are not sensitive to testosterone. Thus, the American crow (*Corvus brachyrhynchos*), for example, which uses its song all year long in multiple contexts to facilitate affiliative behaviors among group members (Brown 1985) is not expected to show a correlation between T-levels in contrast with species that use their song as a secondary sexual trait during breeding season in which song is modulated by testosterone as in the common chaffinch (*Fringilla coelebs*) (Thorpe 1958) or canaries (Fusani et al. 2000). Further studies on the seasonality of black jacobin song and the behavioral context of their emission are, nevertheless, needed to support this hypothesis.

Hummingbirds have the highest metabolic rates amongst homeotherm vertebrates (Suarez 1992), which may constrain their daily activities towards finding and defending nectar-rich food sources. Thus, we speculate that, in black jacobin, the song is used by both sexes primarily to defend food sources. Although it remains to be seen whether the testosterone-insensitivity of black jacobin's vocalizations is life-stage dependent, it is possible that the non-reproductive use of song implied the loss of the activation effect of testosterone in their vocal control system. The courtship display of the black jacobin is a joint flight of both sexes in which males chase the female around 100 m upward in a stepwise fashion followed by a synchronously straight dive (Ruschi 1949). Thus, we speculate that, in black jacobin, mate choice is primarily based on visual displays and visual sensory processing. Thus, it needs to be seen if the non-reproductive use of song implied the loss of the activation effect of testosterone in their vocal control system, and in turn, the evolution of testosterone-sensitive visual displays and visual processing.

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## SUPPLEMENTAL DATA

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## SUPPLEMENTAL DATA

### *Supplemental statistical analyses*

Generalized Linear Mixed Models (GLMMs) were fitted to test whether the testosterone treatment (T-treatment) affected the number of songs and chirps emitted by the black jacobins, their feeding behavior and aggressiveness in the aviaries.

For the song and chirp counts, we fitted the GLMMs using the ‘glmmTMB’ package which model has three formulas: a conditional, a zero-inflation and a dispersion model formula (Brooks et al. 2017). The ‘glmmTBM’ offers an adequate approach to analyze the black jacobin's songs and chirps count data which are zero-inflated and overdispersed. The best-fitting model for song count as a response variable was fitted with the distribution negative binomial 1 (variance increases linearly with the mean) and for chirp count with the distribution negative binomial 2 (variance increases quadratically with the mean). The predictors for the conditional, zero-inflated and dispersion formulas of the best-fitting models are specified in the main text Table 4 (song) and Table 5 (chirp). We validated the best-fitting models for song and chirp counts using the DHARMa package (Hartig 2020). Given the methodological constraints to combine the information of GLMM’s conditional, zero-inflated and overdispersion formula, we tested whether the testosterone treatment affected the song and chirp counts by using the Likelihood Ratio (LR) test (Bolker et al. 2009; Winter 2013). The LR test is used to compare the likelihood of two models: the model without (null model) and with the variable of interest (full model), if the difference between the likelihood of these two models is significant, then the variable of interest is significant (Winter 2013). We excluded the testosterone treatment as a predictor of all formulas of the best-fitting models to create the null models, then we performed the LR test using the ‘anova’ function. We estimated marginal means and the predicted 95% confidence interval for song and chirp counts given by the best-fitting models with the random effects set to zero using the ‘ggpredict’ function implemented by the package ‘ggeffects’ (Lüdecke 2018). In the results, we reported the estimated mean 95% CI [lower, upper confidence interval].

For the feeding (feeds from the feeders and fruit-flies capture) and attack counts, we fitted the GLMMs using the ‘lmer’ function implemented by the ‘lme4’ package (Bates et al. 2015). The best-fitting models for each of the feeding counts (feeders and fruit flies) and attack count as response variables were fitted with the Poisson distribution. The predictors for the best-fitting models are specified in Table S3 (feeding) and Table 6 (attack). We validated the best-fitting models for feeding and attack counts by inspecting whether the models' assumptions were met: normality of the residuals, homoscedasticity, and lack of remaining patterns. Then, for each best-fitting model, we simulated 1,000 values from the joint posterior distributions of the model parameters using the function ‘sim’ implemented by the package ‘arm’ (Gelman & Su 2016) with an uninformed prior distribution. We estimated the means of the simulated values from the posterior distributions and the predicted 95% credible intervals (CrI). In the results, we reported the estimated mean 95% CrI [lower, upper credible interval]. Following a significant effect in a predictor or interaction of interest, we performed a post-hoc analysis of interactions using the function ‘testInteractions’ implemented by the package ‘phia’ (Rosario-Martinez et al. 2015) and adjusted the *P*-values with the Bonferroni method.

*Supplemental figures and tables*

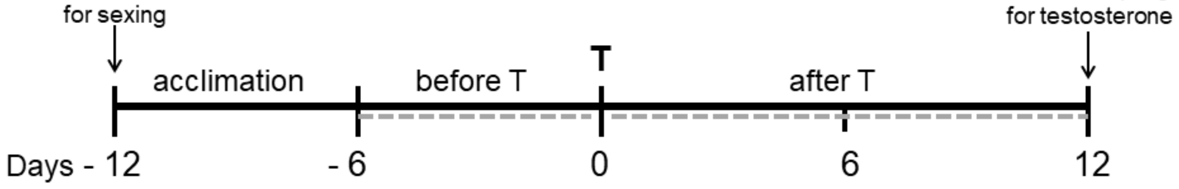

Fig. S1. — Timeline of the experiment conducted with captive black jacobins (females n = 10 and males n = 14). The birds were housed and kept in aviaries for 4 weeks. The first blood sampling for sexing was obtained from hallux's nail 2 weeks before the testosterone implantation (day – 12). They were monitored for their adaptation to the captive conditions, for example, it was observed whether they feed and bathe regularly as it is expected for the species for 1 week (from day – 12 to – 6). The behavioral observations were conducted for the following 3 weeks (dashed line, from day – 6 to 12). For the analysis, the behavioral observations were divided into 3 time-points: before (before T, day – 6 to – 1), 1 (T1, day 1 to 6) and 2 weeks after the testosterone treatment (T2, day 7 to 12). The birds were implanted with testosterone or placebo on day 0. Body weight was obtained on day – 12, 0 and 12.

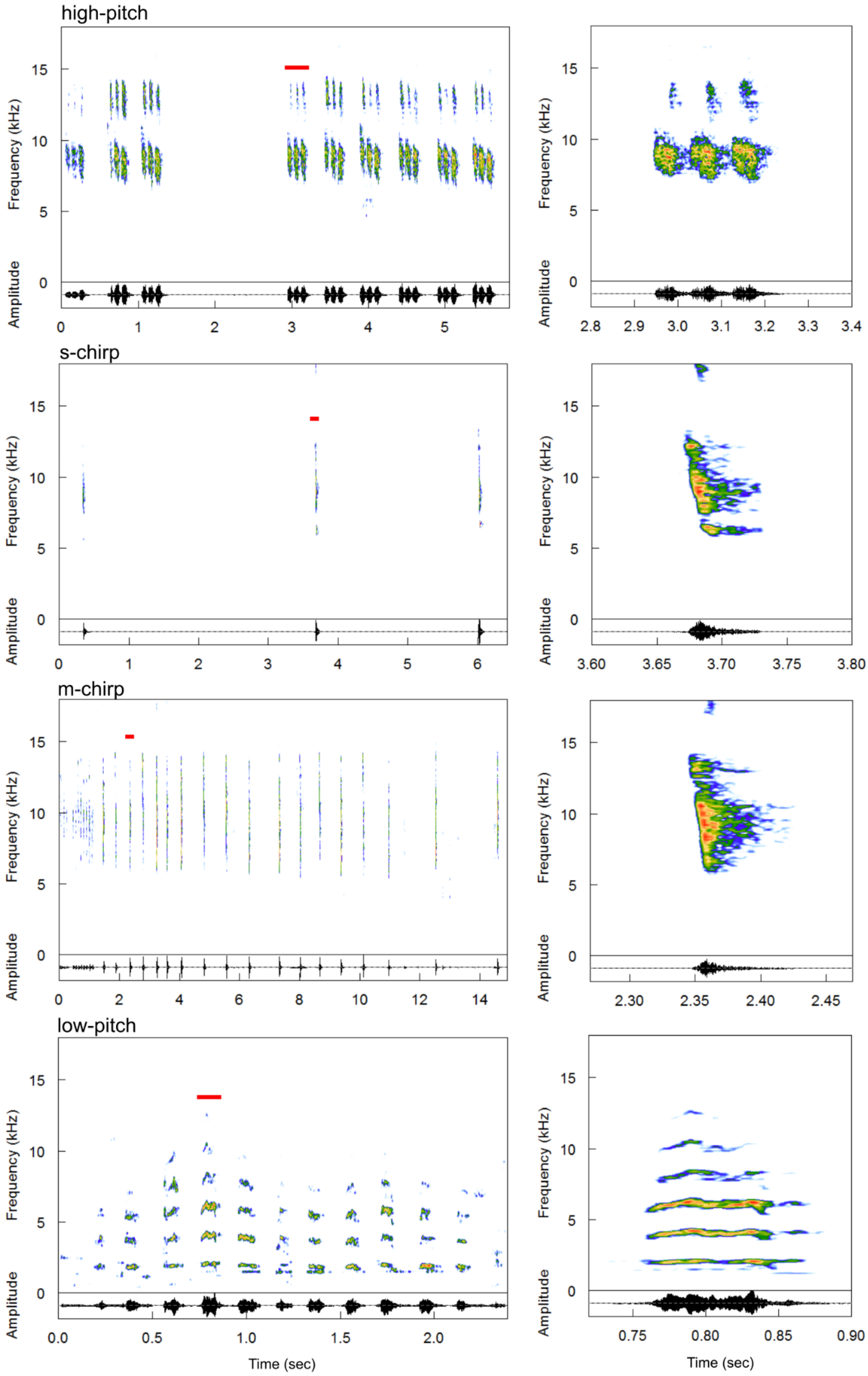
high-pitch
s-chirp
m-chirp
low-pitch
Frequency (kHz)
Amplitude
Time (sec)
15
10
5
0
0
1
2
3
4
5
6
2.8
2.9
3.0
3.1
3.2
3.3
3.4
3.60
3.65
3.70
3.75
3.80
8
10
12
14
2.30
2.35
2.40
2.45
0.0
0.5
1.0
1.5
2.0
0.75
0.80
0.85
0.90

Fig. S2. — Black jacobins emit vocalizations that can be grouped into three spectrally distinct types: high-pitch, chirp and low-pitch. The high-pitch, which is likely the black jacobin's song, is a fast modulated unit that is usually composed by three subunit (triplet). The chirp is a short-duration unit (~ 25 msec) in a wide frequency range (from ~ 6 to 13 kHz). Chirps can be classified as a single chirp (s-chirp) and multiple chirp (m-chirp), the former is composed by one chirp emitted alone or a few with variable silent interval between them and the latter by many sequential chirps with progressively longer silence interval between them. Given that m-chirp is rare, s- and m-chirps were analyzed jointly. Finally, the low-pitch is composed by the repetition of many units with from 3 to 6 frequency bands with more energy between 2 to 5 kHz. Spectrogram (frequency) and oscillogram (relative amplitude) examples of each vocalization is depicted. The red bar indicates the unit that is zoomed on the right panel.

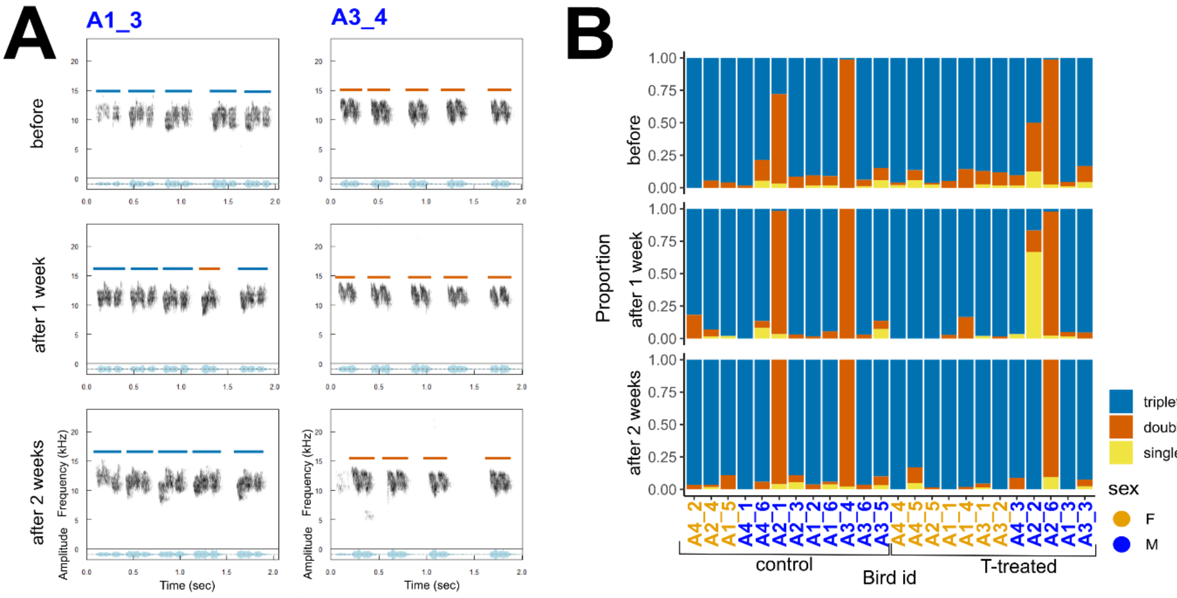

Fig. S3. — Black jacobin song can be composed by one (single), two (double) or three (triplet) subunits which is highly repetitive within birds. (A) Spectrogram (frequency) and oscillogram (relative amplitude) examples of song bouts of a male (bird id A1_3) which song is composed mainly by triplets (indicated by a dark blue bar) and a male (bird id A3_4) which song is composed mainly by doubles (indicated by a red bar) before, 1 and 2 weeks after testosterone treatment. (B) Proportion of singles, doubles and triplets emitted per bird before, 1 week and 2 weeks after testosterone. The bird ids are color-coded by sex (females in orange and males in light blue) and grouped by treatment.

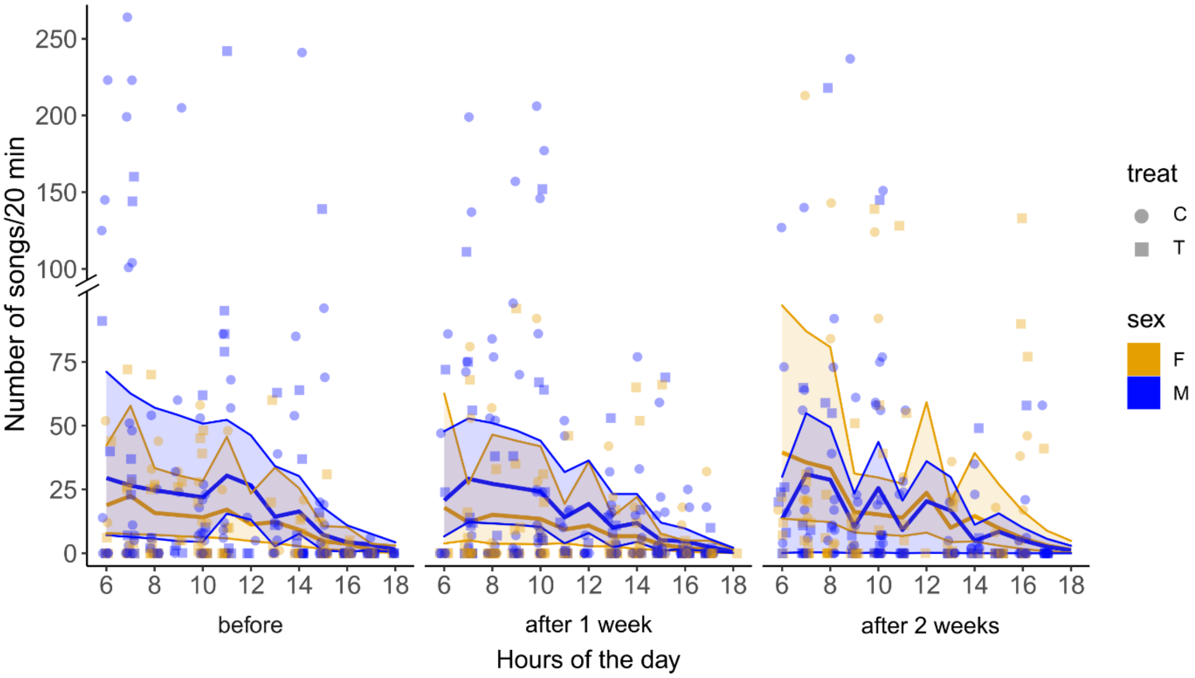

Fig. S4. — Black jacobins tended to sing less throughout the day. The data points represent song counts obtained per bird in 20 min of observation. Data from testosterone-treated (squares) and control birds (circles) were plotted together because they show no difference. The curves represent the estimated values given by the generalized linear mixed model and the shaded areas the respective 95% credible intervals for females (lighter) and males (darker).

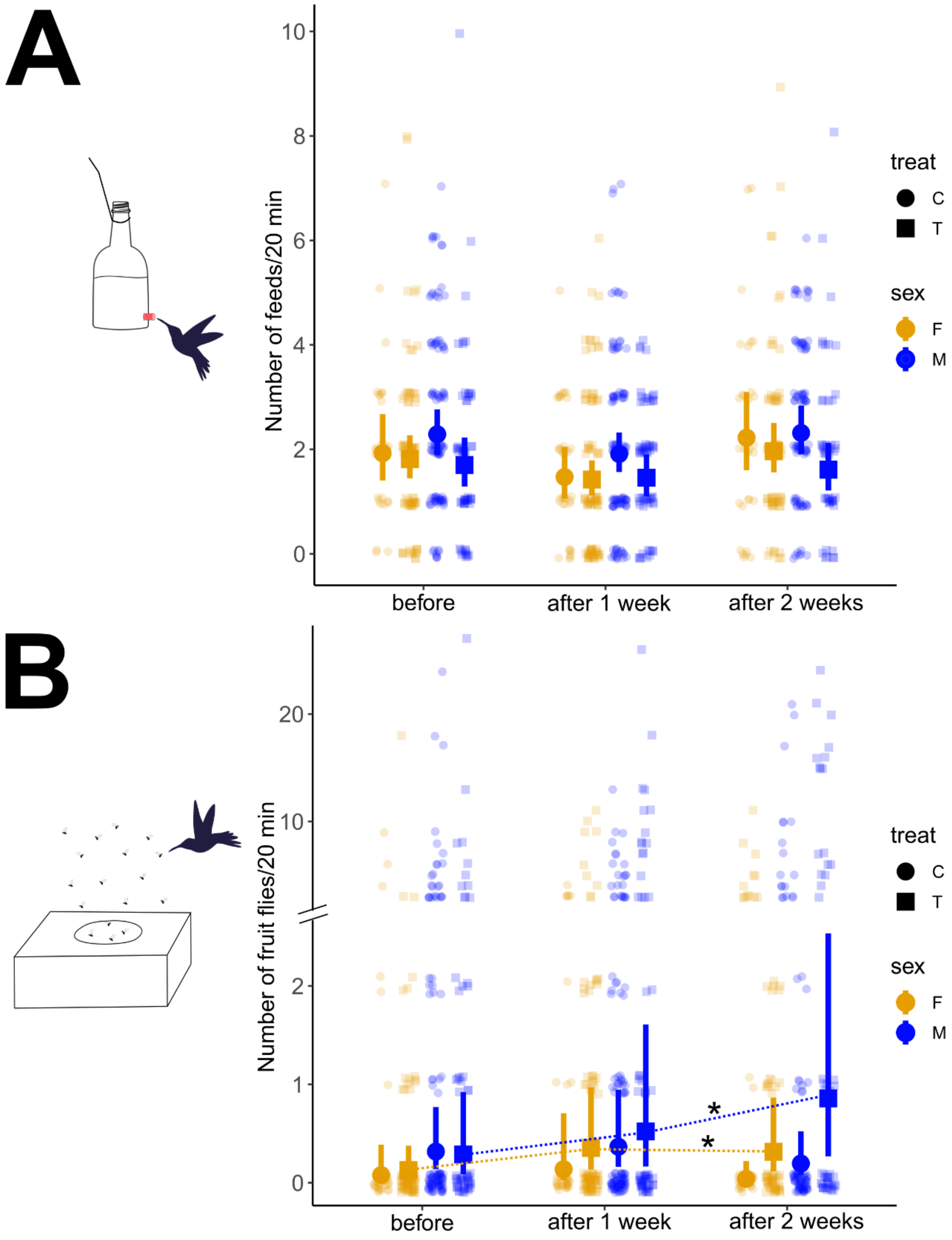

Fig. S5. — Black jacobin's feeding counts grouped by sex and testosterone-treatment across time. Whereas in (A) black jacobins show no differences in the number of times they feed from the feeders with sugary water, in (B) Testosterone-treated black jacobins tended to capture more fruit-flies as longer they spent in captivity. The data points represent feeding counts obtained per bird in 20 min of observation. The error bars represent the fitted mean and the 95% credible interval given by the generalized linear mixed model. The asterisk (*) represents *P* < 0.05, but > 0.01.

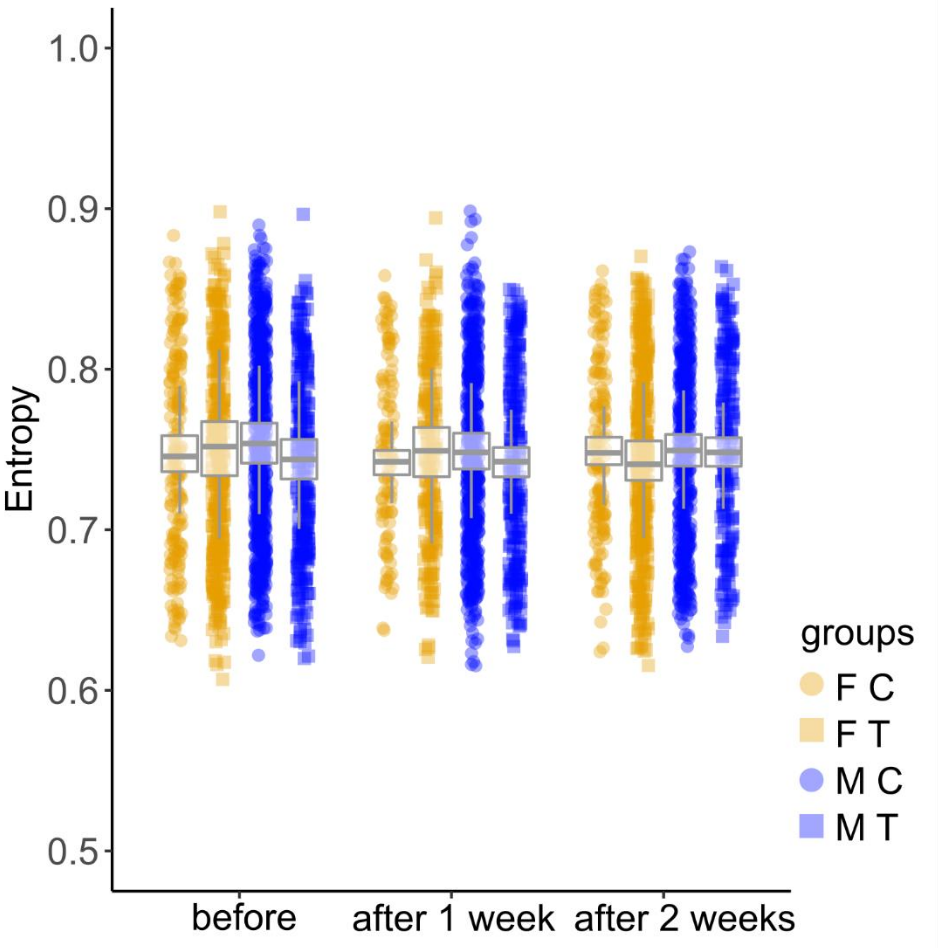

Fig. S6. — The entropy of black jacobin’s song in both sexes of control and T-treated groups did not differ before, 1 or 2 weeks after T treatment. Entropy ranges from 0 when the song is closer to a pure tone to 1 when is noisy. All box plots indicate the median and interquartile range (25% to 75%). Each data point indicates an individual song measurement: FC, female control (3 females, 178 songs before, 107, 1, and 146, 2 weeks after T); FT, female T-treated (7 females, 342 songs before, 201, 1, and 370, 2 weeks after T); MC male control (5 males, 624 songs before, 545, 1, and 466, 2 weeks after T) and MT, male T-treated (9 males, 268 songs before, 228, 1, and 185, 2 weeks after T).

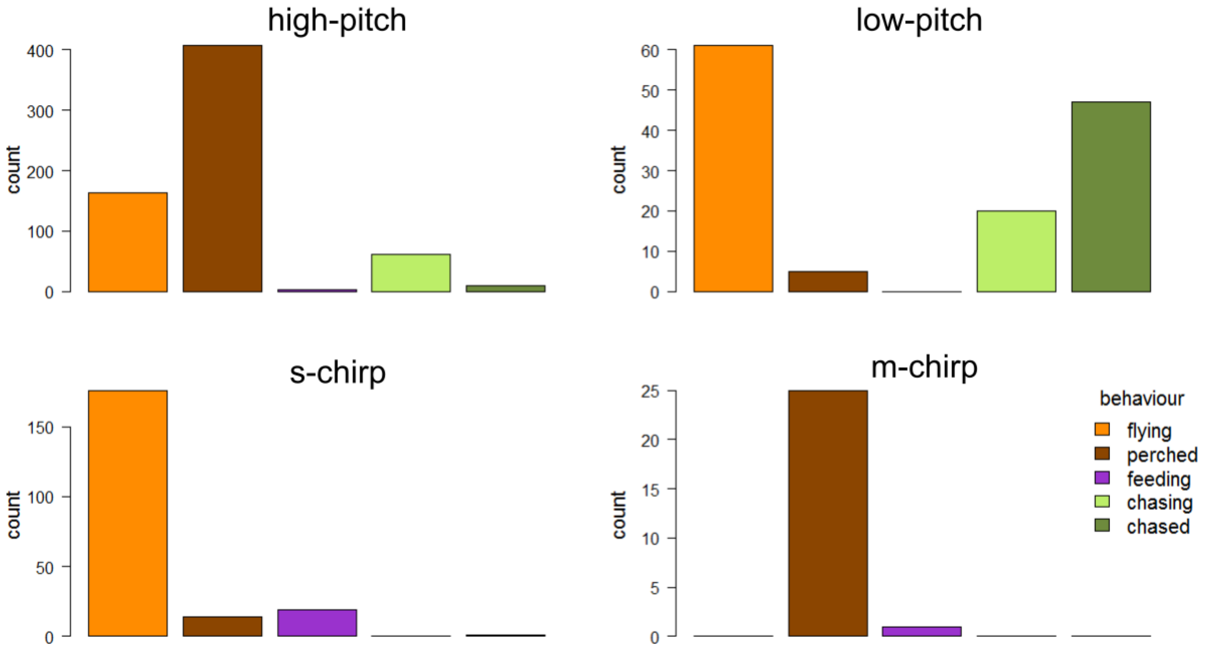

Fig. S7. — Each of the black jacobin's vocalization was consistently uttered in a specific behavioral context. Summary of all emissions in the aviaries during 1 week before testosterone treatment jointly for both sexes. The behaviors of the birds during vocalization were flying (orange), perched (brown), feeding (purple), chasing (light green) or being chased (dark green) by a conspecific. The behaviors were quantified during observation sessions of 20 min each (see Methods). The song (high-pitch) was mainly produced when the bird was perched or in flight sometimes when chasing another bird away. The low-pitch was produced in agonistic contexts when the bird was flying either chasing or being chased by a conspecific. The s-chirp was produced mainly during flight sometimes shortly before feeding, whereas m-chirp was produced when the bird was perched alone. Low-pitch and m-chirp were rare.

Table S1.

List of the 26 acoustic parameters measured for the song of the black jacobin with the function "specan" implemented by the package "warbleR" (Araya-Salas & Smith-Vidaurre 2017).

| Parameter | Description |
|---|---|
| duration | length of signal |
| meanfreq | mean frequency (in kHz) |
| SD | standard deviation of frequency |
| freq.median | median frequency (in kHz) |
| freq.Q25 | first quantile (in kHz) |
| freq.Q75 | third quantile (in kHz) |
| freq.IQR | interquantile range (in kHz) |
| time.median | time in which the median of the frequency was obtained (in sec) |
| time.Q25 | time in which the first quantile of the frequency was obtained (in sec) |
| time.Q75 | time in which the third quantile of the frequency was obtained (in sec) |
| time.IQR | difference between time.Q75 and time.Q25 (in sec) |
| skew | skewness, a measure of asymmetry. skew < 0 indicated that the spectrum is skewed to left; skew = 0 when the spectrum is symmetric and skew > 0 when the spectrum is skewed to right |
| kurt | kurtosis, a measure of peakedness. kurt < 3 indicates that spectrum is platikurtic, i.e. it has fewer items at the center than the normal curve; kurt = 3 when the spectrum shows a normal shape and kurt > 3 when the spectrum is leptokurtic, i.e. it has more items near the center. |
| sp.ent | spectral entropy. Energy distribution of the frequency spectrum. Pure tone ~ 0; noisy ~ 1 |
| time.ent | time entropy. Energy distribution on the time envelope. Pure tone ~ 0; noisy ~ 1 |
| entropy | spectrographic entropy. Product of time and spectral entropy |
| sfm | spectral flatness. Pure tone ~ 0; noisy ~ 1 |
| meandom | average of dominant frequency |
| mindom | minimum of dominant frequency |
| maxdom | maximum of dominant frequency |
| dfrange | range of dominant frequency |

| | |
|---|---|
| modindx | modulation index (accumulated absolute difference between adjacent measurements of fundamental frequencies divided by the frequency range) |
| startdom | dominant frequency measurement at the start of the signal |
| enddom | dominant frequency measurement at the end of the signal |
| dfslope | slope of the change in dominant frequency through time calculated as enddom – startdom/duration |
| meanpeakf | mean peak frequency. Frequency with highest energy from the mean frequency spectrum |

Table S2.

Results of linear models explaining variation in the circulating testosterone plasma levels (T plasma levels) and body weight of wild black jacobins including age (1st year-juveniles × adults) as a predictor. As age have no effect on T plasma levels neither on body weight, first-year juveniles and adults were analyzed together. In bold are the significant predictors (*P* < 0.01).

| | Estimate | SE | t value | *P*-value |
|---|---|---|---|---|
| T plasma levels | | | | |
| Intercept | 5.729 | 0.927 | 6.260 | < **0.001** |
| Sex (ref. female) | 0.525 | 0.136 | 3.823 | < **0.001** |
| Age (ref. 1st year) | 0.224 | 0.199 | 1.125 | 0.262 |
| Body weight | 0.327 | 0.119 | 2.736 | **0.007** |
| Day | – 0.290 | 0.021 | – 13.836 | < **0.001** |
| Body weight | | | | |
| Intercept | 6.304 | 0.369 | 17.074 | < **0.001** |
| Sex (ref. female) | 0.263 | 0.077 | 3.403 | **0.001** |
| Age (ref. 1st year) | 0.179 | 0.112 | 1.596 | 0.112 |
| Log T | 0.104 | 0.038 | 2.736 | **0.007** |
| Day | 0.034 | 0.016 | 2.090 | **0.038** |

Table S3.

Results of the generalized linear mixed model explaining variation in the feeding counts (feeds from the feeders and fruit-fly captures) per observation session (20 min) of captive black jacobins. The number of feeds from the feeders was affected by the number of fruit-flies captured, whereas the number of fruit-flies captured was affected by the number of feeds from the feeder and the interaction between testosterone-treatment and time. In bold are the significant predictors (*P* < 0.01).

| | Estimate | Std. Error | z value | p value |
|---|---:|---:|---:|---:|
| Number of feeds from the feeders | | | | |
| Intercept | 0.655 | 0.170 | 3.850 | **< 0.001** |
| T treatment (ref. control) | – 0.060 | 0.196 | – 0.305 | 0.761 |
| Time after 1 week (ref. before T-treatment) | – 0.270 | 0.140 | – 1.929 | 0.054 |
| Time after 2 weeks (ref. before T-treatment) | 0.144 | 0.136 | 1.059 | 0.290 |
| Sex male (ref. female) | 0.173 | 0.190 | 0.909 | 0.363 |
| Fruit flies | 0.033 | 0.007 | 4.873 | **< 0.001** |
| T treatment : Time after 1 week | 0.026 | 0.137 | 0.188 | 0.851 |
| T treatment : Time after 2 weeks | – 0.064 | 0.136 | – 0.471 | 0.638 |
| T treatment : Sex male | – 0.238 | 0.232 | – 1.024 | 0.306 |
| Time after 1 week : Sex male | 0.089 | 0.140 | 0.637 | 0.524 |
| Time after 2 weeks : Sex male | – 0.133 | 0.138 | – 0.961 | 0.336 |
| Number of fruit flies | | | | |
| Intercept | – 3.08 | 0.85 | – 3.62 | < 0.001 |
| T treatment (ref. control) | 0.50 | 0.96 | 0.52 | 0.604 |
| Time after 1 week (ref. before T-treatment) | 0.56 | 0.44 | 1.29 | 0.198 |
| Time after 2 weeks (ref. before T-treatment) | – 0.68 | 0.48 | – 1.40 | 0.162 |
| Sex male (ref. female) | 1.39 | 0.94 | 1.48 | 0.138 |
| Hour | – 0.15 | 0.08 | – 2.00 | 0.046 |
| Feeds | 0.27 | 0.05 | 5.39 | **< 0.001** |
| T treatment : Time after 1 week | 0.45 | 0.39 | 1.15 | 0.250 |
| T treatment : Time after 2 weeks | 1.57 | 0.43 | 3.68 | **< 0.001** |
| T treatment : Sex male | – 0.59 | 1.17 | – 0.51 | 0.614 |
| Time after 1 week : Sex male | – 0.42 | 0.42 | – 0.99 | 0.321 |
| Time after 2 weeks : Sex male | 0.20 | 0.45 | 0.44 | 0.661 |

Table S4.

Predicted means, lower and upper values from the 95% credible interval given by the generalized linear mixed models for the song counts per sex, treatment across time.

| Sex | Treatment | n | Time | Mean | Lower | Upper |
|---|---|---|---|---|---|---|
| Female | Control | 3 | Before T | 16.62 | 6.49 | 42.53 |
| | | | After 1 week | 18.73 | 7.09 | 49.47 |
| | | | After 2 weeks | 24.83 | 9.94 | 62.03 |
| | T-treated | 7 | Before T | 12.75 | 6.40 | 25.39 |
| | | | After 1 week | 14.52 | 7.41 | 28.46 |
| | | | After 2 weeks | 12.98 | 6.78 | 24.85 |
| Male | Control | 9 | Before T | 26.23 | 14.75 | 46.66 |
| | | | After 1 week | 17.52 | 9.19 | 33.42 |
| | | | After 2 weeks | 15.78 | 8.24 | 30.22 |
| | T-treated | 5 | Before T | 14.81 | 6.28 | 34.95 |
| | | | After 1 week | 10.00 | 4.54 | 22.00 |
| | | | After 2 weeks | 6.07 | 2.64 | 13.97 |

Table S5.

Predicted means, lower and upper values from the 95% credible interval given by the generalized linear mixed models for the chirp counts per sex, treatment across time.

| Sex | Treatment | n | Time | Mean | Lower | Upper |
|---|---|---|---|---|---|---|
| Female | Control | 3 | Before T | 4.61 | 1.45 | 14.68 |
| | | | After 1 week | 1.26 | 0.35 | 4.45 |
| | | | After 2 weeks | 1.77 | 0.53 | 5.84 |
| | T-treated | 7 | Before T | 15.67 | 7.26 | 33.81 |
| | | | After 1 week | 7.19 | 3.25 | 15.90 |
| | | | After 2 weeks | 12.73 | 6.02 | 26.93 |
| Male | Control | 9 | Before T | 8.14 | 4.02 | 16.47 |
| | | | After 1 week | 4.89 | 2.46 | 9.71 |
| | | | After 2 weeks | 7.34 | 3.71 | 14.55 |
| | T-treated | 5 | Before T | 3.31 | 1.27 | 8.58 |
| | | | After 1 week | 3.34 | 1.25 | 8.95 |
| | | | After 2 weeks | 6.32 | 2.38 | 16.81 |

Table S6.

Eigenvalues obtained from a PCA of the acoustic parameters of the black jacobin’s song.

| | PC1 | PC2 | PC3 | PC4 | PC5 | PC6 | PC7 | PC8 |
|---|---|---|---|---|---|---|---|---|
| SD | 3.074 | 2.427 | 2.069 | 1.502 | 1.221 | 0.868 | 0.771 | 0.683 |
| Proportion of Variance | 0.364 | 0.227 | 0.165 | 0.087 | 0.057 | 0.029 | 0.023 | 0.018 |
| Cumulative Proportion | 0.36 | 0.59 | 0.75 | 0.84 | 0.90 | 0.93 | 0.95 | 0.97 |

Table S7.

Eigenvectors obtained from a PCA of the acoustic parameters of the black jacobin’s song. PC1 described mainly spectral parameters, whereas PC2 described temporal parameters. In bold the heavily loading characters within each vector. The abbreviation of the parameters are described in Table A.1. where is Table A1?

| Parameters | PC1 | PC2 | PC3 |
|---|---|---|---|
| duration | – 0.125 | – **0.343** | 0.112 |
| meanfreq | **0.263** | – 0.161 | – 0.140 |
| SD | **– 0.282** | 0.021 | – 0.140 |
| freq.median | **0.262** | – 0.184 | – 0.101 |
| freq.Q25 | **0.281** | – 0.153 | – 0.032 |
| freq.Q75 | **0.229** | – 0.182 | – 0.209 |
| freq.IQR | – 0.160 | – 0.040 | – 0.386 |
| time.median | – 0.133 | – **0.313** | 0.164 |
| time.Q25 | – 0.076 | – 0.185 | 0.183 |
| time.Q75 | – 0.134 | – **0.350** | 0.106 |
| time.IQR | – 0.128 | – **0.337** | 0.074 |
| skew | 0.006 | 0.055 | **0.435** |
| kurt | – 0.006 | 0.050 | **0.424** |
| sp.ent | **– 0.230** | – 0.117 | – 0.298 |
| time.ent | 0.112 | **0.345** | – 0.105 |
| entropy | – **0.207** | – 0.014 | – 0.346 |
| sfm | – **0.263** | – 0.041 | – 0.162 |
| meandom | **0.276** | – 0.141 | – 0.088 |
| mindom | **0.275** | 0.066 | – 0.041 |
| maxdom | – 0.112 | – 0.105 | – 0.042 |
| dfrange | – **0.252** | – 0.097 | 0.010 |
| modindx | 0.058 | – 0.258 | – 0.110 |

| | | | |
|---|---|---|---|
| startdom | **0.214** | – 0.049 | – 0.136 |
| enddom | 0.194 | – 0.227 | – 0.032 |
| dfslope | – 0.021 | – 0.241 | 0.095 |
| meanpeakf | 0.245 | – 0.182 | – 0.010 |

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# Chapter 4

# Absence of androgen receptors in the putative vocal control system of hummingbirds

## Abstract

Hummingbirds evolved brain specializations analogous to songbirds and parrots that allow them to learn their songs. In songbirds, most brain areas of their song control system express androgen receptors (AR). In non-songbirds, ARs are expressed in midbrain and/or brainstem vocal areas. In songbirds, testosterone and its metabolites play an important role in the ontogeny of vocal learning and the production of learned songs in adulthood. In most studied species, including songbirds and non-songbirds, testosterone increases the singing rate. However, in a previous study we showed that the song of an early-branched hummingbird, the black jacobin (*Florisuga fusca*) is testosterone insensitive. In order to verify whether this insensitivity is related to a lack of ARs in their vocal control system, we performed RNA scope in-situ hybridization for AR mRNA on histological sections of female and male black jacobin. We compared these AR mRNA distributions to further eight hummingbird species with different degrees of phylogenetic distance. Male and female black jacobins shows weak expression or lacked AR mRNA in their putative vocal control system but have strong expression in other areas of the thalamus and midbrain, in particular visual areas such as the nucleus pretectalis (Pt). Surprisingly, a similar paucity of AR expression in putative vocal areas was found in four further species while AR mRNA was expressed in the putative vocal area VAN (analogous to songbird LMAN) of males sombre hummingbird (*Aphantochroa cirrochloris*), versicolored emerald (*Amazilia versicolor*) and blue-chinned sapphire (*Chlorestes notatus*). These species belong to the hummingbird clade Emeralds, which, among the studied species, is the most distantly related to black jacobins', the clade Topazes. The lack of neural expression of

androgen receptors in vocal control areas suggests that singing in some hummingbird species are likely testosterone sensitive while testosterone insensitive in other species. Thus, hormonal control and sexual function of singing might be more heterogenous in hummingbirds as compared to songbirds.

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## Introduction

The need to undergo a learning process early in life to communicate using sounds is not a human exclusivity. Three groups of birds, songbirds (Suborder *Passeri*) (Nottebohm et al., 1976), parrots (Order *Psittaciformes*) (Jarvis & Mello, 2000) and some hummingbirds (Family Trochilidae) (Araya-Salas & Wright, 2013; Baptista & Schuchmann, 1990; Gahr, 2000; Jarvis et al., 2000; K. E. Johnson & Clark, 2020) learn how to produce their songs early in life by a process which holds striking similarities to the process of speech acquisition, the vocal-production learning (Doupe & Kuhl, 1999; Jarvis, 2004; Marler, 1970). Interestingly, these vocal learners independently evolved of a set of discrete but interconnected nuclei in the forebrain related to the acquisition, perception and production of their learned songs, the vocal control system (reviewed in Nottebohm, 2005). The vocal control system shows similar pattern of androgen receptor (AR) expression amongst bird vocal learners which suggests that androgens play an important role in vocal learning (Frankl-Vilches & Gahr, 2018; Gahr, 2000; Gahr & Metzdorf, 1997; Matsunaga & Okanoya, 2008).

Androgen receptors (AR) are binding site for testosterone and to dihydrotestosterone (DHT), a more biologically active metabolite. Testosterone (T) can be converted by the enzyme 5α-reductase to DHT or by aromatase to estradiol which in turns binds to estrogen receptors (ER) (reviewed in Davey, and Grossmann, 2016). In nearly all vertebrate classes, including fishes, amphibians, reptiles, birds and mammals, the distribution of AR and ER are highly conserved in certain brain areas such as the septal-preoptic, hypothalamus and midbrain (Pfaff, 1980). The binding of androgens and estrogens in these areas are related to the activation of a wide range of reproductive behaviors such as copulation, pre- and postcopulatory displays, aggression, territorial defense and singing (reviewed in Balthazart, 1989).

In avian vocal learners, the AR and ER in their vocal control system enable the action of androgen and estrogens on the brain development either through organization or activation (reviewed in Filová et al., 2013). An organization effect influences early development promoting irreversible changes, for example, the zebra finch (*Taeniopygia guttata*) undergoes a highly level of sex differentiation in their vocal control system resulting in atrophied nuclei in adult females which are unable to sing unless females are treated with estradiol when juvenile and DHT or T when adults (Gurney & Konishi, 1980). An activation effect influences behavior by transient action in the brain, for example, male canaries (*Serinus canaria*) enlarge their vocal control system and revise their songs every breeding season under higher circulating levels of T (F. Johnson & Bottjer, 1992). Canaries and probably most songbird species studied so far present a lower degree of sex difference in their vocal control systems compared to the zebra finch and singing females can be occasionally observed or induced by T treatment (Gahr, 2014; Gahr & Metzdorf, 1997). Interestingly, male zebra finches under continuous T treatment from hatching until 40 days old develops abnormal song (Korsia & Bottjer, 1991) which reinforce the idea that the role of T and its metabolites differ across learning stages.

The distribution of AR mRNA delineates the main vocal control areas (HVC, proper name; RA, robust nucleus of the archipallium and LMAN, lateral magnocellular nucleus of the anterior nidopallium) in both sexes of all songbird species investigate to date (e.g.: Gahr, and Metzdorf, 1997; Balthazart et al., 1992). In parrots, such delimitation was found in the analogous nuclei in both sexes of budgerigars (*Melopsittacus undulatus*) (NLC, central nucleus of the lateral nidopallium; AAC, anterior arcopallium and NAO, oval nucleus of the anterior nidopallium) (Matsunaga & Okanoya, 2008). In hummingbird, such delimitation was found strongly in the analogous of the songbird LMAN (VAN, vocal nucleus of the anterior nidopallium), weakly in the analogous of HVC (VLN, vocal nucleus of the lateral nidopallium) and not detected in the analogous of RA (VA, vocal nucleus of the arcopallium) in a male Anna's and Amazilia hummingbirds (Gahr, 2000). In Gahr's study (2000), the expression of AR mRNA was localized by in situ hybridization with cRNA probes of the zebra finch AR which also did not detect any AR expression in Budgerigars. Thus, the AR expression in the vocal control system in hummingbirds remains unclear. Furthermore, in a previous study we found that the song of an early-branched hummingbird, the black jacobin (*Florisuga fusca*), during the breeding season seems insensitive to testosterone (Monte et al., 2023). Therefore, we investigated the AR distribution in the brain of both sexes of black jacobin and then compared to further eight species with different degrees of phylogenetic distance.

The most recent hummingbird phylogeny placed the black jacobin in the clade Topazes (tribe *Topazini*) (McGuire et al., 2009, 2014) which is a sister taxon of all other hummingbirds (McGuire et al., 2014) and can give insights into the ancestral condition in the family. The black jacobin occur in the Atlantic forest on the extent of the Brazilian coast until Uruguay and Argentina (Schuchmann & Bonnan, 2019; Sick, 1997). The black jacobin produce a high-pitched song with a high fundamental frequency (Monte et al., 2020; Olson et al., 2018), as commonly found in hummingbirds (Duque et al., 2018), and harmonics above the human hearing range, the ultrasonic elements (Olson et al., 2018). This high-pitched song is composed of extremely fast frequency oscillations and is suggested to be learned (Monte et al., 2020; Olson et al., 2018). In a previous study, testosterone implantation in both sexes during the breeding season indicated that their song is most likely testosterone-insensitive (Monte et al., 2023). Therefore, we investigated the presence of androgen receptors in the putative neural substrate of singing in vocal learners, the vocal control system. We found that AR mRNA was not differentially expressed in the putative vocal control system of the black jacobin, and this pattern is rather widespread in the hummingbird family with exception of the VAN in males of two phylogenetic distant species. AR were highly expressed in some thalamic regions as is also found in other bird species which are non-vocal learners (Gahr & Wild, 1997).

## Materials and Methods

### *Animals and tissue sampling*

We sampled hummingbirds of five from nine of major clades (Topazes, one species; Hermits, one species; Mangoes, one species; Coquettes, one species and Emeralds, five species) described in the most comprehensive hummingbird phylogeny to date (McGuire et al., 2009, 2014). We conducted the capture of free-living hummingbirds in two locations: Amazon Rainforest in the Gunma Ecological Park, Santa Barbara, Para, Brazil (01° 13' 00.86" S, 048° 17' 41.16" W) and Atlantic Rainforest in the park of the Museum of Biology Prof. Mello Leitão in Santa Teresa, Espírito Santo, Brazil (19° 56' 08.30" S, 040° 35' 59.10" W). The sampling happened on June and July in the Amazon Rainforest and November and December in the Atlantic Rainforest during their respective dry seasons to optimize the capture of birds in breeding condition (A. Ruschi, 1964; Sick, 1997); females with brood patch were released.

We placed feeder bottles filled with sugary water (solution of water with 20 % sugar), then we captured the birds during visit to the feeder with a trap called “Ruschi net trap” developed and optimized to capture hummingbirds (P. A. Ruschi, 2009). The birds were killed by decapitation, the brain was dissected, flash frozen in dry ice and stored at -80 ºC until use. The procedure from decapitation to freezing lasted less than five minutes.

We sampled five species in the Amazon Rainforest: one female and one male reddish hermit (*Phaethornis ruber*), two females and three males black-throated mango (*Anthracothorax nigricollis*), one female and one male grey-breasted sabrewing (*Campylopterus largipennis*), one female and one male fork-tailed woodnymph (*Thalurania furcata*), one female and two males blue-chinned sapphire (*Chlorestes notata*) and four species in the Atlantic Rainforest: three females and three male black jacobin (*Florisuga fusca*), two males frilled coquette (*Lophornis magnificus*), one female and two males sombre hummingbird (*Aphantochroa cirrochloris*) and one male versicoloured emerald (*Amazilia versicolor*). We sampled a minimum number of birds to minimize the impact on the natural populations which is in conformity with the exploratory scope of this study.

The procedures of this study were conducted in conformity with the instruction n° 03/2014 of the Chico Mendes Institute for Biodiversity Conservation (ICMBio) and with the permission of the Brazilian Institute of Environment and Renewable Natural (IBAMA) under the Biodiversity Information and Authorization System (SISBio) licenses numbers 30319-1, 41794-2 and 49097-1.

### ***Histology***

The brains of both sexes were sectioned with the same procedure. Before sectioning, the brain was thawed under -20 ºC for 10 minutes; then, it was sectioned on a Leica Jung CM3000 cryostat (Leica, Germany) into 20 µm sections and mounted onto Fisher Superfrost Plus slides (Fisher Scientific, Germany). The brain was sectioned transversally (black jacobin) or sagittal (other species). The brain sections were mounted onto 15 series of slides, so that adjacent sections could be analyzed by different methods. One series of sections was Nissl-stained. The other series of sections were stored at -80 ºC until use. For the staining procedure, the brain slides were air-dried for two hours, rehydrated, stained with 0.1% Thionin (Sigma-Aldrich, USA) for six seconds, dehydrated, immersed in xylene and cover-slipped with Roti histokitt II mounting medium (Carl Roth, Germany).

In the Nissl-stained brain sections from both sexes of black jacobin, we identified, by visualization of cytoarchitectonic features, three distinct structures analogous to the main vocal control system (VCS) nuclei previously described for males of rufous-breasted hermit (*Glaucis hirsuta*), sombre hummingbird (*Aphantochroa cirrhochloris*) (Jarvis et al., 2000), Anna's hummingbirds (*Calypte anna*) and Amazilia hummingbirds (*Amazilia amazilia*) (Gahr, 2000). These nuclei were the vocal nucleus of the lateral neostriatum (VLN or HVC-like when comparing to songbirds), the vocal nucleus of the archistriatum (VA or RA-like) and the vocal nucleus of the anterior neostriatum (VAN or LMAN-like). The boundaries of VA and VAN are easily distinguishable on Nissl-stained sections due to their oval disposition, darkly staining, and higher cell density than the surrounding. The VA is located at the central portion of the arcopallium and limited on the upper part by the lamina pallio-subpallialis (LPS), whereas the VAN is located at the anterior portion of the nidopallium, limited on the upper part by the lamina frontalis superior (LFS) and the lower part for the dorsal part of the striatum lateralis (StL). The delimitation of the VLN is not as clear as the VA and VAN; it is limited on the upper part by the overlying ventricle (V), but the lower boundary is unclear on the Nissl-stained slides. Thus, we further confirmed the boundaries of the VLN using the synaptosomal-associated protein 25 kDa (SNAP-25) as a marker (Supplementary Material Fig. S1). SNAP-25 has shown to be differently expressed throughout the vocal control system of the zebra-finches including the HVC (Voigt et al., 2004).

Brain sections of a male canary exposed to a short-day photoperiod (9/15 h light/dark cycle), which is typical of the nonbreeding season, and a male zebra-finch were prepared with the same procedure of the black jacobin's and included in the analysis to control for methodological artifacts.

### *In situ hybridization and image analysis*

Sections of the brain were used for in situ hybridization to localize the androgen receptor (AR) mRNA expression in cells.

The in situ hybridization was performed using the kit RNAscope 2.5 HD Assay-brown (Advanced Cell Diagnostics, USA) following the manufacturer's protocol (Wang et al., 2012). Chromogenic detection was carried out using chromogen diaminobenzidine (DAB) for 10 min at room temperature. Counterstaining was performed using 50% Hematoxylin. Given that the AR fragment of the black jacobin has a 97% homology with the AR of the zebra finch, one series of slides were hybridized using the probe RNAscope™ Probe- Tgu-AR (Cat. No. 469741, Advanced Cell Diagnostics, USA) and another series using a species-specific probe designed from isolated cDNA fragment of black jacobin AR. Although the species-specific probe for black jacobin had greater signaling than the zebra finch probe, the expression pattern obtained by the hybridization with both probes was consistent.

For the black jacobin, we quantified the AR mRNA expression in brain sections hybridized with the species-specific black jacobin probe. First, we imaged slides at 20x magnification using a Leica CTR 6000 microscope equipped with a DM6000 B camera (Leica Camera AG, Germany). Then, we quantified AR mRNA expression of two females and two males in four brain nuclei: (*i*) vocal nucleus of the lateral neostriatum, VLN (HVC-like); (*ii*) vocal nucleus of the archistriatum, VA (RA-like); (*iii*) vocal nucleus of the anterior neostriatum, VAN (LMAN-like) and (*iv*) nucleus pretectalis (Pt). VLN, VA and VAN were selected due to their analogy with vocal control system of songbirds and represent the main nuclei of the putative vocal control system of hummingbirds. To compare to the vocal control system, we visually inspected all slides, identified and quantified a non-vocal area that showed greater expression which was the Pt. As counting area, we used the entire area within the boundaries of the nuclei for VLN and Pt and an area of 74 x 74 µm placed in the center VA and VAN. Given that AR mRNA was labeled by chromogenic particles, a positive cell had at least one dot or a dot cluster of chromogenic grains. Using the “multi-point” tool of the ImageJ2 software (Rueden et al., 2017), we counted the number of positive cells and the total number of cells within a counting area. The level of AR mRNA expression was given by the ratio between the number of positive cells and the total number of cells. We quantified up to four serial sections for each nucleus per bird of two females (bird ids: FFU10 and FFU23) and two males black jacobin (bird ids: FFU12 and FFUA4_6) and averaged the result per nuclei and bird. Results were represented as ratio average (± standard deviation, bird id, number of quantified slices per bird).

For the other species, we realized a semiquantitative assessment of RNAscope staining by visual inspection of the AR mRNA hybridized slides in a bright field of a light microscope (DM6000 B, Leica, Germany). We categorize the AR mRNA expression in each brain nucleus based on the number of positive cells within its boundaries. A positive cell had at least one dot or a dot cluster of chromogenic grains visible at 20-40 x magnification. We classified the AR mRNA expression according to the following scoring guidelines: low expression (representing by +) when less than 25 % of the cells within the nucleus boundaries were positive; medium expression (++) when between 25 and 75 % of the cells were positive and high expression (+++) when above 75 % of the cells within the nuclei boundaries were positive.

The complete list of samples analyzed, and all images generated in the present study are available in the public repository in the following link: https://osf.io/eaz6j/

## Results

### *Lack of androgen receptors in the putative vocal control system of male and females black jacobin*

In both sexes of black jacobins, the main nuclei of the putative vocal control system, the vocal nucleus of the lateral neostriatum (VLN or HVC-like), vocal nucleus of the archistriatum (VA or RA-like) and vocal nucleus of the anterior neostriatum (VAN or LMAN-like) were not delineated by AR mRNA expression but the nucleus pretectalis (Pt) was (Fig. 1A). When we quantified AR mRNA expression in these nuclei as the ratio between AR positive cells and the total of cells, in both sexes, Pt showed the highest levels ranging from 0.59 (bird id: FFU10, n slices = 1) to 0.83 (FFU23, n = 1); VAN showed intermediate levels ranging from 0.35 (FFU10, n = 1) to 0.43 (± 0.08, FFU12, n = 4); VLN and VA low expression ranging from non-detected (FFUA4_6, n = 1) to 0.21 (± 0.06, FFU10, n = 3) (Fig. 1B). The HVC (proper name) of a male zebra finch and a male canary, which slices were hybridized together with the black jacobins was delineated by AR mRNA expression (Fig. 1C).

Two areas of the black jacobin forebrain in both sexes strongly expressed AR mRNA: the septum (Fig. 2A) and the in the caudomedial neostriatum (NCM) along the lateral ventricle (Fig. 2B). The AR mRNA expression in further areas of the black jacobin's forebrain and midbrain for both zebra finch and black jacobin probes and the high expression observed in HVC of zebra finch and canary (Fig. 1C) indicates that non-detected levels of AR mRNA in the putative vocal control system is unlikely to be a methodological artifact.

In both sexes of black jacobin, the thalamus showed areas with low to non-detected AR mRNA expression, for example, the nucleus rotundus (Rt) and areas with high AR mRNA expression, for example, the Geniculate nucleus (GLv) (Fig. 3A). The midbrain had a few areas with high expression, for example, the nucleus intercollicularis (ICo) (Fig. 3B) and the nucleus preopticus medialis (POM) (Fig. 3C).

### *Lack of androgen receptors in the putative vocal control system of species from other clades, except for Emeralds*

A pattern of AR mRNA expression similar to the black jacobins (clade Topazes) was found in the main nuclei of the putative vocal control system (VLN, VA and VAN) in both sexes of further eight hummingbird species (clades Hermits, Mangoes, Coquettes and Emeralds) with a few exceptions in the clade Emeralds (Fig. 4, Table 1). The nucleus VLN showed non-detectable AR expression in male reddish hermit (*Phaethornis ruber*), male and female black-throated mango (*Anthracothorax nigricollis*), male and female fork-tailed woodnymph (*Thalurania furcata*) and females grey-breasted sabrewing (*Campylopterus largipennis*) and blue-chinned sapphire (*Chlorestes notata*). The male grey-breasted sabrewing had a few positive AR mRNA cells clearly in the boundaries of VLN but the male blue-chinned sapphire had a agglomeration of positive cells in the ventral boundary of VLN which is unclear whether they all belong to the nucleus (Fig. 4A). The nucleus VA also showed consistent non-detected levels of AR expression in both sexes of all species analysed in this study (Fig. 4B) except for females black-throated mango and blue-chinned sapphire in which VA was not found. The nucleus VAN showed differences among species: both sexes of reddish hermit, black-throated mango and grey-breasted sabrewing had non-detectable levels of AR expression, whereas female sombre hummingbird showed some positive AR mRNA expressing cells and males sombre hummingbird, versicoloured emerald and blue-chinned sapphire had the nuclei completely delimitated by AR expression such in songbirds (Fig. 4C).

Similar to black jacobin, a pattern of high AR mRNA expression was observed in both sexes of all species in which NCM was found (Fig. 5A). This consistency was not the case for the nucleus Pt which showed high expression as in black jacobins only in the female reddish hermit, whereas both sexes of black-throated mango, sombre hummingbird and blue-chinned sapphire showed only a few positive cells and male reddish hermit, male frilled coquette (*Lophornis magnificus*), both sexes grey-breasted sabrewing and male fork-tailed woodnymph showed non-detected level of AR mRNA expression (Fig. 5B). In the midbrain the nucleus intercollicularis (ICo) showed high expression consistently in both sexes of all species samples in this study (Fig. 5C). A summary of the AR mRNA expression on eight hummingbird species is provided in Table 1 and Figure 6.

## Discussion

Androgen receptor (AR) mRNA expression is a robust marker used to outline the boundaries of the vocal control system in all avian vocal learners investigated to date independently of sex or breeding condition (Frankl-Vilches & Gahr, 2018; Gahr, 2014; Gahr & Metzdorf, 1997; Matsunaga & Okanoya, 2008). Surprisingly, this differential expression does not seem to be the case in hummingbirds because, consistently in both sexes of all investigated hummingbirds, AR mRNA did not delimitate the putative vocal control system but was expressed in midbrain areas that are well-known to have AR in other birds and mammals. Furthermore, males of different species of the clade Emerald showed AR mRNA only in one nucleus of their putative vocal control system, the VAN. It is important to keep in mind that only a few birds per species were inspected and further investigations are necessary to confirm the results of this study, nevertheless, the findings presented in here suggests a paucity of AR in the putative vocal control system which is maybe widespread in hummingbirds.

In songbirds, a differential expression similar to AR mRNA is obtained in the vocal control system by other methods that target the AR molecule. For example, HVC, RA and LMAN of zebra finch and canary were labeled by immunocytochemistry performed with a rabbit antibody (AR32) which binds to the N-terminus of the androgen receptor (Balthazart et al., 1992). In agreement with this data are the labels obtained by autoradiography with tritiated dihydrotestosterone [$^3$H] which binds to available AR as well as estrogen receptors (ER) after aromatization of testosterone and also the labels with a nonaromatizable androgen dihydrotestosterone [$^3$H]DHT (reviewed in Ball, 1994). Although it is unlikely that methods that target the AR molecule would differ from the labeling obtained by AR mRNA, it is still possible that in hummingbirds the amount of AR mRNA underrepresents the available AR in the cells. Immunocytochemistry is necessary to elucidate this possibility.

The putative vocal control system of hummingbirds was previously labeled by other markers such as the glutamate receptor subunits NR2A and mGluR2 in sombre hummingbird (Wada et al., 2004) and the immediate early gene EGR1 in sombre hummingbird and rufous-breasted hermit (*Glaucis hirsutus*) (Jarvis et al., 2000). Except for the analogous of songbird Area X, we could clearly identify the VLN, VA and VAN by the cytoarchitectural analysis of histologically stained sessions in most of the species investigated in this study similar to those described in previous studies. The VLN of a male black jacobin was labeled by SNAP-25 (Fig. S1) which indicates that the areas we identified are most likely the putative vocal control system of the hummingbirds. Nevertheless, confirmation of the putative vocal control system in both sexes using further markers remains to be seen.

The paucity of AR mRNA in hummingbirds excludes the possibility of an activation effect of testosterone and its metabolites in singing of adults via vocal control system. All hummingbirds investigated in this study show AR mRNA in the medial intercollicular nucleus (ICo), a midbrain area known to play an important role in the activation of vocal behavior in vocal learners but also in non-vocal learners such as chicken (Lanerolle & Andrew, 1974) and doves (Cohen & Cheng, 1982). However, an activation effect via midbrain is also unlikely because, in black jacobins of both sexes, high plasma levels of testosterone after implantation did not affect singing whatsoever (Monte et al., 2023). An effect of estrogens is also unlikely because, in treated black jacobins, testosterone could have been aromatized in the brain and rather bonded to estrogens receptors (ER) and affected their song which did not happen (Monte et al., 2023). It is possible to speculate that if hummingbirds have a mechanism to activate singing, it is independent of androgens and estrogens. To further support this possibility, it is necessary first to map the presence of ER and the enzyme aromatize (ARO) in their brains.

Interestingly, the AR gene was found in black jacobin with high similarity to that of zebra finch (Frankl-Vilches, unpublished data) so that black jacobin-specific cRNA probes label zebra finch's HVC (Fig. 1C). Hummingbirds have an AR gene which seems functional, but adults probably have a mechanism to avoid AR gene expression or transcriptional alterations that produce inactive AR splice variants. Nevertheless, the lack of AR in adult hummingbirds does not exclude the possibility that AR is transiently expressed in juveniles. If so, then androgens may have organization effects during song ontogeny such as the sex differentiation of their vocal control system.

Songbirds and parrots do not show qualitative differences of AR distribution in their vocal control system regardless of sex or singing phenotypes (Gahr, 2007; Matsunaga &

Okanoya, 2008). Such lack of sex differences was found in black jacobins and all other close related clades (Hermits and Mangoes), however, the most distant clade Emeralds showed AR in the VAN of males only. Black jacobins and the Emeralds species differ in sex-specificity of their songs; whereas in black jacobins both sexes sing a spectrally similar song at similar rates (Monte et al., 2023), in Emeralds, except for the blue-chinned sapphire in which females sing (Carvalho, 1958), most of other species it is unknown whether females sing (Fig. 6). This sex difference in AR of Emeralds is probably regardless of singing phenotypes. The presence of AR in Emeralds indicates that in this clade perhaps the song of males is sensitive to androgens. In relation, this song-sensitivity may be also the case in species of the clade Bees because AR was found in the vocal control system of Anna's hummingbird (Gahr, 2000).

We know from songbirds that the LMAN, nuclei analog to the VAN of hummingbirds, plays an important role in the development of song in juveniles most likely by forming a sort of auditory-motor transcription of the song (Bottjer et al., 1984). In adults, lesion of LMAN in intact birds do not affect their learned song but prevent the impairment of song in deafened birds (Brainard & Doupe, 2000). In Emeralds, VAN but not the other nuclei of the vocal control system have AR probably because testosterone and its metabolits may play a role in the seasonal update of their song. This idea is further supported by the evidence that hummingbirds are able to revise their learned song when adults, thus, they seem to be open-learners (Araya-Salas & Wright, 2013). However, this evidence was found in long-billed hermits (*Phaethornis longirostris*) and the reddish hermit that we investigated in the present study do not show AR in their vocal control system. A mapping of AR distribution in the brain of long-billed hermits can shed light to this question. In addition, for mGluR2, the posterior part of the hummingbird VAN nucleus (VAN-P) had lower expression, whereas the anterior part (VAN-A) had higher expression relative to the surrounding nidopallium, and this dual pattern was unique to hummingbirds (Wada et al., 2004). A similar pattern was found in the VAN of male sombre hummingbird and blue-chinned sapphire, but the implication of this apparent segmentation remains to be seen.

The expression of AR in caudomedial neostriatum (NCM) was found in songbirds but not in parrots (Matsunaga & Okanoya, 2008; Metzdorf et al., 1999). In zebra finch, breeding are associated with an increase in estrogen and the amplitude of the female's neural auditory responses in NCM (Adreani et al., 2020), this question has not yet been tackled in parrots. In both sexes of all hummingbird species investigated in this study was found high levels of AR mRNA in NCM which suggests auditory plasticity as well but, in both sexes, and rather modulated by androgens.

When identified, visual areas such as the retinoreceptors nucleus geniculatus lateralis pars ventralis (GLv) and the nucleus lentiformis mesencephali (LM) show AR mRNA in most of the species studies here. GLv are LM are related to optokinetic reflex and visuomotor responses and LM is hypertrophied in hummingbirds to meet the increased optic flow processing demanded by the hovering flight (Vega-Zuniga et al., 2018; Wylie et al., 2015). In long-billed hermits (*Phaethornis longirostris*), geographical variation in their visual displays that cannot be explained by genetic differences support the idea that not only their song but visual displays are learned (Araya-Salas et al., 2019). The neural substrate for visual learning remains unknown, but the presence of AR in visual areas, particularly in Hermit species, can give hints of areas potentially related to organization and activation effects mediated by androgens in visual learning.

## Conclusion

Androgen receptors (AR) delimitated the vocal control system of all songbirds and parrots investigated to date (reviewed in Frankl-Vilches, and Gahr, 2018; Gahr, 2014) but not the putative vocal control system of hummingbirds. Except for one nucleus, this paucity was consistent in nearly all species investigated in here with different degrees of phylogenetic distance. If confirmed, these results suggest that AR is not an intrinsic feature of the vocal control system, and that the convergent evolution of a vocal control system did not necessarily imply convergence of androgen sensitivity. Hovering flight and nectarivory evolved in the hummingbird radiation shortly after the split between hummingbirds and swifts which seem related to an early the loss of a gluconeogenic muscle enzyme (McGuire et al., 2014; Osipova et al., 2023; Prum et al., 2015). It may be the case that the challenges that androgens, particularly testosterone (Wingfield et al., 1990), pose to the metabolism constrained the evolution of their androgen sensitivity. The past two-decade of comparative studies among avian vocal learners showed mainly convergences in their vocal control system despite the independent evolution of vocal production learning. Nevertheless, as more hummingbirds are investigated from a phylogenetic perspective, the more differences are found in their putative vocal control system that can unveil a distinct, yet interesting, pathway that the evolution of vocal production learning might have taken.

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## Statement of Ethics

All procedures of this study were conducted following the national regulations in Brazil. The samples for this study were collected with the permission of 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) licenses numbers 30319-1, 41794-2 and 49097-1. All sampling procedures were performed 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). The bird handling and housing procedures were conducted in accordance with the Law nº 11.794 / 2008, Decree 6899 of July 15, 2009 as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was approved by the Ethics Committee on Animal Use of the Federal University of Para (CEUA/UFPA) under the protocol number CEUA 5937130718 (ID 000953). The genetic data of the black jacobin presented in this study were accessed in compliance with the Law nº 13.123/2015 of the Ministry of the Environment and Genetic Heritage Management Council, the activities were registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the registration number AF0CC37.

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

AM, MG and CF conceived the idea of the paper; AM collected the samples and analyzed the data; AB and CF performed the laboratory analysis of the samples; MLS and MG supervised the research; AM, MLS, and MG wrote and/or substantially edited the manuscript; and MG secured funding for this work.

## Data Availability Statement

The complete list of samples analyzed, and all images generated in the present study are available in the public repository in the following link: https://osf.io/eaz6j/

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## Figure Legends

Fig. 1. Black jacobin of both sexes did not have the main nucleus of their vocal control system delimited by androgen receptor (AR) mRNA expression. A) Transversal brain sections showing the VLN, VA, VAN and Pt in wild male and female black jacobin labeled by species-specific AR cRNA (right panel). Schematic drawing indicating nuclei location (left panel). Black arrows indicate the delimitation of the nuclei and red arrows examples of mRNA-expressing cells. B) Low AR mRNA expression in the main nuclei of their putative vocal control system (VLN, VA and VAN) in comparison to a nucleus nonrelated to vocal production (Pt). Ratio between the number of AR mRNA positive cells and the total of cells within the nucleus (VLN and Pt) or a counting frame placed in the middle of the nucleus (70 x 70 mm, VA and VAN). Each data point represents the measurements obtained of one bird. C) The HVC of a male zebra finch and a male canary was delimited by AR mRNA expression although labeled with black jacobin specific probe. A, arcopallium; H, hyperpallium; HVC, formal name, located in nidopallium; LFS, lamina frontalis superior; LPS, lamina pallio-subpallialis; M mesopallium; MD, mesopallium dorsal; MSt, medial striatum; MV, mesopallium ventral; Pt, nucleus pretectalis; StL, striatum laterale; V, ventricle; VA, vocal nucleus of the archistriatum; VAN, vocal nucleus of the anterior neostriatum; VLN, vocal nucleus of the lateral neostriatum.

Fig. 2. Black jacobin of both sexes had similarly high androgen receptor (AR) mRNA expression in further areas of the forebrain other than the vocal control system: A) in the septum and B) in the area corresponding to the caudal neostriatum (NCM). Transversal brain sections showing the septum and the NCM in wild male and female black jacobin labeled by species-specific AR cRNA (right panel). Schematic drawing indicating the location of the section (left panel). Red arrows indicate examples of mRNA-expressing cells. A, arcopallium; H, hyperpallium; N, nidopallium; NCM, caudomedial neostriatum; St, striatum.

Fig. 3. Black jacobin of both sexes had similar androgen receptor (AR) mRNA expression in thalamic and midbrain areas. A) In the nucleus rotundus (Rt), a low expression and in the nucleus geniculate (GLv), a medium expression was observed. B) In the nucleus intercollicularis (ICo), a high expression was observed. C) In the medial preoptic nucleus (POM), a high expression was observed. Transversal sections of the wild male and female showing the AR mRNA expression (right panel). Schematic drawing indicating nuclei location (left panel). Red arrows indicate examples of mRNA-expressing cells.

Fig. 4. Hummingbirds of the clades Hermits, Mangoes, Coquettes did not have the main nucleus of their vocal control system delimited by androgen receptor (AR) mRNA expression similarly in both sexes, whereas males of the clade Emeralds had one nucleus (VAN) delimited by AR mRNA expression. Sagittal brain sections showing the A) VLN, B) VA and C) VAN labeled by species-specific AR cRNA (right panel). Schematic drawing indicating nuclei location (left panel). Black arrows indicate the delimitation of the nuclei and red arrows examples of mRNA-expressing cells. Red arrows indicate examples of mRNA-expressing cells. The clades are represented by the color of the squares: Hermits, red; Coquettes, purple; Mangoes, blue and Emeralds, green. A, arcopallium; DLM, nucleus dorsolateralis anterior thalami, pars medialis; E, entopallium; GP, globus pallidus; H, hyperpallium; HA, hyperpallium apicale; HD, hyperpallium densocellular; ICo, nucleus intercollicularis; M, mesopallium; N, nidopallium; St, striatum; TrO, tractus opticus; VA, vocal nucleus of the archistriatum; VAN, vocal nucleus of the anterior neostriatum; VLN, vocal nucleus of the lateral neostriatum.

Fig. 5. Hummingbirds of the clades Hermits, Mangoes, Coquettes and Emeralds show high expression of androgen receptor (AR) mRNA expression in the area corresponding to the caudal neostriatum (NCM) consistently among species and in both sexes; low to medium AR expression in the nucleus pretectalis (Pt) and high AR expression in the nucleus intercollicularis (ICo) also consistently among species and in both sexes. Sagittal brain sections showing the A) NCM, B) Pt and C) ICo labeled by species-specific AR cRNA (right panel). Schematic drawing indicating nuclei location (left panel). Red arrows indicate examples of mRNA-expressing cells. The clades are represented by the color of the squares: Hermits, red; Coquettes, purple; Mangoes, blue and Emeralds, green. DM, nucleus dorsomedialis of the midbrain; GP, globus pallidus; HA, hyperpallium apicale; HD, hyperpallium densocellular; ICo, nucleus intercollicularis; M, mesopallium; MLd, nucleus mesencephalicus lateralis, pars dorsalis; N, nidopallium; NCM, caudomedial neostriatum; Pt, nucleus pretectalis; St, striatum; TrO, tractus opticus.

Fig. 6. Summary of the androgen receptor (AR) mRNA expression in the brain of hummingbirds of the clades Topazes, Hermits, Mangoes, Coquettes and Emeralds investigated in this study. Close related clades show similar paucity in the delimitation of the main nuclei of the putative vocal control system nuclei, except for males of the clade Emeralds. All clades show consistently high AR expression in the NCM in both sexes. The prevalence of AR mRNA expression in the clades seems independent of singing. Schematic representation of the hummingbird phylogeny (McGuire et al., 2014) (right panel). Prevalence of AR mRNA expression in females and males for the species investigated in this study per clade (middle panel). A green tick mark represents presence in most of the species, a red x represents absence in all species and a question mark, unknown data. References for the song prevalence: black jacobin (Monte et al., 2023), reddish hermit (Nicholson, 1931; Snow, 1973; Vielliard, 1983),

black-throated mango (Castro, 2017), frilled coquette (A. Ruschi, 1973) and sombre hummingbird (Ferreira et al., 2006). Hummingbird pictures were reproduced with permission of the rights owners: black jacobin and sombre hummingbird (Amanda Monte); reddish hermit (Jayrson Araújo); black-throated mango (Felipe Castro) and frilled coquette (Rodrigo Matos).

# Figures

Figure 1

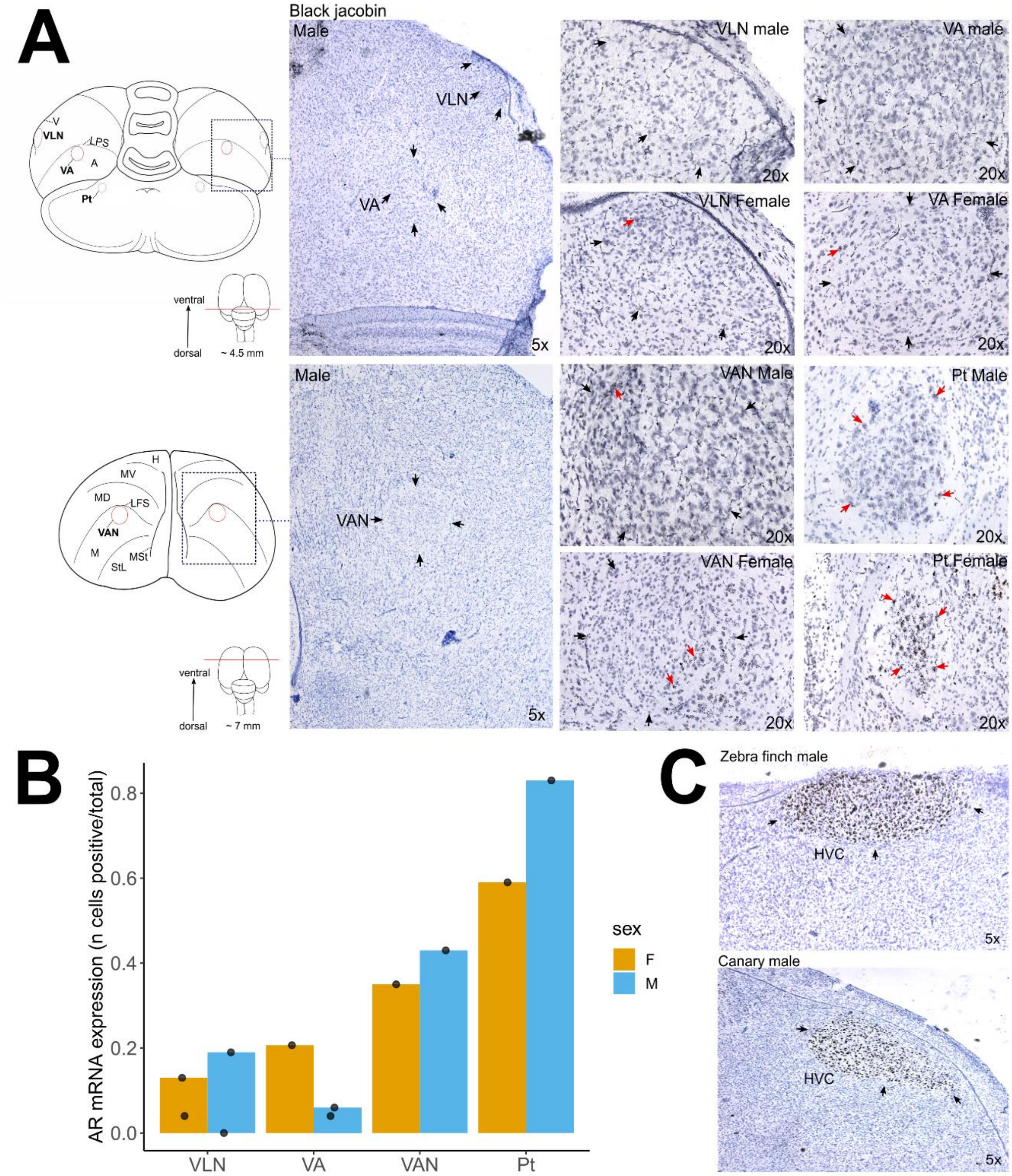


Figure 2

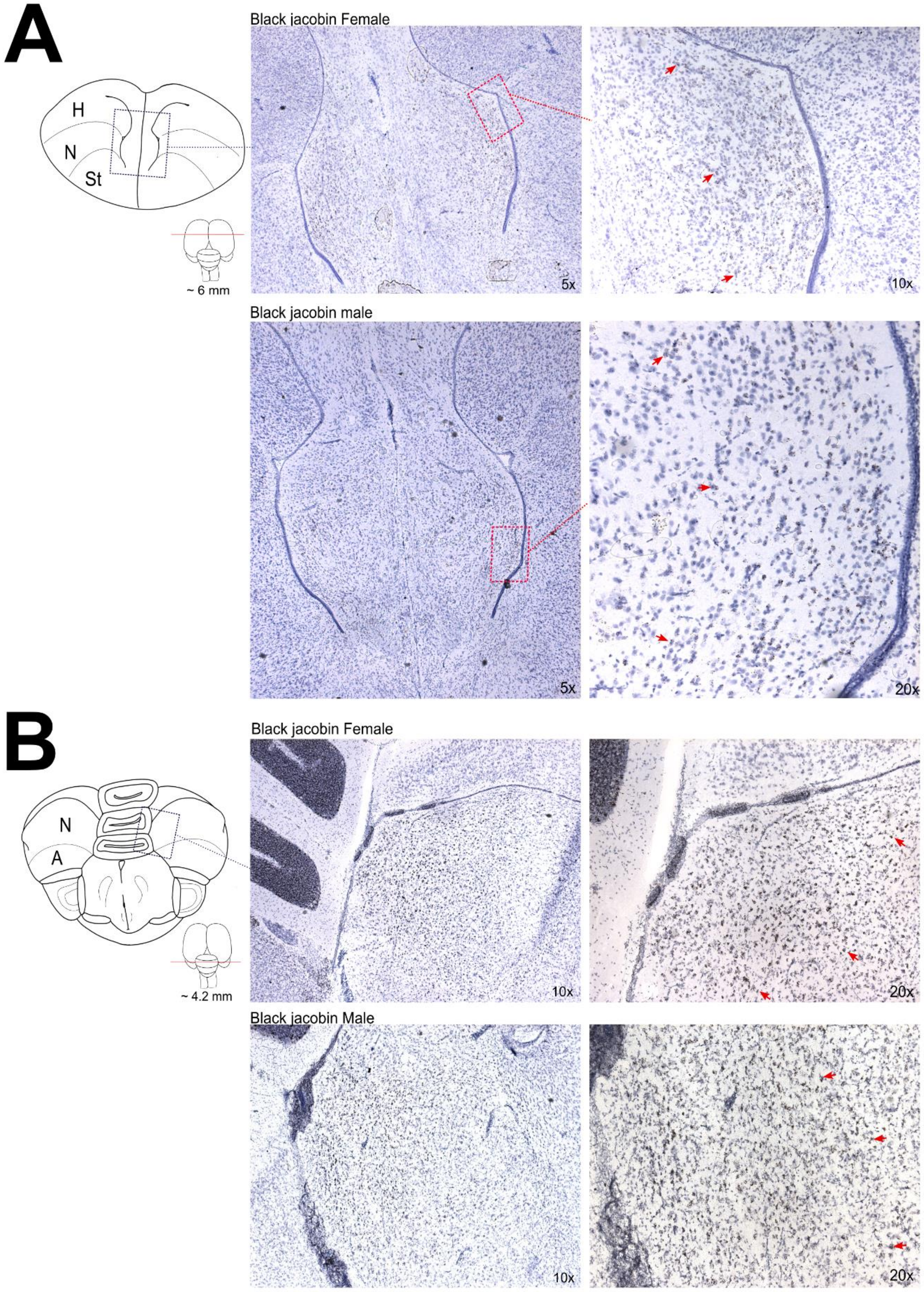

A
H
N
St
~ 6 mm
Black jacobin Female
5x
10x
Black jacobin male
5x
20x
B
N
A
~ 4.2 mm
Black jacobin Female
10x
20x
Black jacobin Male
10x
20x

Figure 3

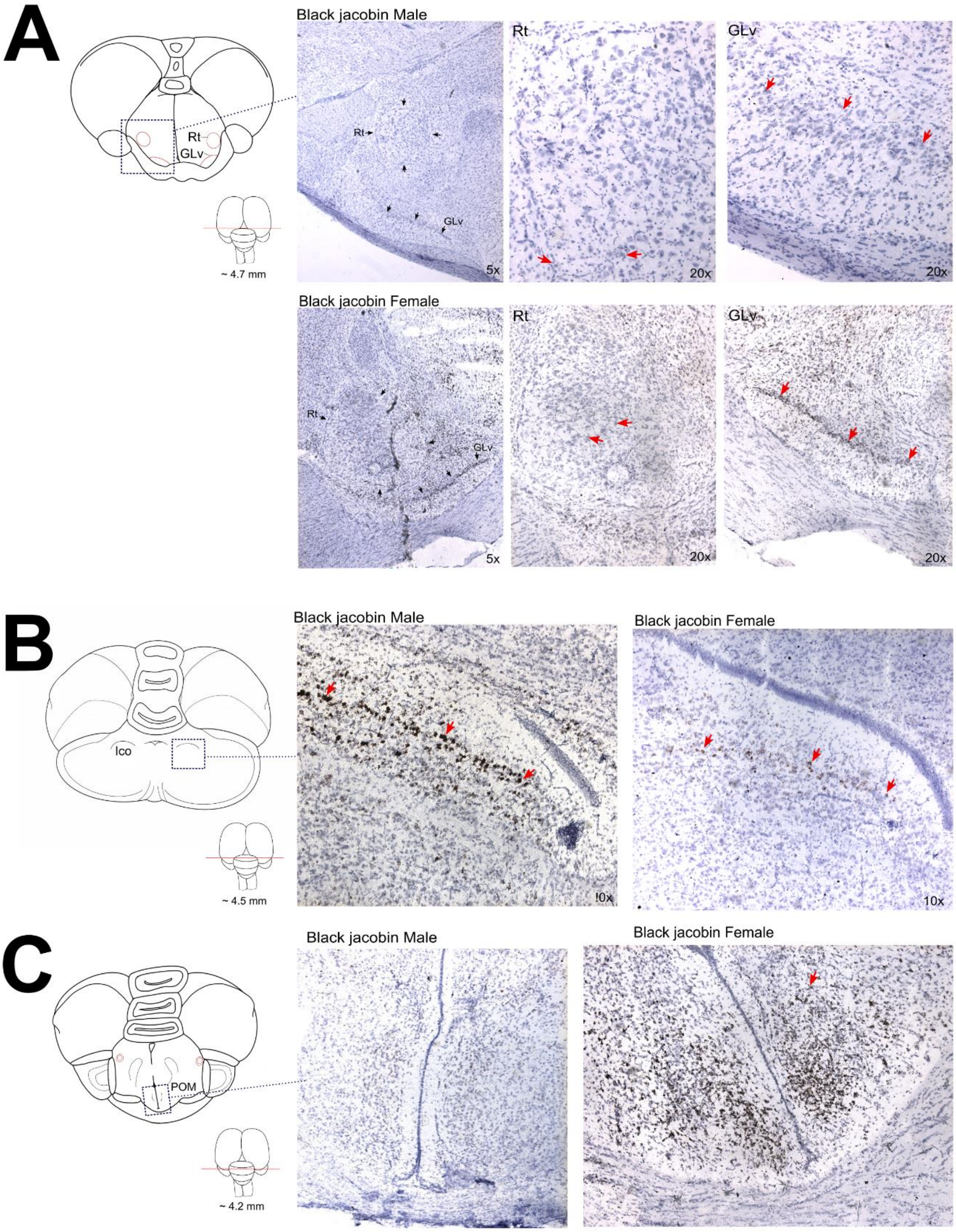

A
Rt
GLv
~ 4.7 mm
Black jacobin Male
Rt
GLv
5x
Rt
20x
GLv
20x
Black jacobin Female
Rt
GLv
5x
Rt
20x
GLv
20x
B
Ico
~ 4.5 mm
Black jacobin Male
!0x
Black jacobin Female
10x
C
POM
~ 4.2 mm
Black jacobin Male
Black jacobin Female

Figure 4

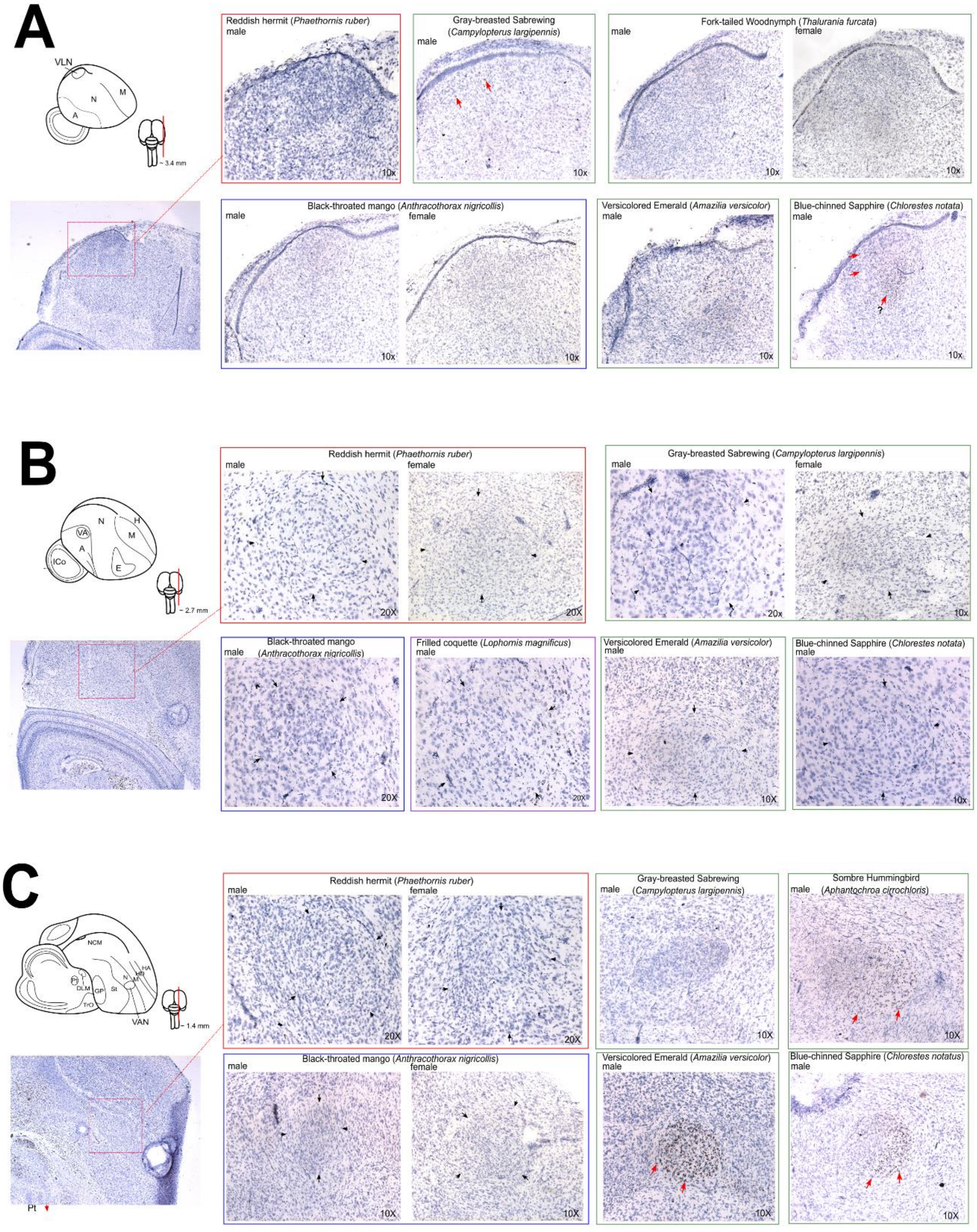

A
VLN
N
M
A
~ 3.4 mm
Reddish hermit (Phaethornis ruber)
male
10x
Gray-breasted Sabrewing (Campylopterus largipennis)
male
10x
Fork-tailed Woodnymph (Thalurania furcata)
male
female
10x
10x
Black-throated mango (Anthracothorax nigricollis)
male
female
10x
10x
Versicolored Emerald (Amazilia versicolor)
male
10x
Blue-chinned Sapphire (Chlorestes notata)
male
?
10x
B
N
H
VA
M
A
ICo
E
~ 2.7 mm
Reddish hermit (Phaethornis ruber)
male
female
20X
20X
Gray-breasted Sabrewing (Campylopterus largipennis)
male
female
20x
10x
Black-throated mango (Anthracothorax nigricollis)
male
20X
Frilled coquette (Lophornis magnificus)
male
20X
Versicolored Emerald (Amazilia versicolor)
male
10X
Blue-chinned Sapphire (Chlorestes notata)
male
10x
C
NCM
HA
Hp
N
M
Pt
DLM
GP
St
TrO
VAN
~ 1.4 mm
Pt
Reddish hermit (Phaethornis ruber)
male
female
20X
20X
Gray-breasted Sabrewing (Campylopterus largipennis)
male
10X
Sombre Hummingbird (Aphantochroa cirrochloris)
male
10X
Black-throated mango (Anthracothorax nigricollis)
male
female
10X
10X
Versicolored Emerald (Amazilia versicolor)
male
10X
Blue-chinned Sapphire (Chlorestes notatus)
male
10X

Figure 5

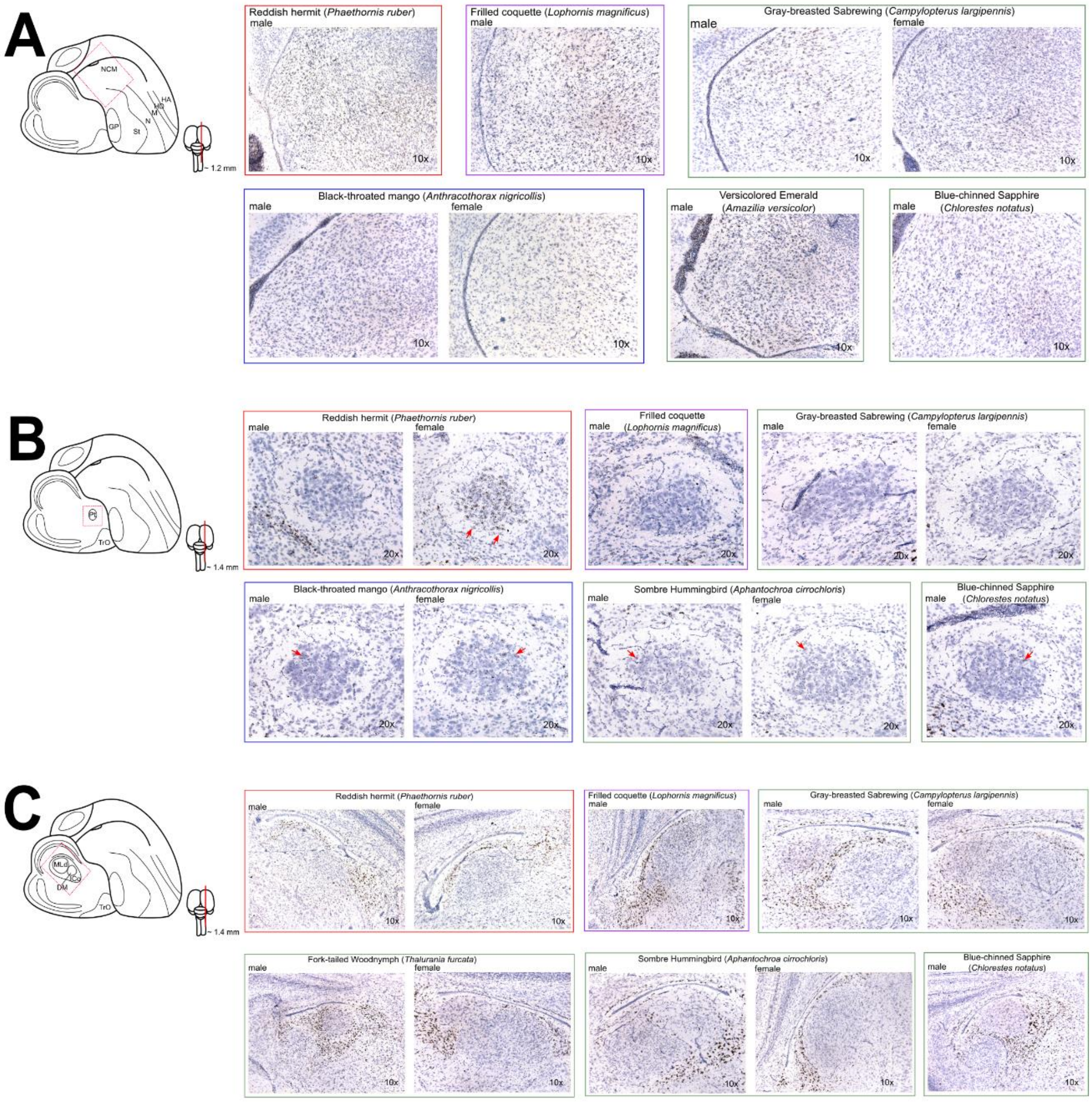
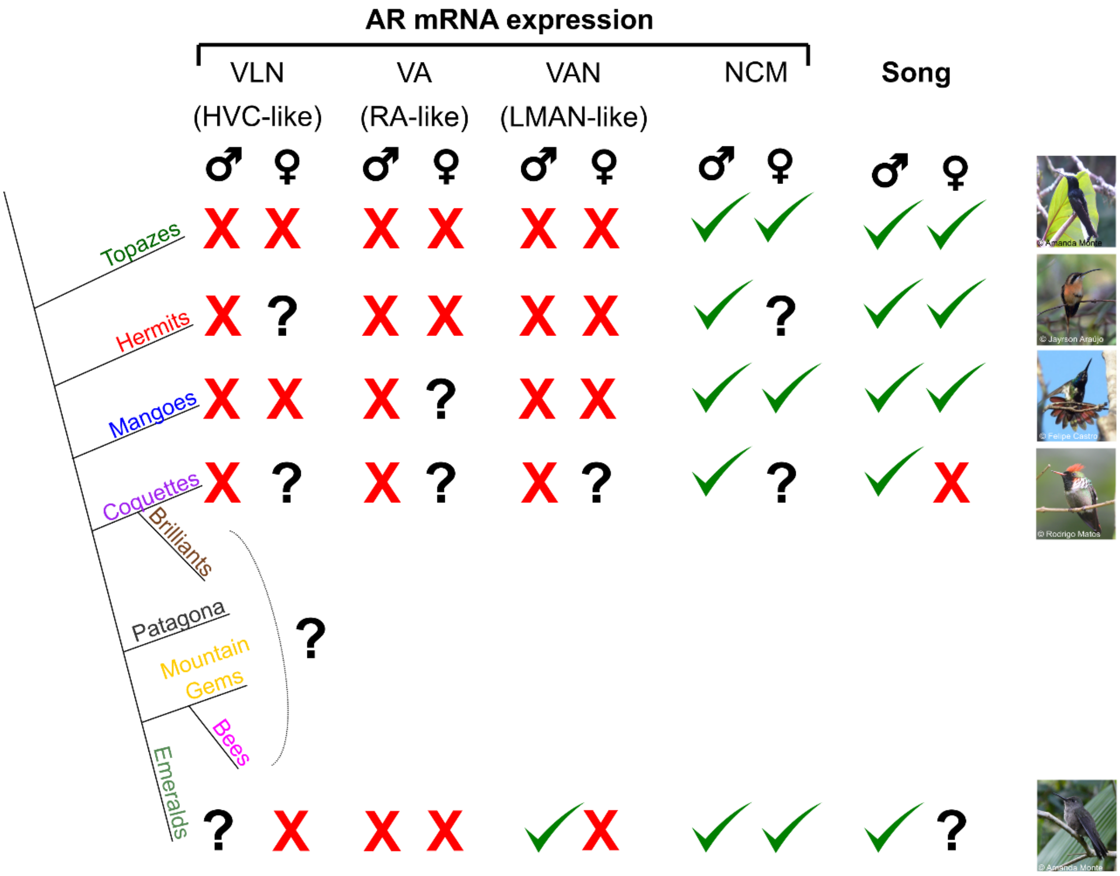


Figure 6


AR mRNA expression
VLN (HVC-like)
VA (RA-like)
VAN (LMAN-like)
NCM
Song
♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀ ♂ ♀
Topazes X X X X X X ✓ ✓ ✓ ✓
Hermits X ? X X X X ✓ ? ✓ ✓
Mangoes X X X ? X X ✓ ✓ ✓ ✓
Coquettes X ? X ? X ? ✓ ? ✓ X
Brilliants
Patagona
Mountain Gems
Bees
?
Emeralds ? X X X ✓ X ✓ ✓ ✓ ?
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## Supplementary Material

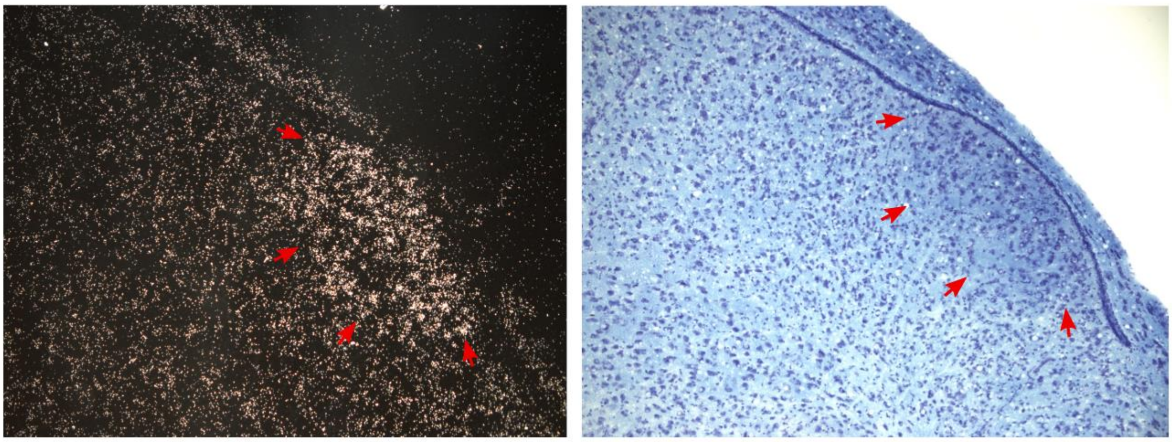

Fig. S1. Black jacobin had the boundaries of the vocal nucleus of the lateral neostriatum (VLN or HVC-like) delimitated by synaptosomal-associated protein 25 kDa (SNAP-25) mRNA. On the left, the darkfield photomicrographs of the in-situ hybridizations for SNAP-25 mRNA and on the right, the brightfield photomicrographs of the adjacent Nissl-stained section. Transversal sections. Red arrows indicate the ventral delimitation of the nuclei. Images produced by Cornelia Voigt.

## Tables

Table 1 – Androgen receptor (AR) mRNA expression in the brain of females (F) and males (M) in seven hummingbird species. The AR expression of male canary (songbird) and ring dove (a nonsongbird) according to Metzdorf et al. (1999) is also shown. A semiquantitative assessment of the AR expression within the boundaries of each area is represented by + for low expression (less than 25% positive cells); ++ for medium expression (between 25 and 75 % positive cells) and +++ for high expression (above 75 % positive cells). Paucity is indicated by – (absence or only background expression), unclear result by a question mark and inexistent brain area by a blank field. The highest expression found among the accessed brain sections is depicted. For black jacobin, the brain of three birds per sex was inspected; for reddish hermit, grey-breasted

sabrewing and fork-tailed woodnymph: one bird per sex; black-throated mango: two females and three males; frilled coquette: three males; sombre hummingbird: one female and two males, versicoloured emerald: one male and blue-chinned sapphire: one female and three males.

| | black jacobin | | reddish hermit | | black-throated mango | | frilled coquette | grey-breasted sabrewing | | fork-tailed woodnymph | | sombre hummingbird | | versicoloured emerald | blue-chinned sapphire | | canary | ring dove |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | M | F | M | F | M | M | F | M | F | M | F | M | M | F | M | M |
| Forebrain | | | | | | | | | | | | | | | | | | |
| VLN (HVC-like) | - | - | - | ? | - | - | ? | + | - | - | - | ? | ? | - | + | ? | ++ | |
| VA (RA-like) | - | + | - | - | - | ? | - | - | - | - | - | - | - | - | - | ? | + | |
| VAN (LMAN-like) | + | + | - | - | - | - | ? | - | - | ? | ? | + | ++ | +++ | +++ | - | ++ | |
| NCM | +++ | +++ | ++ | ? | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ? | ? | +++ | +++ | ++ | + | |
| Tn | ? | ++ | ? | ? | ? | ? | ++ | ? | ? | ? | ? | ? | ? | ? | ? | ? | | |
| Hp | ? | ? | ? | ? | + | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | + | + |
| S | +++ | +++ | ? | ? | ++ | ? | + | ? | ? | ? | ? | ? | ? | ? | ? | ++ | | |
| Thalamus | | | | | | | | | | | | | | | | | | |
| Rt | - | - | ++ | ++ | + | ++ | ++ | ? | ? | ? | ? | ? | ? | ? | ++ | ? | ? | ? |
| GLv | +++ | +++ | ? | +++ | ++ | +++ | ? | +++ | ? | ? | ? | ? | ? | ++ | ++ | ? | ? | ? |
| DLM | ? | ? | +++ | ? | ? | ? | ? | ? | | ? | ? | ? | ? | ? | ? | ? | | |
| Midbrain | | | | | | | | | | | | | | | | | | |
| Pt | +++ | +++ | - | +++ | ++ | ++ | - | - | - | - | - | + | + | ? | + | + | ? | ? |
| SpL | - | - | | | ? | | | ? | | | | | | ? | | ? | ? | ? |
| SpM | + | ++ | ? | ? | + | ? | ? | ? | ? | ? | ? | ? | ? | ++ | + | ? | ? | ? |
| LM | ? | ++ | ? | ? | ? | ++ | ++ | ? | ? | ++ | ? | ? | ? | ? | ++ | ? | ? | ? |
| ICo | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ | ++ | ++ |
| MLd | ? | ? | ++ | + | + | | ++ | + | ++ | + | + | ? | + | ? | + | ? | ? | ? |
| DM | ? | +++ | ? | ? | | ? | | ++ | ? | + | ++ | ? | | ? | | ? | ? | ? |
| POM | +++ | +++ | ? | ? | +++ | ? | +++ | ? | ? | ? | ? | +++ | ? | ? | +++ | ? | ++ | ++ |
| PMH | ? | ? | ? | ? | ++ | ? | ? | ? | ? | ? | ? | +++ | ? | ? | ? | ? | + | |
| Hindbrain | | | | | | | | | | | | | | | | | | |
| nXIIts | ? | ? | ? | | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ++ | ? | +++ | + |
| Cerebellum | | | | | | | | | | | | | | | | | | |
| Purkinje cells | +++ | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | +++ | + |
| CbI | +++ | ? | ? | ? | + | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | ? | | |

Nucleus interfascialis (Nif), caudalmedial neostriatum (NCM), Nucleus taeniae (Tn), Hippocampus (Hp), Septum (S), Ovoidalis (Ov), Nucleus rotundus (Rt), Medial portion of the dorsolateral nucleus of the thalamus (DLM), N. pretectalis (Pt),

N. spiriformis lateralis (SpL), N. spiriformis medialis (SpM), Pretectal nucleus lentiformis mesencephali (LM), Geniculate nucleus (GLv), N. intercollicularis (ICo), Dorsal lateral nucleus of the mesencephalon (MLd), N. dorsomedialis of the midbrain (DM), N. preopticus medialis (POM), N. posterioris hypothalamic medialis (PMH), N. posterioris hypothalamic lateralis (PLH), tracheosyringeal portion of the hypoglossal motor nucleus (nXIIts), N. cerebellaris internus (CbI).

# Chapter 5

# Map of an immediate-early gene expression in the brain of the long-tailed hermit when singing spontaneously in the lek

## Abstract

At least 14% of all hummingbird species join a male aggregation to sing, the so-called lek. In the lek, males perform learned songs and learned visual displays to repel competitors and attract females. Although the ecological relevance of lek for hummingbirds, the brain processes underlying singing in lek remains unknown. Mapping the expression of the immediate early gene EGR-1 has been shown as a powerful tool to investigate the brain areas active during behaviors unreproducible in captivity. We mapped the EGR-1 expression of long-tailed hermit (*Phaethornis superciliosus*) males when singing spontaneously in lek in comparison with males kept in silence. Singing in the lek induced up to 4-fold EGR-1 expression in some of the telencephalic nuclei of the vocal control system (VLN, VA, and VAN) and an auditory area (NCM), however, when compared to the immediate surroundings, this expression was unspecific. EGR-1 expression was locally proportional to different aspects of the song: syllable rate in VLN and mean syllable duration in VAN. A unique pattern of expression was observed in the thalamus which is potentially related to visual displays. The pattern of EGR-1 expression found in the long-tailed hermits highly differs from non-lekking hummingbirds and suggests that, as songbirds, hummingbirds show context-dependent EGR-1 expression. Furthermore, these differences in EGR-1 expression give insights into alternative brain pathways that may be associated with complex multi-modal signaling in a natural context such as in lek.

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## Introduction

A remarkable behavior of an important group of birds that is unexplored from the neural processing perspective is the formation of leks in hummingbirds. Broadly defined, a lek is an aggregation of males with distancing for the purpose of finding a mate (e.g.: Stiles and Wolf, 1979). From each small individual territory, a male may vocalize and perform characteristic visual displays such as rhythmic head and tail movements to attract females (Nicholson, 1931; Wiley, 1971), thus resulting in complex audible-visual displays that contain information for their prospective mates. Lek formation is widespread within hummingbirds (Bleiweiss, 1998; Stiles & Wolf, 1979). Leks are reported in 49 species, 14% of all hummingbird species, including almost all major clades (Martínez-García et al., 2013; McGuire et al., 2014; Schuchmann, 1999), but leks are especially frequent in two of them: hermits and emeralds (Bleiweiss, 1998; Martínez-García et al., 2013; McGuire et al., 2014).

Interestingly, hummingbirds are also able to learn vocalizations, a trait that is rare in birds in mammals, but which allows for the cultural transmission of information across generations (e.g. in hummingbirds: Araya-Salas and Wright, 2013; Engeln, 2013; González and Ornelas, 2014; Feo et al., 2015). Among birds, this ability to learn vocalizations – referred here as vocal learning – and its related brain specialization evolved independently in songbirds, parrots and hummingbirds, the vocal learners (Gahr, 2000; Jarvis et al., 2000; Jarvis & Mello, 2000; Nottebohm et al., 1976). Only in vocal learners, the basic mechanism to control vocal production, which consists of distinct brainstem nuclei that control muscles of the syrinx, muscles of respiration and secondary structures, receives motor commands from a set of anatomically discrete but interconnected nuclei of the forebrain, the vocal control system (Gahr, 2000; Nottebohm, 1991; Schmidt & Ashmore, 2008).

In songbirds, the vocal control system includes two nuclei of the motor pathway (HVC, proper name and the RA, robust nucleus of the arcopallium) which makes a direct projection from the arcopallium onto the vocal motor-neurons in the brainstem and four nuclei in an anterior forebrain pathway loop which interconnects the nidopallium (HVC and the lMAN, lateral magnocellular nucleus of the anterior nidopallium) with the striatum (Area X) and thalamus (DLM, nucleus of the dorsolateral thalamus) which projects back to the nidopallium (reviewed in Brainard and Doupe, 2002). Whereas the motor pathway is necessary for normal song production throughout life, the anterior forebrain pathway is important for song learning early in life and song plasticity in adult birds (reviewed in Farries and Perkel, 2008). The vocal control system found in birds holds many parallels to the neural basis of spoken language in humans (reviewed in Petkov and Jarvis, 2012).

In fact, much of the behavioral evidence for vocal learning in hummingbirds were obtained from studies of lekking species (Araya-Salas & Wright, 2013; Kroodsma & Baylis, 1982). Recently, it was demonstrated in a lekking hummingbird that not only songs, but also their visual displays, show more similarity within leks than between leks and that this similarity was explained by social learning rather than genetic similarities (Araya-Salas et al., 2019). Furthermore, in the lek, males can modify the silent intervals between their songs to allow songs to alternate the songs of immediate neighbors, rather than overlap (Araya-Salas et al., 2017). The number of males per lek varies widely from 2 - 23 (D. S. Brito, 2012; Stiles & Wolf, 1979) with around 50% of them annually replaced (Stiles & Wolf, 1979). Hummingbird leks can be active year-round and throughout the day in the tropics, where individual males sing up to 50% of daylight hours, continuously for periods of up to 30 minutes (D. S. Brito, 2012; Stiles & Wolf, 1979; Vielliard, 1983) – a behavior which may demand a mechanism to deal with acoustic habituation. Thus, males face many challenges when singing in the lek, including (*i*) the production of learned visual displays concurrently with learned songs, (*ii*) the constant attentiveness to the songs of the neighbors to avoid overlap of songs and (*iii*) long-lasting song production and the need to learn and relearn song across seasons. However, how the neuronal vocal control system facilitates singing in the context of being in a lek remains unknown.

The expression of the gene EGR-1, an immediate-early gene, is a powerful tool to investigate the connection between brain and behavior (C. V. Mello et al., 1992); especially those behaviors that animals do not produce under laboratory conditions (Jarvis et al., 1997). EGR-1 is a highly sensitive marker for neuronal activation because this gene is expressed rapidly and transiently in response to neuronal depolarization (reviewed in Mello, 2002a). The expression of EGR-1 increases within minutes after stimulus onset and peaks at around 30 min before it decreases back to basal levels by 1 h due to habituation (C. Mello et al., 1995; C. V. Mello & Clayton, 1994). Since the 1990s, EGR-1 has been largely used to map neuronal activation triggered by vocal behavior in birds (C. V. Mello, 2002b). Comparative analysis of the EGR-1 expression in unrelated species of birds has revealed striking similarities in the organization of brain systems for production and perception of learned vocalizations (Jarvis et al., 1998, 2000; Jarvis and Mello, 2000; but see Mischler et al., 2020).

During the production of learned vocalizations, i.e., songs or learned calls, the vocal control system shows a differential pattern of EGR-1 expression which is strikingly similar among vocal learners (Jarvis et al., 2000; Jarvis & Mello, 2000; Jarvis & Nottebohm, 1997). Nevertheless, studies on songbirds indicated that the patterns of EGR-1 expression can be dramatically different depending on whether the male sings to a female, to another male, or by themselves (Jarvis et al., 1998). The investigation of the context-dependent expression of EGR-1 comparatively in species of vocal learners with different biologically relevant behaviors can shed light on the functionality and plasticity of the vocal control system in adult birds.

To investigate the neural activity of the vocal control system in lekking hummingbirds, we mapped the EGR-1 expression in the brains of male long-tailed hermits (*Phaethornis superciliosus*) when singing spontaneously in the lek in comparison with silent controls. The EGR-1 expression in the brains of male rufous-breasted hermits (*Glaucis hirsuta*), a non-lekking species (Ornelas et al., 2002), was previously investigated. When the non-lekking hermit was presented with playbacks of conspecific song, EGR-1 was expressed in some telencephalic areas possibly related to auditory perception, including the caudal medial nidopallium (NCM). Whereas, when the non-lekking hermit sang in response to the playback, EGR-1 was expressed in different brain areas including the putative telencephalic vocal control system of hummingbirds, such as the vocal nucleus of the lateral nidopallium (VLN or HVC-like), the vocal nucleus of the arcopallium (VA or RA-like) and vocal nucleus of the anterior nidopallium (VAN or lMAN-like) (Jarvis et al., 2000). EGR-1 expression in these nuclei was proportional to the amount of song produced during the singing period (Jarvis et al., 2000). The comparison of the EGR-1 expression between lekking and non-lekking hermits may indicate whether there is context-dependent EGR-1 expression in the vocal control system of hummingbirds, such as found in songbirds.

Thus, we verified whether there is: (*i*) song-induced EGR-1 expression in the vocal control system of spontaneously singing hermits, compared to silent controls; (*ii*) a differential EGR-1 expression in the vocal control system in relation to the surrounding area and (*iii*) a correlation of the EGR-1 expression and the number of songs produced by the hermits. We found that in a lekking hummingbird there is more generalized forebrain expression and many other forebrain areas are also involved in song production, thus establishing a lek-specific context-dependent EGR-1 expression in the vocal control system of hummingbirds. This pattern occurred differently from songbirds in which the expression was observed in fewer areas during singing to a female. This comparison of EGR-1 expression in lekking hermits to what is known for singing and hearing-only patterns in non-lekking hummingbirds, thus illuminates our understanding of brain areas involved in multimodal communication, including the integration of vocal and visual signaling.

## Methods

### *Animals and field procedures*

Ten wild long-tailed hermit males were used; six for the singing group and four for the silent control group. The birds were captured in the Gunma Ecological Park, an Amazon Rainforest fragment with around 400 hectares of primary forest in Brazil (1º13'S, 48º41'W), and its surroundings. The samples were collected in July 2014 and July/August 2015 during the dry season when the highest singing activity of the long-tailed hermits was registered (D. da S. Brito & da Silva, 2013). All procedures were approved by the Brazilian authorities (Ministry of Environment and Chico Mendes Institute for Biodiversity Conservation) via Biodiversity Authorization and Information System (Sistema de Autorização e Informação em Biodiversidade – SISBIO) permit number 41794-1.

The males of the singing group were localized by their singing activity in a male aggregation (lek) where they can continuously sing for about 30 min (Stiles & Wolf, 1979). An individual focal male was visually localized on its display perch with binoculars in a larger group of other males that were perched and performing characteristic head and tail movements and producing song. After visual localization of the focal male on its perch, its song was recorded for 30 - 90 seconds, and the focal male was shot with an air pressure gun during singing. Within the 6 - 8 minutes, the head was located, the brain was dissected and flash-frozen in dry ice. In most of the cases, the activity of the non-focal birds in the lek was not disrupted, or if disrupted, the immediate neighbor males resumed singing within the dissection period. The singing males were sampled from four leks with at most two males per lek, not exceeding 35% of the lek's composition. In the lek, the highest singing rate was observed between 8:00 and 10:30 a.m. (D. S. Brito, 2012). The birds were captured at least one hour after, between 9 and 10:30 a.m., so that the singing males in the lek would have reached the peak of the EGR-1 mRNA levels, which occurs from 30 min to 1 hour after the exposure to a stimulus (C. V. Mello

& Ribeiro, 1998). The males of the control group were captured outside the lek with the Ruschi net trap, a trap with sugar water (20%) developed for hummingbird capture (Ruschi, 2009) or a mist-net. Males captured out of the lek were kept in quiet conditions without species-specific auditory stimuli for at least a half-hour. No song production was observed in the silent control group.

### *Histology and EGR-1 in situ hybridization*

Brains were sectioned sagittally starting at the lateral surface at 20 µm thickness. One hemisphere was processed per bird: right hemispheres of three singing group and two silent control group birds and left hemispheres of the other three singing and two control birds. No gross differences were observed between hemispheres (see supplementary material, Fig. S1). Sections were thaw mounted onto Superfrost® Plus slides (Thermo Fisher Scientific, United States). One set of tissue from each bird was Nissl stained at 60 µm intervals to allow for the identification of anatomy.

The second set of tissue was processed for the visualization of the mRNA expression of the immediate early gene EGR-1 by *in situ* hybridization (ISH; referred to hereafter as EGR-1 expression). The ISH was performed on 13 to 22 sections per bird, spaced at ~160 µm using the RNAscope® 2.5 High Definition (HD) Brown Assay (Advanced Cell Diagnostics, United States) according to the manufacturer's instructions. Briefly, the sections were fixed (4% PFA in 1X PBS for 15 minutes at 4°C ), dehydrated (EtOH at 50%; 70% and 2 x 100% for 5 minutes each at room temperature) and then air-dried dried at room temperature. The targeted sections were isolated by a hydrophobic barrier manually traced with an Immedge™ pen (Advanced Cell Diagnostics, United States). Then, the sections were pretreated, hybridized to the target probe RNAscope® 2.5 Probe Tgu-EGR1 (Advanced Cell Diagnostics, United States) and submitted to multiple steps of amplification. The signal detection was given by diaminobenzene

(DAB)-HRP that labeled each mRNA transcript as a distinct dot of chromogen precipitate visible in a bright field microscope. The sections were counterstained with 50% Hematoxylin.

### *Image analysis*

The stained tissue sections were imaged using a Leica CTR 6000 microscope equipped with a DM6000 B camera (Leica Camera AG, Germany). For quantitative analysis, five brain regions of interest were imaged at 20x magnification, including the (*i*) vocal nucleus of the lateral nidopallium, VLN (HVC-like); (*ii*) vocal nucleus of the arcopallium, VA (RA-like); (*iii*) vocal nucleus of the anterior nidopallium, VAN (LMAN-like); (*iv*) caudo medial nidopallium, NCM and (*v*) the entopallium, E. The regions of interest were selected due to their suggested analogy with songbird nuclei of the posterior pathway involved in the motor control of vocal production (VLN and VA), nuclei of the anterior pathway involved in the vocal learning processes (VLN and VAN), area involved in the auditory processing (NCM) and entopallium, a telencephalic visual area that should show no EGR-1 induction specifically from the auditory stimuli to correct for variation in the background. Counts of EGR-1 expression were made in an area of 95 x 95 µm for VLN, 126.5 x 126.5 µm for VA, VAN and NCM, and 95 x 126.5 µm for entopallium. The counting frame was delimited by boxes placed in the center (field in) of each region (Fig. 3a) on up to three serial sections per bird and the results were averaged. For VLN, VA and VAN, an additional counting frame of the same size was obtained in the same region but outside (field out) the boundaries of the nuclei on its immediate surrounding (Fig. 3a) to verify the specificity of the EGR-1 expression.

For each counting frame, the area covered by cells and the area covered by mRNA EGR-1 molecules labeled by chromogen precipitate were quantified. Using ImageJ2 software (Rueden et al., 2017) available by Fiji distribution (Schindelin et al., 2012), cells and EGR-1 chromogen dots were separated from the background with the plugin Trainable Weka Segmentation version 3.2.34 (Arganda-Carreras et al., 2017), a machine learning tool, using the default classifier “FastRandomForest”. The output of the segmentation for cells and EGR-1 chromogen dots were each transformed into binary images. The area covered by cells was then estimated automatically by the analyze particles tool which was set to detect particles above 1 $\mu m^2$ with circularity 0.08 – 1 [defined as $4\pi$ (area/perimeter$^2$)] and the area covered by EGR-1 chromogen dots by the same tool but set to detect particles above 0.02 $\mu m^2$ with circularity 0.10 – 1. When the cells were not visible on the same slide of the EGR-1 chromogen dots due to tissue damage occurring during the hybridization, they were quantified on the adjacent Nissl stained slice. To estimate the intensity of EGR-1 mRNA molecules expressed in the counting area, the ratio between the area covered by cells divided by the area covered by EGR-1 chromogen dots was calculated.

***Statistics***

The EGR-1 expression in the long-tailed hermits – calculated as the ratio between the area covered by EGR-1 chromogen dots and the area covered by cells – was compared between singing and silent controls for each of the brain areas of interest (VLN, VA, VAN, NCM and E) using beta regressions. Beta regressions are a suitable approach for data within the standard unit interval (0, 1) such as the EGR-1 expression in the long-tailed hermits. The beta regressions were fit using the function “betareg” implemented by the package “betareg” (Cribari-Neto & Zeileis, 2010) in the R software version 4.0.2 (R Core Team, 2020). The regressions had the EGR-1 expression as the response variable, the experimental group (singing or silent control) and the field (inside or outside) as predictors, logit link and maximum likelihood estimator. Additional regressions with the number of syllables produced before capture (syllable rate) as a predictor were run for all areas of interest except for the entopallium. For each regression, the diagnostic plots were used to assess the regression assumptions, however, none showed any functional dependency in the residuals. Pseudo $R^2$ was obtained to estimate the goodness of fit. In the results, we reported the marginal means and standard errors obtained from the regressions with the function “emmeans” implemented by the R package “emmeans” (Lenth et al., 2021). Significance was set to $\alpha < 0.05$.

## Results

Long-tailed hermits singing spontaneously in the lek have a high overall EGR-1 expression level in several brain regions (Fig. 1a, b) when compared with silent controls (Fig. 1c). In singing hermits, EGR-1 expression intensified in the nido- and mesopallium towards the medial part of the brain, reached its highest level in the thalamic nucleus, dorsolateralis anterior thalami, pars medialis (DLM) and nucleus dorsomedialis anterior (DMA) but was absent in the entopallium (E), globus pallidus (GP) and field L2 (Fig. 1a, b). In silent hermits, EGR-1 expression was low but present in the frontal mesopallium towards the lateral part of the brain and the nucleus basorostralis pallii (Bas) (Fig. 1c). Although more intense in singing hermits, both singing and silent hermits have EGR-1 expression in the nucleus mesencephalicus lateralis, pars dorsalis (MLd) and in the nucleus intercollicularis (ICo) (Fig. 1b, c).

Singing in the lek induced a 3- to 4-fold EGR-1 expression in the telencephalic nuclei of the putative vocal control system VLN (HVC-like nucleus), VA (RA-like), VAN (MAN-like) and in the caudal medial nidopallium (NCM) when compared to the silent condition (Fig. 2). According to the beta regressions with the EGR-1 expression ratio as the response variable, this difference in the EGR-1 expression was significant for VLN ($p = 0.03$), VA, VAN and NCM ($p < 0.001$), but not for the entopallium (E), as expected (Table 1, Fig. 2).

Although higher when compared to silent controls, EGR-1 expression in singing hermits was not specific in the telencephalic nuclei of the putative vocal control system (Fig. 3). According to the beta regressions, there were no differences between the EGR-1 expression inside the boundaries of the nuclei and outside in the immediate surrounding of the nuclei for VLN ($p = 0.47$), VA ($p = 0.68$) and VAN ($p = 0.78$) (Table 1, Figs. 3b, c and 4). VAN showed an interesting pattern of EGR-1 expression which was consistent among singing males: the posterior part of the hummingbird VAN nucleus (VAN-P) had a lower expression, whereas the anterior part (VAN-A) had a higher expression relative to the surrounding nidopallium (see Fig. 3b).

The songs emitted by the singing hermits were composed of two syllables repeated multiple times in a song bout (Fig. 5a). The number of syllables sang per second (syllable rate) and the average duration of the syllables were obtained as a proxy for the singing activity of the males before the capture. The syllable rate ranged from 0.83 (bird id = PSU11) to 2.04 (bird id = PSU1) and the average duration of the syllables ranged from 0.14 seconds (± 0.01, n = 129 syllables, bird id = PSU1) to 0.29 seconds (± 0.06, n = 27 syllables, bird id = PSU10). According to the beta regressions, there were significant correlations between EGR-1 expression and the syllable rate only in VLN ($p = 0.015$, Fig. 5b) and between EGR-1 expression and the average of the syllable duration only in VAN ($p = 0.014$, Fig. 5c). There was no correlation between EGR-1 expression and syllable rate or syllable duration in the NCM and VA (Table 2).

## Discussion

The map of EGR-1 expression in the long-tailed hermit (*Phaethornis superciliosus*) obtained in this study provides further support for the functionality of the vocal control system during spontaneous singing, in the species' natural setting and without playback stimulation. Singing spontaneously in the lek is a behavior of ecological relevance that evolved multiple times in hummingbirds, but is not possible to measure in captivity. EGR-1 expression was up to 4-fold higher in the main nuclei of the putative vocal control system (VLN or HVC-like, VA or RA-like and VAN, or LMAN-like) in singing males relative to silent controls. However, the expression was not restricted to the boundaries of the nuclei. High EGR-1 expression was also observed in some areas in the thalamus (nucleus dorsolateralis anterior thalami, pars medialis, DLM; and nucleus dorsomedialis anterior, DMA) and in the midbrain (nucleus mesencephalicus lateralis, pars dorsalis, MLd; and nucleus intercollicularis, ICo).

The map of the EGR-1 expression of a non-lekking hermit, the rufous-breasted hermit (*Glaucis hirsuta*), showed that the hearing-induced areas were caudal medial nidopallium (NCM); a couple of areas adjacent to NCM (caudal medial mesopallium, CMM and caudal striatum, CSt); one adjacent to VLN (dorso caudal nidopallium, Ndc); one to VA (intermediate arcopallium, Ai); one thalamic (dorsointermediate nucleus of the posterior thalamus, DIP) and MLd, whereas the singing-induced areas were another couple of areas adjacent to NCM (vocal nucleus of the medial hyperpallium ventrale, VMN and vocal nucleus of the medial nidopallium, VMH); the putative vocal control nuclei (VLN, VA, VAN and VAP or Area X-like) and dorsomedial nucleus (DM) in the mesencephalon (Jarvis et al., 2000). When singing in response to playback, rufous-breasted hermit showed EGR-1 expression in both hearing- and singing-induced areas (Jarvis et al., 2000). When spontaneously singing, long-tailed hermit showed EGR-1 expression in the same areas as the singing rufous-breasted hermit and many additional areas including the medial part of the nidopallium; areas of the visual Wulst such as hyperpallium apicale (HA) and hyperpallium dorsale (HD); a couple of areas in the thalamus (DLM and DMA) and ICo. This difference in song-induced expression of EGR-1 between a non-lekking and a lekking hermit indicated that, as in songbirds, hummingbirds differ in EGR-1 expression depending on the social context in which song is emitted.

### *Context-dependent expression of EGR-1 in the anterior forebrain pathway*

In songbirds, the context-dependent expression of EGR-1 varied from low when males sang in the presence of females (direct song) to high when they sang by themselves (undirect song) mainly in Area X (lateral part), lMAN and RA (core), part of the anterior forebrain pathway (Jarvis et al., 1998). The anterior forebrain pathway was, therefore, suggested to be divided into lateral and medial sections, where the lateral seems to introduce versatility to the undirected song which can function as a rehearsal, and the medial induces stereotypy to the directed song which is subjected to sexual selection (Jarvis et al., 1998; C. V. Mello & Jarvis, 2008). Although the striatothalamic projection (Area X to DLM), found only in birds (Farries & Perkel, 2008), is part of the anterior forebrain pathway, EGR-1 was not expressed in the DLM during song production in any of the contexts investigated so far (Jarvis et al., 1998). Thus, it was suggested that in DLM, such as in the receptors of thalamic sensory projections field L2 and entopallium, neuronal activation and EGR-1 induction are intrinsically uncoupled (C. V. Mello, 2002b; C. V. Mello & Jarvis, 2008). In hummingbirds, while a non-lekking hermit singing in response to the playback of a conspecific song showed EGR-1 expression similar to songbirds in the same context, lekking hermits when spontaneously singing in the lek showed a thalamic EGR-1 expression, including in the DLM, which is unexpected given what was previously reported in non-lekking hummingbirds. It can thus be suggested that the thalamus and further unique patterns of expression found in lekking males are related to the challenges that males meet when singing in lek.

### ***EGR-1 expression and the challenges of singing in lek***

On top of learned songs, lekking hummingbirds produce learned visual displays (Araya-Salas et al., 2019). When singing perched in the lek, long-tailed hermits concomitantly produce rhythmic movements in the horizontal with the head and in the vertical with the tail (head-tail perched display) that is modulated according to the song rhythm and intensify in the presence of an intruder (D. S. Brito, 2012; Stiles & Wolf, 1979). In long-billed hermit (*Phaethornis longirostris*), fine-scale parameters such as duration of specific displays and repetition rate of specific movements within displays were demonstrated to be more similar between individuals of the same lek than individuals from different leks; a greater similarity that was not explained by genetics (Araya-Salas et al., 2019). Given the phylogenetic proximity and apparent plasticity in the head-tail perched display of the long-tailed hermits, it is possible to speculate that this visual display is also learned.

Head-tail perched display is usually produced at the same time as when singing and was observed in all long-tailed hermits before capture. It is likely that in addition to hearing- and singing-induced areas, EGR-1 is also expressed in brain areas involved in the production of this learned visual display. The thalamus is potentially involved as the DMA projects directly to many telencephalic areas including parts of the visual Wulst (Montagnese et al., 2003). It is possible that EGR-1 expression in DMA and visual Wulst observed in the long-tailed hermits are induced by the head-tail visual display. Future experiments with one-eye-covered males in the field can indicate whether DMA and visual Wust expression is induced by visual input. Based on the pattern of connectivity of DMA, it was suggested that DMA plays an important role in the regulation of, among other functions, attention and memory formation (Montagnese et al., 2003). We can speculate that if related to visual display, these areas can be also involved in the learning of visual displays. Another non-excluding possibility is that the adjacent areas of the vocal control system are involved in the production, and possibly motor learning, of the head-tail visual display which explains the lack of differential expression of EGR-1 found in long-tailed hermits. During limb and body movements, vocal learners express EGR-1 in discrete brain areas that are adjacent to their telencephalic nuclei of the vocal control system (Feenders et al., 2008). Based on this pattern of expression, it was hypothesized that the vocal control system in each bird order evolved independently, but from a common ancestor motor pathway as substrate (Feenders et al., 2008). Given that in a non-lekking hermit the expression is restricted to the boundaries of the vocal control system and in the long-tailed hermit, a lekking hermit, is not, it is conceivable to speculate that, if the adjacent areas are related to visual learning, the evolution of visual displays and the motor learning of these displays anteceded vocal learning in hummingbirds.

On top of visual displays, lekking hummingbirds can also discriminate the song of close neighbors to avoid overlapping vocalizations (Araya-Salas et al., 2017). The high EGR-1 expression in all areas known to be hearing-induced without any playback stimuli indicates that long-tailed hermits are likely to be constantly processing auditory information during singing in the lek. Long-tailed hermits can aggregate in a lek with up to 20 males singing at the same time (Ramjohn et al., 2003), nevertheless, they avoid overlapping songs of only their closest neighbors (Araya-Salas et al., 2017). Thalamus and striatum are among the subcortical structures that are highly conserved across vertebrate evolution and that are involved in an ancestral form of selective attention in those animals that lack a neocortex, including birds (Krauzlis et al., 2018). Given that the hearing-induced thalamus and striatum are active in the long-tailed hermit, the auditory discrimination might come about in two complementary ways. First, auditory areas may hold a mechanism to discriminate acoustic signals of different intensities, whereas, secondly, the thalamus and striatum provide the focus on the most relevant ones. Behavioral paradigms with playback of neighbor songs in different intensities can be used to test this hypothesis.

On top of acoustic discrimination, lekking hummingbirds can be found singing for many hours at a time, throughout the day, and throughout the year (Stiles & Wolf, 1979). When repeatedly exposed to the same song in a laboratory setting, songbirds show signs of habituation given by the rapid and persistent decline in EGR-1 expression that they demonstrate (C. Mello et al., 1995). In the long-tailed hermit, singing was always linked to a generalized telencephalic expression of EGR-1 although they were persistently hearing the song of the same neighbors. It may be the case that lekking hummingbirds are resistant to habituation, possibly by a mechanism of EGR-1 induction, including receptors, that differ from songbirds and non-lekking hummingbirds. A difference in the receptors can cause variation in the properties and time of response in EGR-1 induction and may prevent habituation. Interestingly, the pattern of EGR-1 expression found in the VAN – the posterior part had a lower expression than the anterior – of the long-tailed hermit was not observed in non-lekking hummingbirds and was identical to the pattern of mGluR2 expression in the VAN found to be unique to hummingbirds (Wada et al., 2004). Except for the low level of mGluR2 in the VA, the telencephalic pattern of mGluR2 expression almost overlapped with the EGR-1 expression of the long-tailed hermits, including the lack of differentiation of VASt (Wada et al., 2004). This coincidental pattern of EGR-1 and mGlu2 expression may indicate that, at least in the VAN, the receptor inducing EGR-1 expression in lekking hummingbirds may be mGluR2. mGluR2 is a metabotropic receptor of the group II subtype and has a potent inhibitory action on both $Ca^{2+}$ and $K^{+}$ channels (reviewed in Anwyl, 1999). The administration of mGlu2 receptor antagonist induces widespread c-Fos expression, another immediate-early gene, in the brains of mice (Linden et al., 2005). In songbirds, a similar effect is obtained in EGR-1 induction by metrazole, a potent g-aminobutyric acid (GABA)- antagonist that leads to overall brain depolarization (C. V. Mello & Clayton, 1995). It is unknown whether metrazole causes such induction in hummingbirds. Thus, maps of EGR-1 expression after the administration of metrazole and mGlu2 receptor antagonists comparatively in lekking and non-lekking hummingbirds may indicate whether a difference in receptors can explain resistance to habituation during sustained bouts of behavior.

Alternatively to differences in the mechanism of EGR-1 induction, lekking hummingbirds may avoid habituation simply by regularly absenting from the lek. In songbirds, EGR-1 induction significantly declines after 30 minutes of song exposure (C. Mello et al., 1995). Interestingly, long-tailed hermits were reported to sing for a continuous period of up to 30 minutes (Stiles & Wolf, 1979). It is possible that long-tailed hermits fly away from aural contact with other singing males in the lek every 30 minutes. Nevertheless, in the lek, males are constantly challenged by intruders (Stiles & Wolf, 1979), therefore, a long period of absence may be at the cost of their territory. Thus, tracking studies of lekking males should elucidate this question.

### *Local correlation of EGR-1 expression and song activity*

In the lekking hermit, EGR-1 expression was locally proportional to different aspects of singing. VLN EGR-1 expression was positively correlated to the number of syllables sang per minute, VAN EGR-1 expression to syllable duration, whereas VA and NCM did not show any correlation. In non-lekking hummingbirds, VLN EGR-1 expression was also proportional to the amount of song (Jarvis et al., 2000). Similarly, in songbirds, HVC EGR-1 expression was proportional to the amount of song disregarding whether the song was directed or undirected (Jarvis et al., 1998). It seems, therefore, that activity in VLC, as in HVC, is universally positively correlated to song activity. The lack of EGR-1 expression in hearing-induced areas such as NCM was also observed in non-lekking hummingbirds (Jarvis et al., 2000).

In songbirds, RA and LMAN EGR-1 activity were proportional to the amount of song during undirect song, however, during direct song RA was not correlated and LMAN, although significant, was only weakly correlated (Jarvis et al., 1998). Differently from HVC, not only the pattern of EGR-1 expression but also the correlation between expression and amount of song was context-dependent in RA and LMAN. This dependency may be similar in hummingbirds, which would help to explain the correlation to song activity observed in VA and VAN of non-lekking hummingbirds (Jarvis et al., 2000), but not in the long-tailed hermit. Interestingly in long-tailed hermits, VAN EGR-1 expression correlated to the average syllable duration. The syllable duration showed low standard deviation within each male, ranging from 0.01 to 0.06 seconds, which corroborates with previous data of long-tailed hermits obtained in the same study site that showed low variation within males and a significant difference between males (D. S. Brito, 2012). Thus, syllable duration is potentially a song parameter that encodes individual signatures within the lek. Keeping the low effect and sample size in mind, this idea requires further investigation, nevertheless, it is possible that VAN activity and further nuclei of the anterior forebrain pathway are associated with individual signatures.

### ***Future directions***

It is important to keep in mind that much of the generalized EGR-1 expression in the telencephalon of the long-tailed hermit can be attributed to the awake state of the birds. Moreover, there were differences in the time of the day when lekking and non-lekking hermits were sampled and a daytime effect should be considered. Nevertheless, the results are highly consistent among individuals. Thus, when interpreted with care, some of these remarkable patterns can give insights into the neural activity underlying the multi-modality of natural behaviors such as singing in lek. Future experimental approaches targeting specific aspects of singing in a lek may include (i) neural tracing to unveil the connectivity of thalamic regions and potential subdivisions of VAN; (ii) lesion studies targeting areas of high EGR-1 expression such as DLM, DMA and the visual Wust and their effect into behavior to address their potential relation to visual learning; and (iii) electrophysiological recordings in different subdivisions of VAN to identify whether the lack of EGR-1 expression in the anterior part of the VAN represents lack of activity. These future studies have the potential to shed light on the function of the anterior pathway and the relevance of the striatothalamic projection in adult vocal learners.

## Conclusion

The comparison of the maps of EGR-1 expression in hummingbirds indicates that singing in an aggregation of males, the so-called lek, implicates a pattern of brain activity that differs from singing in a laboratory setting, or even in response to the playback of a conspecific song in the field. This pattern suggests that many telencephalic nuclei, such as some nuclei of the anterior forebrain pathway and visual Wust, and thalamic areas seem to be involved in the multi-modal, auditory and visual, signaling in lek. Leks evolved many times within hummingbirds and may be related to the evolution of vocal learning in the family. The EGR-1 expression patterns presented in this study give valuable insights into the brain pathways associated with complex learned behaviors such as singing and the visual displays performed in lek that is of evolutionary relevance but unreproducible outside of a natural context.

## Tables

Table 1. Beta regressions outputs for the effects of singing on the EGR-1 expression in the long-tailed hermits. The EGR-1 expression was obtained as the ratio between the area covered by EGR-1 chromogenic particles and the area covered by cells in each counting frame per brain area of interest (VLN, VA, VAN, NCM and E). For VLN, VA and VAN regressions, the predictors were treatment (singing x silent) and field (in x out), whereas, for NCM and E regressions, the only predictor was treatment. In bold are predictors which $p < 0.05$. In all quantified nuclei of the putative vocal control system, singing hermits had higher expression of EGR-1 mRNA than controls, however, this expression was not different related to the surrounded forebrain

| Nuclei | | Estimate | Std. Error | z value | p value |
|---|---|---|---|---|---|
| VLN | Intercept | -1.376 | 0.600 | -2.292 | 0.022 |
| | **Treat. song (ref. silence)** | 1.594 | 0.721 | 2.212 | **0.027** |
| | Field out (ref. in) | -0.403 | 0.553 | -0.728 | 0.466 |
| VA | Intercept | -2.242 | 0.436 | -5.137 | < 0.001 |
| | **Treat. song (ref. silence)** | 1.722 | 0.489 | 3.521 | **< 0.001** |
| | Field out (ref. in) | 0.128 | 0.305 | 0.419 | 0.675 |
| VAN | Intercept | -1.553 | 0.281 | -5.527 | < 0.001 |
| | **Treat. song (ref. silence)** | 1.839 | 0.344 | 5.341 | **< 0.001** |
| | Field out (ref. in) | -0.080 | 0.279 | -0.286 | 0.775 |
| NCM | Intercept | -1.878 | 0.344 | -5.463 | < 0.001 |
| | **Treat. song (ref. silence)** | 1.664 | 0.397 | 4.196 | **< 0.001** |
| E | Intercept | -2.328 | 0.229 | -10.157 | < 0.001 |
| | Treat. song (ref. silence) | -0.110 | 0.296 | -0.371 | 0.711 |

Table 2. Beta regressions outputs for the effects of song activity on the EGR-1 expression in the long-tailed hermits. The EGR-1 expression was obtained as the ratio between the area covered by EGR-1 chromogenic particles and the area covered by cells in each counting frame per brain area of interest (VLN, VA, VAN and NCM). The predictors were syllable rate measured as the number of syllables per second and the average duration of the syllables emitted per bird just before capture. In bold are predictors which $p < 0.05$

| Nuclei | | Estimate | Std. Error | z value | p value |
|---|---|---|---|---|---|
| VLN | Intercept | -1.864 | 0.403 | -4.629 | < **0.001** |
| | **Syllable rate** | 0.955 | 0.392 | 2.435 | **0.015** |
| | Average syllable duration | 4.174 | 2.891 | 1.444 | 0.149 |
| VA | Intercept | -2.140 | 0.449 | -4.763 | < **0.001** |
| | Syllable rate | 0.676 | 0.402 | 1.680 | 0.093 |
| | Average syllable duration | 3.310 | 3.107 | 1.065 | 0.287 |
| VAN | Intercept | -1.528 | 0.292 | 5.241 | < **0.001** |
| | Syllable rate | 0.396 | 0.340 | 1.165 | 0.244 |
| | **Average syllable duration** | 6.308 | 2.564 | 2.460 | **0.014** |
| NCM | Intercept | -1.853 | 0.336 | -5.524 | < **0.001** |
| | Syllable rate | 0.639 | 0.359 | 1.778 | 0.075 |
| | Average syllable duration | 3.751 | 2.656 | 1.413 | 0.158 |

## Figure captions

Figure 1. EGR-1 expression map in the brain of males long-tailed hermit. (a) Anatomical map representing the regions shown in the sagittal brain sections on the right. Dashed lines indicate nuclei with identifiable boundaries. Highlighted in pink are the regions that consensually show EGR-1 expression in all males (n = 6) when singing spontaneously in the lek; the intensity of the marker represents the relative intensity of the EGR-1 expression. Diagrams on the left and respective numbers indicate the distance of the sections from the midline. Microphotographs of sagittal brain sections (1.25x magnification) hybridized for EGR-1 mRNA expression in (b) a male singing spontaneously in lek and (c) a male kept in silence. mRNA EGR-1 molecules were labeled by chromogen precipitation (dark brown dots) and counterstained with Hematoxylin. Notice increased EGR-1 expression in the nido-, meso- and hyperpallium, and thalamus of the male singing in lek compared with the silent control. Rostral orientation is on the right and dorsal is on the top. A, arcopallium; APH, area parahippocampalis; Bas, nucleus basorostralis pallii; DLM, medial nucleus of the dorsolateral thalamus; DMA, nucleus dorsomedialis anterior; E, entopallium; GP, globus pallidus; H, hyperpallium; HA, hyperpallium apicale; HB, habenula; HD, hyperpallium dorsale; ICo, nucleus intercollicularis; L2, subfield of auditory field; LSt, lateral striatum, M, mesopallium; MLd, nucleus mesencephalicus lateralis, pars

dorsalis; MSt, medial striatum; N, nidopallium; NCM, caudal medial nidopallium; Pt, nucleus pretectalis; Rt, nucleus rotundus; TrO, tractus opticus; VA, vocal nucleus of the arcopallium; VAN, vocal nucleus of the anterior nidopallium; VLN, vocal nucleus of the lateral nidopallium.

Figure 2. Quantification of EGR-1 expression in five brain areas of males long-tailed hermit when singing in lek in comparison with silent controls. EGR-1 expression was quantified as the ratio between the area covered by EGR-1 chromogen precipitation (dark brown dots) and the area covered by cells. Significant differences were found in the telencephalic nuclei of the putative vocal control system (VLN, $p = 0.027$ indicated by *; VA and VAN, $p < 0.001$ indicated by **) and in the auditory area (NCM, $p < 0.001$). As expected, no difference was found in the entopallium ($p = 0.711$). The bars represent the marginal means and standard error given by the beta regressions (see Methods). Each data point indicate the expression value per male (song, $n = 6$; silence, $n = 4$). E, entopallium; NCM, caudal medial nidopallium; VA, vocal nucleus of the arcopallium; VAN, vocal nucleus of the anterior nidopallium; VLN, vocal nucleus of the lateral nidopallium.

Figure 3. EGR-1 expression in the telencephalic nuclei of the putative vocal control system in comparison (field in) with the immediate surrounding (field out) of males long-tailed hermit when singing in lek. (a) Anatomical map representing the regions shown in the sagittal brain sections on the right. Dashed rectangles represent the counting frames in which EGR-1 expression was quantified. Diagrams on the left and respective numbers indicate the distance of the sections from the midline. Microphotographs of sagittal brain sections (20x magnification) hybridized for EGR-1 mRNA expression of a male singing spontaneously in lek (a) inside the boundaries of the nuclei (field in) and outside the boundaries of the nuclei but on the immediate surrounding (field out) in comparison with (c) a male in silence. mRNA EGR-1

molecules were labeled by chromogen precipitation (dark brown dots) and counterstained with Hematoxylin. Notice similar EGR-1 expressions inside and outside of VLN, VA and VAN. Rostral orientation is on the right and dorsal is on the top. A, arcopallium; Bas, nucleus basorostralis pallii; DLM, medial nucleus of the dorsolateral thalamus; DM nucleus dorsomedialis of the midbrain; E, entopallium; H, hyperpallium; HA, hyperpallium apicale; HD, hyperpallium dorsale; ICo, nucleus intercollicularis; L2, subfield of auditory field; LSt, lateral striatum, M, mesopallium; MLd, nucleus mesencephalicus lateralis, pars dorsalis; MSt, medial striatum; N, nidopallium; NCM, caudal medial nidopallium; Pt, nucleus pretectalis; St, striatum; TrO, tractus opticus; VA, vocal nucleus of the arcopallium; VAN, vocal nucleus of the anterior nidopallium; VAN-A, anterior part of the VAN; VAN-P, posterior part of the VAN; VLN, vocal nucleus of the lateral nidopallium.

Figure 4. Quantification of EGR-1 expression in the telencephalic nuclei of the putative vocal control system in comparison (field in) with the immediate surrounding (field out) of males long-tailed hermit when singing in lek. EGR-1 expression was quantified as the ratio between the area covered by EGR-1 chromogen precipitation (dark brown dots) and the area covered by cells. No significant differences were found between the expression inside and outside the boundaries of any nuclei (VLN, $p = 0.466$; VA, $p = 0.675$; VAN, $p = 0.775$). The bars represent the marginal means and standard error given by the beta regressions (see Methods). Each data point indicate the expression value per male (song, $n = 6$; silence, $n = 4$). VA, vocal nucleus of the arcopallium; VAN, vocal nucleus of the anterior nidopallium; VLN, vocal nucleus of the lateral nidopallium.

Figure 5. Examples of the long-tailed hermit song and correlation with EGR-1 expression. (a) Song spectrogram (frequency) and oscillogram (relative amplitude) of three males long-tailed

hermit. The long-tailed hermit song is composed of two syllables (A and B) repeated multiple times in a song bout. The sonograms exemplify variation among males in the number of syllables emitted per second (syllable rate) and in the duration of the syllables. (b) EGR-1 expression positively correlated to syllable rate only for VLN ($p = 0.015$ indicated by *) and (c) to the mean syllable duration only for VAN ($p = 0.014$). The curves were given by the beta regressions (see Methods). The data points indicate expression values per singing male for each brain area (VLN, VA, VAN and NCM). NCM, caudal medial nidopallium; VA, vocal nucleus of the arcopallium; VAN, vocal nucleus of the anterior nidopallium; VLN, vocal nucleus of the lateral nidopallium.

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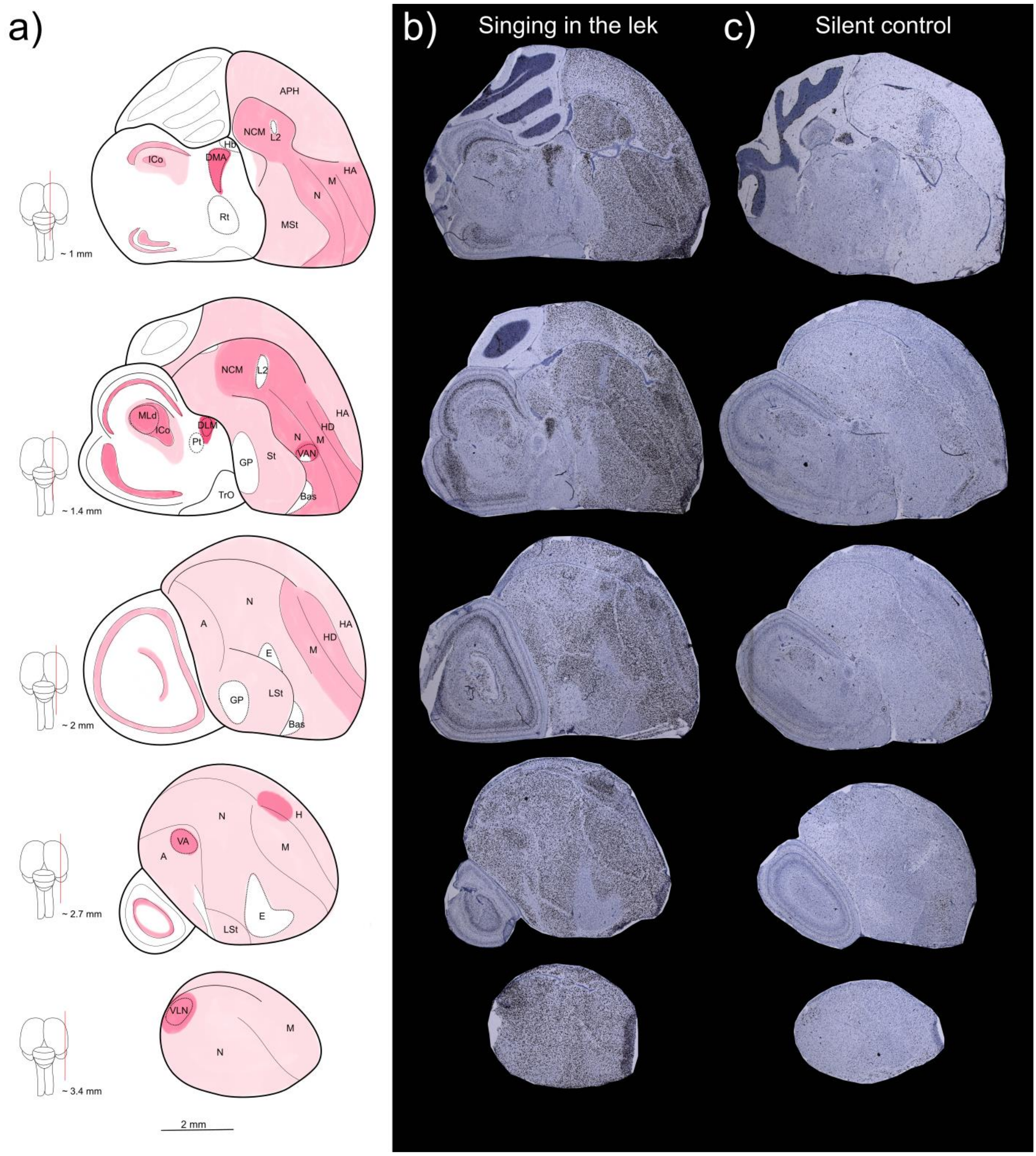
Figure 1


a)
b)
Singing in the lek
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Silent control
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Figure 2

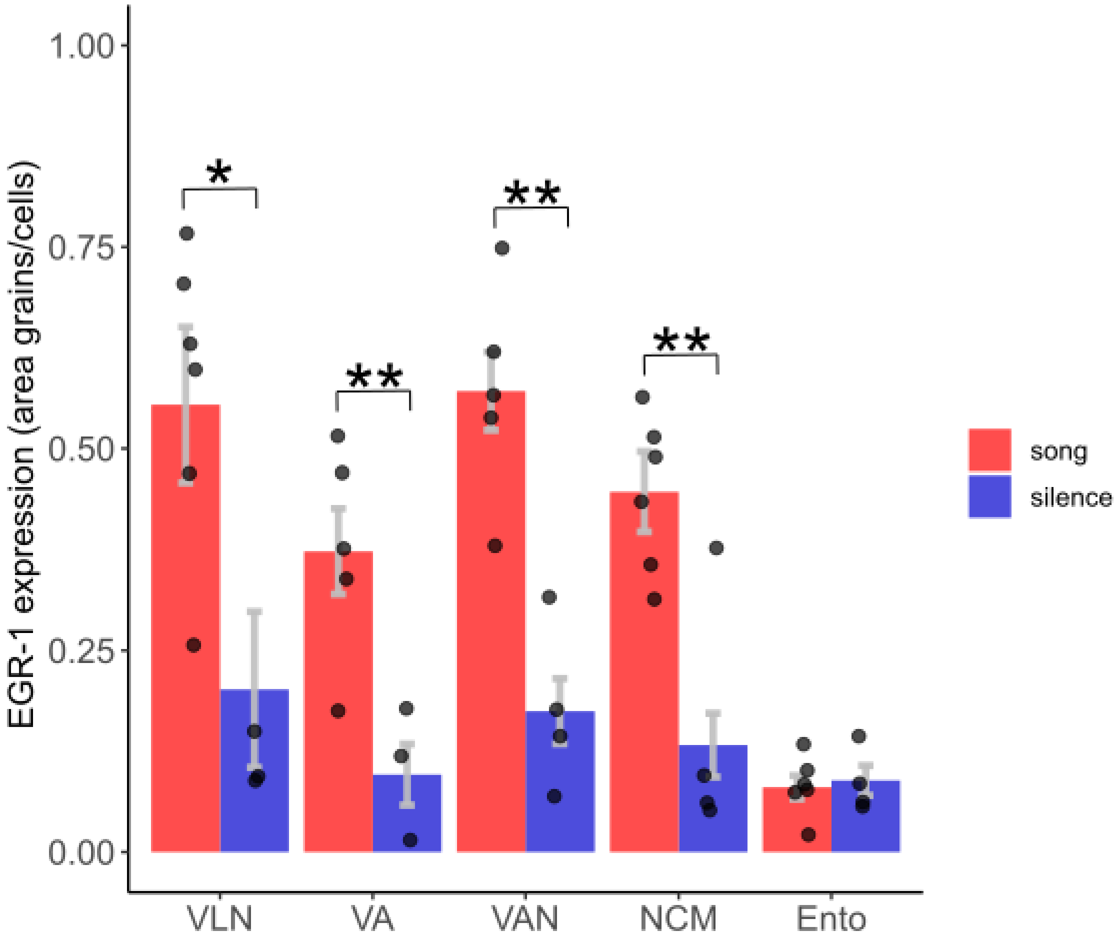
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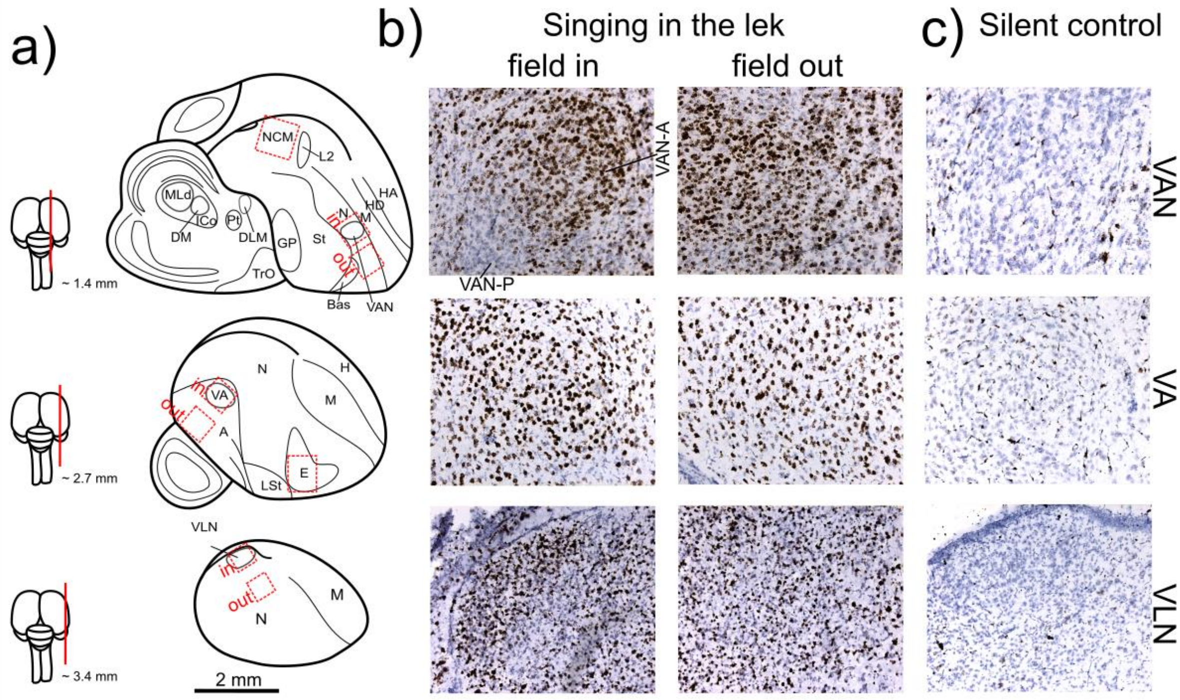
Figure 3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Singing in the lek
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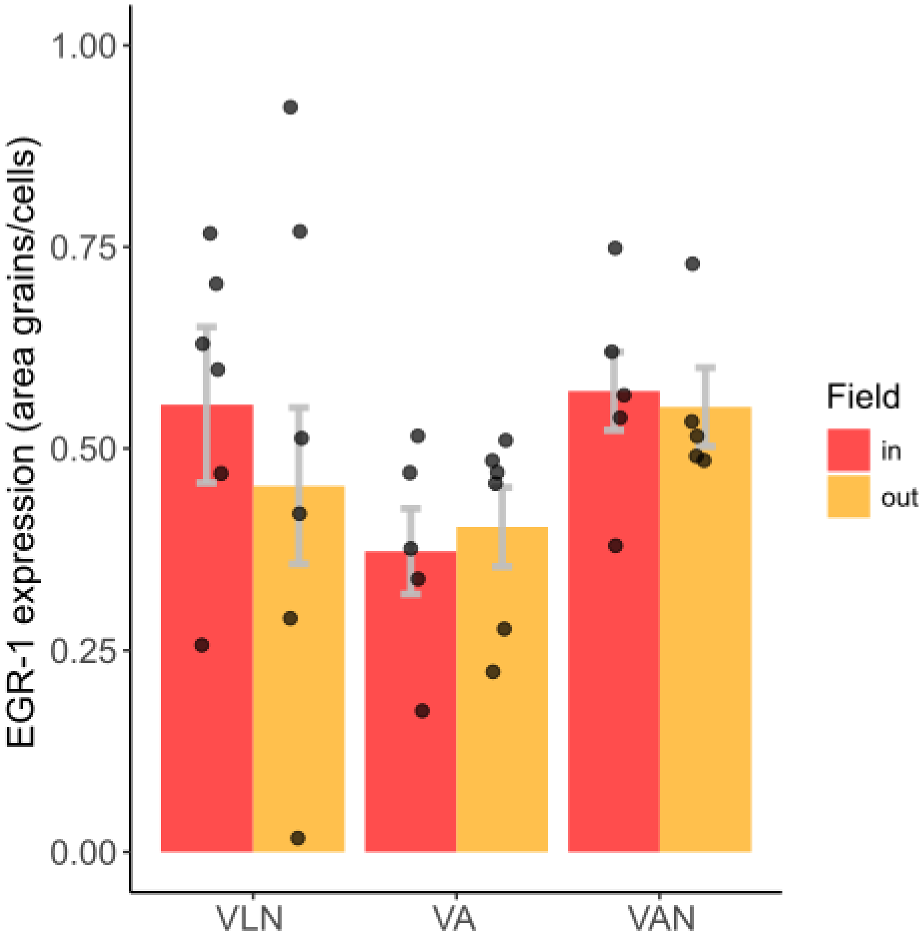
Figure 4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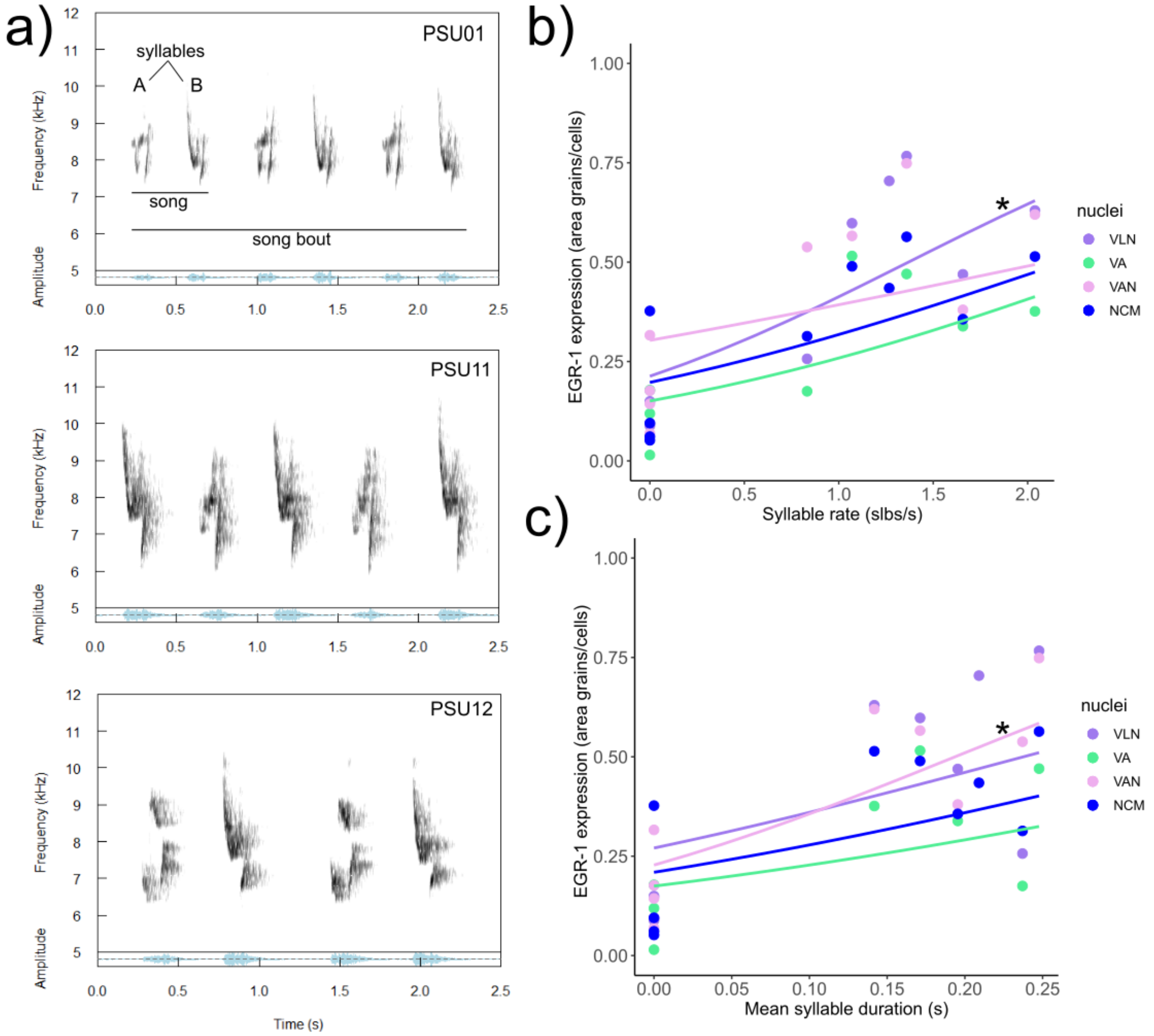
out

Figure 5


a)
PSU01
syllables
A
B
song
song bout
Frequency (kHz)
Amplitude
PSU11
PSU12
Time (s)
b)
EGR-1 expression (area grains/cells)
Syllable rate (slbs/s)
nuclei
VLN
VA
VAN
NCM
c)
Mean syllable duration (s)
*

# General discussion

In this thesis, I investigated the basis of vocal communication in hummingbirds, an unconventional model system in the bioacoustics field, focusing on its anatomical, behavioral and endocrinological aspects. The vast majority of the animals that rely on vocalizations to communicate, including human's closest relatives, can successfully transmit information using exclusively innate sounds. The vocal communication of hummingbirds, nevertheless, includes learned vocalizations that depend on a rare ability to learn which they evolved independently of other vocal learners (Gahr, 2000; Jarvis et al., 2000). Interestingly, hummingbirds live within the limit of energy expenditure, higher than any vertebrate (Suarez, 1992), yet they had to deal with all the costs that came along with their ability to learn how to produce learned vocalizations besides their innate repertoire. One of the main questions that arise is which were the prerequisites and underlying pressures for this independent but convergent evolution? In order to answer this question, a comparative approach to the principles regulating vocal communication among vocal learners would be the best starting point. To this purpose, **Chapter 1** of this thesis presented a systematic study on the vocal communication of hummingbirds at a family level from a phylogenetical perspective. Additionally, in **Chapter 2**, I presented an investigation of the anatomy of the vocal organ in an early-branched hummingbird. This study provided hints on the synapomorphies and suggested prerequisites for the evolution of vocal learning in the family. In **Chapter 3**, I discussed the endocrinological aspects of hummingbirds' vocal communication and uncovered the lack of testosterone sensitivity in their vocalizations. Supporting this finding, **Chapter 4** shows the lack of testosterone sensitivity to be extended to the hummingbird’s vocal control system. And lastly, **Chapter 5** reveals a lack of specificity in the activity of the vocal control system during the production of learned vocalizations in a natural setting.

Ideally, the starting point of this thesis would be a comparative study of the vocal behavior and neuroanatomy in both sexes of singing species of hummingbirds with facultative male song, singing with obligatory male song and non-singing in the lowland of South America. However, in contrast to established model systems with long tradition of captivity (e.g. zebra finches, canaries and budgerigars), in hummingbirds, it was still unclear whether female and male sing because the definition of song in the literature could be ambiguous and observations in the wild is difficult to obtain. For example, a putative non-singing species (amethyst woodstar*, Calliphlox amethystina*) was difficult to observe in the wild due to its sparse distribution whereas putative singing species were abundant but they either lack sexual dimorphism in plumage (e.g.: black jacobin) or male juveniles are similar to adult females (e.g.: black-throated mango). Therefore, in this thesis, I focused on investigating the basis of the vocal communication in hummingbirds from an evolutionary perspective and suggest its implications to the evolution of vocal-production learning. Together, the findings of this thesis, suggest that although hummingbirds evolved neuroanatomical specializations analogous to the other avian vocal learners, the anatomy of their vocal organs, endocrinological control and activation of these specializations followed a distinct evolutionary pathway. These results expand our knowledge about how vocal learning can take place in different organisms.

## Functional aspects of vocal communication in hummingbirds

### *Repertoire of calls*

The hummingbird repertoire of calls is relatively understudied compared to that of songbirds, however, hummingbird species seem to have a relatively poor repertoire considering the number of calls and the variety of behavioral contexts they possess. The sombre hummingbird (*Eupetomena cirrochloris*), for example, is the species with the highest number of calls described so far. Their repertoire is made of six calls mostly related to agonistic contexts including chirp, guttural (expel intruders), vibrato (during direct confrontation or courtship), whistle (mostly when perched sometimes followed by an attack or courtship), crack (only by males) and high-pitch (at the end of a fight) (Ferreira et al., 2006). In contrast, a rich repertoire of calls is commonly observed in gregarious species across all avian phylogenies from early-branched Neoaves to most recent radiations of *Passeriformes* because these calls can be used to fine-tune complex social interactions independently of the species' ability to learn novel sounds (Leroy, 1979; Morton, 1977; Smith, 1972). For example, the red junglefowl (*Gallus gallus*) as well as the domesticated chicken, produce up to 24 different calls including female clucking, two types of food calls such as purring, alarm cackling to ground predator, alarm scream to flying predator, a loud defensive threat to flying predator, hiss by the female on nest, threat calls of low intensity by both sexes and crowing by the male to illustrate a few (Collias, 1987). The Guira cuckoo (*Guira guira*), a *Cuculiform* which is also a basal landbird like the *Caprimulgiformes*, has a repertoire of 15 calls including alarms directed to a conspecific or to inform the presence of an eagle, begging, flight, landing and feeding calls (Fandiño-Mariño, 1989). The orange-winged amazon (*Amazona amazonica*), a *Psittaciform* which is also Australoaves like the *Passeriformes*, has nine calls in reproductive context including flight or perched contact calls, three different types of alarm and two types of distress calls (Moura et al., 2011). Most of the species with rich repertoires seem to have mainly calls of affiliative nature. Hummingbirds, in contrast, have repertoires of a few calls mostly dedicated to agonistic contexts (**Chapter 1**)

Hummingbirds live within the upper limit of energy expenditure of any warm-blooded animal (Suarez, 1992), therefore, they live nearly on the edge of starvation, needing to feed more than their body weight each day (Ruschi, 1973). Their diet is based on nectar and insects (Hoyo et al., 1999; Ruschi, 1973; Sick, 1997) which are often sparsely distributed in their habitat. Thus, the intense search for food probably shapes their interactions and, consequently, their repertoire of calls. This may be also the case in strictly insectivorous species such as the mustached wren (*Pheugopedius genibarbis*), an oscine in which both sexes jointly defend relatively large territories all year round using a duet song, has only further two calls emitted by adults in their repertoire: an alarm and a contact call (A. de A. Monte, 2012).

It is hypothesized that the dependence on energy-rich nectar sources fomented the evolution of aggressive survival strategies (Wolf, 1978) and may explain hummingbird's solitary and aggressive behavior (Schuchmann, 1999). For example, dominant males of the long-billed hermits (*Phaethornis longirostris*) have morphologically specialized bills that serve as weapons (Rico-Guevara & Araya-Salas, 2015). However, due to their lack of pointed bills, the gilded hummingbirds (*Hylocharis chrysura*) and black-throated mangoes (*Anthracothorax nigricollis*) rely on vocalizations rather than on physical attacks to defend certain agglomerations of flowers. It is, therefore, possible that for some hummingbird species, agonistic calls are a way of “resource winning without paying the cost of an escalated fight” (Bradbury & Vehrencamp, 2011).

The resolution of conflicts is facilitated by aggressive calls in many other bird groups (Marler, 2004). For example, four calls of the red junglefowl repertoire of 24 calls are used in aggressive encounters (Collias, 1987). On the other hand, in social birds, such as parrots, calls are used to coordinate group activities; the Carnaby's black-cockatoo (*Calyptorhynchus latirostris*) produces 15 calls, none of which directly arises from an agonistic context (Saunders, 1983). In songbirds (*Passeriformes*), such as the chaffinch (*Fringilla coelebs*) and the great tit (*Parus major*), calls are used in aggressive contexts, including territorial defense (Marler 1956, 2004b, Gompert 1961). In addition to calls, songbirds also use songs, for example, to signal distinct levels of aggressiveness (reviewed in Searcy and Beecher 2009). Some species can lower the amplitude of their songs or match a singing conspecific in frequency to emphasize their aggressiveness (Searcy & Beecher, 2009). Others have different song types and match these to the same type of a conspecific to signal diminished aggression, or they switch song types to signal either escalation or de-escalation, and some use their song performance to signal aggressive escalation (Searcy & Beecher, 2009). Hummingbirds seem to mainly use their calls to resolve agonistic encounters.

In addition to agonistic calls, many hummingbird species have a call spectrally similar in their repertoire which is commonly named "chirp" or "chip" and described in almost all clades except for those that are not well studied (Topazes, Brilliant, Patagona and Coquettes). The chirps show strikingly similar temporal and spectral parameters in all species. They all have a relatively short duration combined with a wide frequency bandwidth. For example, a unit of the rufous-breasted hermit (*Glaucis hirsutus*) chirp call is composed of ascending frequency modulation of 6 to 7 kHz during 60 to 90 ms (Ferreira et al., 2006). The function of the chirps is unclear. Rufous-breasted hermits (Ferreira et al., 2006) and blue-throated hummingbirds (Ficken et al., 2002) emit chirps in two different contexts: the bird is perched alone emitting a series of chirps with progressively longer silence intervals, and the bird is only flying or foraging and producing single chirps. Ficken et al. (2002) suggested that the blue-throated hummingbirds' serial chirps served as "long distance territorial advertisement" to complement the function of their short-distance whisper song. Given their spectral and temporal properties, the chirps produced during the flight may be used for spatial orientation and/or the localization of insects during foraging flights. Swiftlets of the genus *Aerodramus* (order *Caprimulgiformes*) produce clicks, with spectral parameters similar to those of the chirps, to navigate in the dark (Thomassen & Povel, 2006). Oilbirds (*Steatornis caripensis*, order *Caprimulgiformes*) also produce clicks to navigate in dark caves (Konishi & Knudsen, 1979). Both oilbirds and swiftlets are related to hummingbirds in the order *Caprimulgiformes* (Jarvis et al., 2014; Prum et al., 2015). Behavioral paradigms are necessary to identify whether hummingbirds can also use sounds, most likely the “chirps”, to navigate such as oilbirds and swiftlets or they might have lost this function.

### *Song*

The song of hummingbirds is mainly produced by perched males in the reproductive context (**Chapter 1**). The importance of being perched during emission in the hummingbird song classification appears to be associated with the fact that most Hermits and Emeralds species are reported to sing in assemblies, so-called leks (Bleiweiss, 1998; B. K. Snow, 1973; D. W. Snow, 1968; Stiles & Wolf, 1979). In the lek, each male holds a certain position or small individual singing territory (which is different from large breeding or food territories) and performs vocally and with complementary visual displays such as rhythmic head and tail movements (Nicholson, 1931; Wiley, 1971). The relative position of each male, its detectability and singing endurance within the lek might be important for female choice.

Some hummingbird species, nevertheless, can produce lower intensity or soft songs with closed beaks that have been reported as audible only within ~ five meters (Feo et al., 2014). These low-intensity songs are so-called whisper songs and are described as usual among hummingbirds (Skutch, 1964a). Whisper song is produced by, for example, Inaguan hummingbird (*Calliphlox evelynae lyrura*), a Bee (Feo et al., 2014), scaly-breasted hummingbird (*Phaeochroa cuvierii*) and the sombre hummingbird (*Aphantochroa cirrochloris*), both Emeralds (Ferreira et al., 2006; Skutch, 1964b). The Allen's hummingbird is recurrently mentioned as a nonsinging species, but they have been reported to emit "mouse-like squeaks" (Basset, 1921) that could potentially be a whisper song. For songbirds, it was hypothesized that in dense populations whisper song is used to address a specific target directly while reducing the risk of conspecific eavesdropping, in other words, it is "used in situations with a need for privacy" (Dabelsteen et al., 1998). For example, a whisper song is used by male common blackbirds (*Turdus merula*) while establishing a territory to address only specific males. This behavior, in turn, prevents broader conflicts and can conceal courtship to avoid interruption by further potential competitors (Snow, 1958). Given the fact that hummingbirds are mainly solitary (Hoyo et al., 1999), this "privacy" hypothesis could be applied only to those species that sing in dense feeding areas, for example, the sombre hummingbird (pers. obs.). Alternatively, a whisper song might signal aggressiveness, as demonstrated in swamp sparrows (*Melospiza georgiana*) (Searcy & Beecher, 2009). This possibility is more likely since many hummingbird species are territorial (Wolf, 1978). Thus, it is also possible that the lower amplitude of the whisper songs is a byproduct of singing with the beak closed. In sombre hummingbirds, singing with a closed beak was suggested to reduce the song's pitch range and tonal quality when compared with long-tailed hermits that sing with open beaks (Ferreira et al., 2006). The starting point to investigate the function of the whisper song in hummingbirds is to verify whether the species that sing whisper song (*i*) emit their song in dense feeding territories, (*ii*) sing with closed beaks and (*iii*) are more likely to attack after singing.

Interestingly, many hummingbirds emit relatively high-pitched songs with a fundamental frequency range of 9-13 kHz (Duque et al., 2018) above the outer extent of the hearing in birds which range is 2–5 kHz with hearing sensitivity falling rapidly after this range and not reaching 10 kHz (Dooling, 2004). Hummingbirds, nevertheless, can perceive and respond to these high-pitched songs but the mechanisms underlying this capacity remains unknown (Duque et al., 2020). Furthermore, two species can even emit ultrasonic sounds ( > 30 kHz): the blue-throated hummingbird (*Lampornis clemenciae*), a mountain gem (Pytte et al., 2004) and the black Jacobin (*Florisuga fusca*), a topaz (Olson et al., 2018). Only the blue-throated hummingbird was investigated concerning its hearing range. While its song contains harmonics up to 30 kHz, surprisingly, its upper hearing threshold is around 7 kHz, and the peak sensitivity is 2 kHz in auditory brainstem responses (ABR) (Pytte et al., 2004). The mismatch between sound production and hearing capacity in the blue-throated hummingbird still requires confirmation since the ABR recording was conducted on birds anesthetized with an intramuscular injection of xylazine/ketamine combination (Pytte et al., 2004) and it was demonstrated that some anesthesia conditions could impair auditory sensitivity in relation to the awake condition with high species-specific variation in the sensitivity to different anesthetics (Thiele & Köppl, 2018). Thus, ABR recordings in awake birds and behavioral tests are needed to verify the extent of this mismatch. If both species are genuinely unable to perceive their song fully, an even more intriguing question arises: why do they produce sounds they cannot hear? It is hypothesized that ultrasonic sounds serve non-communicative purposes (Olson et al., 2018; Pytte et al., 2004); these sounds may disturb insect flight making them easier to be captured (Pytte et al., 2004). Alternatively, ultrasounds are perhaps just a byproduct of their long vocal tracts (Roverud, 1983; Zusi 2013) that lead to the generation of multiple harmonics (Hoese et al., 2000) which range into the ultrasonic range. However, the blue-throated hummingbird song comprises syllables with frequency bandwidth exclusively in the ultrasonic range (Pytte et al., 2004), not only harmonics as the black jacobin's (Olson et al., 2018). Behavioral paradigms using the playback of only the ultrasonic part of the song may reveal whether they are perceived and, therefore, used in conspecific communication.

## Evolution of singing in hummingbirds and implications for vocal production learning

In contrast to the most studied group, the songbirds, singing is heterogeneous in hummingbirds (**Chapter 1**). It is important to mention that in songbirds and parrots, most studies were conducted on a few temperate species. Nevertheless, all studied songbirds so far were reported to sing and have a learned song, therefore the ability of vocal learning is usually extrapolated to the complete suborder *Passeri* or *Oscine*. A similar extrapolation needs caution for the whole *Trochilidae* family because the evolutionary losses of singing are probably associated with losses of vocal-production learning ability (**Chapter 1**).

### *Hypothesis on the evolution of vocal-production learning*

The sexual selection hypothesis was suggested to explain the evolution of learned songs in hummingbirds (Jarvis, 2006). This hypothesis predicts that the proximate effect of vocal-production learning is the expansion of song repertoires (Nowicki & Searcy, 2014). Female preference for diverse vocal repertoires and male vocal-production learning would allow songs to become more varied in terms of syllable types or complex syllables to signal male quality (Nowicki & Searcy, 2014). However, the song of the lek-mating hermits tends to be simple, repetitive, loud and long-lasting (Araya-Salas & Wright, 2013; Macdougall-Shackleton & Harbison, 1998; Skutch, 1964a) driven by endurance and detectability rather than the size of the song repertoire. Therefore, sexual selection is unlikely to be the major driver of vocal production-learning at least in early-branched lekking hummingbirds such as hermits. Alternatively, the advantages of group signatures might have driven vocal-production learning in these hummingbirds.

Most of the lek-mating species have vocal dialects (e.g. Snow 1968, Wiley 1971, Mirsky 1976, Baptista and Schuchmann 1990). Lekking, which is widespread among hummingbirds (Martínez-García et al., 2013; Pizo, 2012), is restricted to early mornings in some species [e.g. in topazes: the crimson topaz (*Topaza pella*) (Davis, 1958); in emeralds: the swallow-tailed hummingbird (*Eupetomena macroura*) (Pizo & Silva, 2001)] when visibility is low. It is possible that males of a lek can acoustically distinguish neighbors, which are most likely to hold singing territories with stable boundaries within the leks, from newcomers, which are probably seeking a singing territory. Thus, territorial males can use this information to modulate their aggression during territorial responses, according to the risk of the threat. The reduced aggression from territorial animals towards familiar individuals, generally neighbors, with whom relationships have already been established, is called the "dear enemy effect" and has been shown in songbirds (Briefer et al., 2008).

In hummingbirds, males often do not build nests or defend breeding territories, and parental care is performed exclusively by the female (Hoyo et al., 1999; Ruschi, 1973), so it is important to bear in mind that singing territories within the lek context differ from the feeding/breeding territories in which the "dear enemy effect" was demonstrated in songbirds.

Although this effect is awaiting experimental confirmation in hummingbirds, in **Chapter 1**, I hypothesize that vocal-production learning evolved in early-branched hummingbirds to facilitate group signatures that enabled the identification of neighbors and the modulation of aggression in territorial responses. This hypothesis predicts two selective benefits: males can (*i*) increase their detectability and consequently their mating success and (*ii*) decrease the costs of a fight, thus increasing their survival odds.

Although previous studies suggest that it is unlikely that group signatures drove the evolution of vocal learning because the former could not have evolved before the latter (Nowicki & Searcy, 2014), it is possible that, in a highly belligerent bird as the hummingbirds, the evolution of lekking behavior was correlated to the development of group signatures as a strategy to mitigate aggression and increase survival. Probably this pressure for encoding group and individual signatures in the song was also the main driver in other non-songbirds that show evidence for vocal-production learning such as some lekking cotingas (D. Kroodsma et al., 2013) and, most recently, the Australian musk ducks (*Biziura lobata*), also known to display in leks (ten Cate & Fullagar, 2021). However, given that lekking manakins are relatively well studied but up to date they lack sound evidence for vocal-production learning (ten Cate, 2021), this hypothesis may be limited to hummingbirds. Comparisons of the vocal-production learning between lekking and non-lekking hummingbird species have the potential to elucidate this hypothesis.

Lekking, therefore, seems to be related to the evolution of vocal-production learning in at least the six most-studied clades (except Coquettes, Brilliants and Patagona, which are rarely studied). However, extraordinary cases of song complexity were reported for some hummingbird species, particularly among the mangoes, mountain gems and emeralds, all of which perform lek-singing. Thus, it is possible that vocal-production learning evolved in lekking hummingbird species to form group signatures. Once present, vocal-production learning allowed the coding of other information in various clades and species. Alternatively, vocal-production learning evolved under different pressures in different hummingbird clades.

Furthermore, it remains unknown whether the songs of topazes species are learned. Since it is known that swifts are non-vocal learners (Gahr, 2000) and topazes is a sister clade of all hummingbirds (McGuire et al., 2014), investigations of vocal-production learning in topazes can shed light on the ancestral condition of the hummingbird family.

### *Evolutionary loss of singing and probably vocal-production learning*

Evolutionary losses of singing were previously reported to have happened across the clade bees (tribe *Mellisugini*) (Clark et al., 2018). Interestingly, these losses seem to correlate with the degenerated development of the putative vocal control system (Gahr, 2000). In males of ruby-throated hummingbird (*Archilochus colubris*) and Allen's hummingbird (*Selasphorus sasin*), the putative vocal-control system has a phenotype similar to that of rudimentary song control areas of female songbirds (Gahr, 2000), such as females zebra finch that are unable to produce learned songs (Nottebohm & Arnold, 1976).

In Bees, dive display is an ancestral trait within the clade (Clark et al., 2018; Clark & Feo, 2010) whereas dive or homologous behaviors seem rare outside the bees (Clark et al., 2018). It was demonstrated for bees that the evolution of singing is negatively correlated to the production of sounds with the wing (wing trills) as they seem functionally equivalent (Clark et al., 2018). Neither song nor wing trill seemed costlier, thus, this replacement was suggested to have happened neutrally (Clark et al., 2018). However, given that most of the species in the two genera of bees (*Selasphorus* and *Archilochus*) that lost the song occupy open habitats, it is, therefore, possible that some bees avoided perched song because it made them more conspicuous to predators. Allen's hummingbird (*Selasphorus sasin*), for example, is a riparian breeder that often perches conspicuously on exposed leafless branches (Clark & Mitchell, 2020). Although ruby-throated hummingbird (*Archilochus colubris*) can be predated by hawks during their migratory stopover (Zenzal et al., 2013), the predation of adults has been seldom observed and predation does not seem a significant risk to hummingbirds (Miller & Gass, 1985). This low predation pressure might have been achieved, in parts, by the difficulty to intercept males in which courtship is composed mainly of dynamic displays.

Predation was already suggested as the main pressure against the evolution of learned songs because predators habituate easily to the constancy of innate calls than to the variation of learned songs; thus, a relaxed predatory pressure seems to precede the evolution of vocal-production learning (Jarvis, 2006). In the clade coquettes, relatively close-related to bees, many species of tropical Central America that occupy dense forest interior and second-growth habitats tend to have static displays on a perch; whereas species that occupy open habitats such as clearings, meadows, and forest edge tend to perform visual displays that resemble a rudimentary dive (Stiles, 1982). Furthermore, the relatively small body sizes of bees may lower the energetic requirements for dive displays and their short wings may facilitate the sophistication of the dive's aerodynamics by increasing agility and maneuverability (Payne, 1984). Overall, the expansion of bees to North America and consequent occupation of open habitats on relatively low altitudes combined with their small body sizes might have favored the enhancement of visual displays and the diversification of dive displays and associated mechanical sounds.

Interestingly, both species of the genus *Atthis* are non-diving and evolved singing (Clark et al., 2018). The evolutionary regain of singing in *Atthis* may be related to the habitat they occupy: both bumblebee hummingbird (*Atthis heloisa*) and wine-throated hummingbird (*Atthis ellioti*) inhabit forests, pine-oak woodlands, and neighboring shrubby areas (Arizmendi et al., 2020; Thurber et al., 2020). Predatory risks must be investigated comparatively between singing and non-singing bees to elucidate this possibility.

### *Comparisons of vocal-production learning in hummingbirds, songbirds and parrots*

The family *Apodidae*, including swifts and swiftlets, is the sister family of hummingbirds (Prum et al., 2015; Thomassen et al., 2005). Swiftlets and swifts produce species-specific vocalizations (Thomassen & Povel, 2006) but are not known to learn their emitted songs (Gahr, 2000; Jarvis, 2006). Furthermore, none of the species of the other families of the *Caprimulgiformes* (Strisores) are known to show vocal-production learning. Thus, it is likely that vocal-production learning is the apomorphic state and singing is the plesiomorphic state of hummingbirds. However, with the current knowledge of vocal-production learning among hummingbirds, it was not possible to resolve if this phenotype had emerged in the lineage leading to the extant hummingbird clades, was lost and regained at least twice in bees, or emerged independently several times during the evolution of the major hummingbird clades and, in some cases, within clades, such as the bees (**Chapter 1**). The evolution of vocal-production learning might have followed independent evolutionary pathways within the family, as some species incorporated group signatures in their simple songs (e.g. lekking hermits), other species imitate their mechanically produced flight sounds (e.g. diving bees), and others develop rich song repertoires composed of many different syllables, which are difficult to achieve by innate mechanisms (e.g. lekking mountain gem and emeralds).

The best-studied group of vocal-production learners is the songbirds (for a species list see, Kroodsma and Baylis 1982). Nevertheless, the selection pressures underlying the evolution of vocal-production learning in songbirds are far from understood. Different aspects of the songs among and even within songbird families cannot be explained by a single hypothesis, for example, the selection of smaller repertoires in some species and larger repertoires in others (Beecher & Brenowitz, 2005). Given that there are roughly 100 songbird families encompassing about 4,500 species (Hoyo et al., 2016), vocal-production learning likely has various functions among songbirds that have evolved in response to various selection pressures.

Likewise, despite their relatively close relatedness (Prum et al., 2015), songbirds and parrots have accumulated many differences in the functional aspects of their learned vocalizations, which suggests that their vocal-learning abilities evolved independently. For example, in wild parrots, vocal-production learning appears related to the maintenance of social bonds and the defense of nest sites rather than sexual selection and mate competition, as it is for many songbirds (Bradbury & Balsby, 2016). Beecher and Brenowitz (2005) suggested that "…many questions about the evolution of song learning will yield to an integrated approach in which comparative data on song-learning strategies are analyzed in a full phylogenetic context." Future comparative studies on vocal-production learning in parrots and songbirds in the light of their phylogenies will most likely reveal that their singing strategies and, maybe, vocal-learning abilities are as diverse as those suggested for hummingbirds. In contrast to hummingbirds, none of the songbird and parrot species investigated to date showed evidence of an evolutionary loss of vocal-learning ability. However, as the studies on vocal-production learning in songbirds and parrots are focused on a few temperate species, the investigation of poorly studied groups in a comparative framework including hummingbirds may reveal whether further vocal learners have lost the ability of vocal-production learning and, most exciting, the probable evolutionary constraints against the development of such high cognitive skill as vocal-production learning. To begin with, do hummingbirds have anatomical specializations that may facilitate the evolution of vocal-production learning?

## Anatomy of the vocal organ and its implication to vocal production learning

Although the syrinx of the hummingbirds is as complex as that of songbirds (Riede & Olson, 2020), hummingbirds have unique features which make sound production possible despite their morphological adaptations for hovering flight (Monte et al., 2020). Hummingbirds are the only group reported to have their syrinx outside of the thoracic cavity (Monte et al., 2020; Riede & Olson, 2020; Zusi, 2013). I proposed that the displacement of the syrinx in hummingbirds may have been driven by the physical constraints generated by the presence of large heart and chest muscles (**Chapter 2**). In the neck, the syrinx does not need lateral stabilization which may explain the loss of sterno-tracheal muscles which are present in almost all other birds. Another consequence of an extrathoracic syrinx may be the orientation of its intrinsic musculature which is horizontal in contrast to that of most bird species (**Chapter 2**).

Independent of its specializations, the syrinx of vocal learners has in common the presence of intrinsic musculature (Gaunt, 1983). In previous studies, the lack of correlation found between the number of intrinsic muscles and the sound complexity rejected the hypothesis of the participation of specific anatomical characteristics of the vocal organ in the evolution of vocal production learning (Jarvis, 2019). For example, most parrots have two pairs of intrinsic muscles, in contrast to up to five in songbirds (Ames, 1971; Gaunt, 1983), yet, as songbirds, parrots produce more complex sounds. Instead of rejecting this hypothesis, in **Chapter 2**, I proposed an update by speculating that a complex vocal system that allows the fine control of the sound source can constitute a prerequisite for vocal-production learning. As a prerequisite, I mean that not all species that have a complex vocal system went to the next evolutionary step and evolved the ability of vocal production learning, but all learners must have a complex vocal system. An analogy in the same logic would be that not everyone that has a good guitar will be able to play the solo of "Sweet Child O’Mine" but Slash would not be able to perform this iconic solo without having a good guitar.

A complex vocal system constitutes a series of anatomic structures that, commanded by the forebrain, can independently control a series of distinct sound parameters. Such anatomic structures can be the intrinsic musculature, the tongue and even the tympanic ossicles of hummingbirds, an ossified structure embedded in the vibratory membrane which is probably under muscular control (**Chapter 2**). In the case of parrots, for example, although they possess two pairs of intrinsic muscles, their lingual articulation has the ability to modify the amplitude of the frequency formants, which in part explains their extraordinary ability to mimic human voice (Beckers et al., 2004).

The complexity of the system may give many degrees of freedom for the trial-and-error attempts inherent to the learning process (**Chapter 2**). Songbirds have many intrinsic muscles and fine control over the vibratory membrane (Düring et al., 2013). Parrots have only two pairs of intrinsic muscles but they articulate their tongue (Beckers et al., 2004). Lastly, hummingbirds possess muscles that are organized in a specific manner which enables them to control their vibratory membranes in at least three different ways (A. Monte et al., 2020). The similarities found between the syrinx of the black jacobin and that of their distantly related species Anna's hummingbird (Riede & Olson, 2020) indicate that the prerequisite for the evolution of vocal production learning may constitute an ancestral and conserved trait within hummingbirds. The neuronal control of this vocal organ and endocrinal control of their vocalizations were the subject of **Chapter 3**, **4** and **5**.

## Neuroanatomy of the vocal control system and implications for vocal production learning

In avian vocal learners, the vocal organ can be controlled by a set of nuclei in their forebrain, the vocal control system (reviewed in Nottebohm 2005). Although all avian vocal learners investigated to date have a vocal control system in their forebrains which can be delimited by androgen receptors (AR) expressing cells (Frankl-Vilches & Gahr, 2018; Gahr & Metzdorf, 1997; Matsunaga & Okanoya, 2008) and differentially active when singing (Jarvis et al., 2000; Mello, 2002), this is not the case for the putative vocal control system in most of hummingbird species investigated in this thesis (**Chapter 4** and **5**).

Previous studies have shown a vocal control system analogous to songbirds in males of a hermit [rufous-breasted hermit (*Glaucis hirsuta*) (Jarvis et al., 2000)]; a bee [Anna’s hummingbird (*Calypte anna*) (Gahr, 2000)]; and two Emeralds [sombre hummingbird

(*Aphantochroa cirrhochloris*) (Jarvis et al., 2000), and Amazilia hummingbirds (*Amazilia amazilia*) (Gahr, 2000)]. In nearly all the species studied in this thesis and in most cases in both sexes, I successfully identified the vocal control system by its location and cytoarchitecture using Nissl-stained brain slices. However, I could not delimitate these nuclei by the two classical techniques used to delimit the nuclei of the vocal control system in songbirds and parrots: in situ hybridization for the expression of AR mRNA (**Chapter 4**) and the expression of immediate early gene EGR-1 (**Chapter 5**).

After many attempts adapting the protocol of in situ hybridization performed with $^{35}$S-CTP-labeled probes, including the design of a hummingbird-specific that successfully delimited the HVC of a zebra finch but not hummingbirds (**Chapter 4**), finally, the in situ hybridization performed using a highly sensitive and specific kit with chromogenic-labeled probes (RNAscope®, Advanced Cell Diagnostics, United States) (Wang et al., 2012) cloned from zebra finch or hummingbirds consistently revealed that mRNA AR was rather expressed elsewhere in the forebrain and in some areas in the midbrain known to express AR in other non-learning birds as well (Metzdorf et al., 1999). These findings indicated that the absence of AR in the putative vocal control system of hummingbirds is unlikely to be an artifact in histological preparations. Furthermore, the lack of differential EGR-1 mRNA expression in the vocal control system of a hermit hummingbird (**Chapter 5**) was consistent for two different in situ techniques of labeling ($^{35}$S or chromogenic labels) and for birds sampled in different years. It is important to keep in mind that the putative vocal control system of hummingbirds, especially of the Topazes, still needs confirmation with further molecular markers such as those listed in the project ZEBrA database (Oregon Health & Science University, Portland, United States http://www.zebrafinchatlas.org). However, the findings of this thesis indicate that most likely the vocal control system of both sexes in most of the hummingbird species has some remarkable, yet overlooked, molecular differences from the other avian vocal learners.

The ARs are binding sites to androgenic hormones that can structurally affect the brain by inducing changes in the expression of genes related, for example, to the incorporation of new neurons such as BDNF (Dittrich et al., 2014). Thus, the presence of AR and the transcription activation mediated by the ARs can be linked to the plasticity of the vocal control system which is heterogeneous among vocal learners (Frankl-Vilches & Gahr, 2018). This heterogeneity may partially explain how a conservative network of nuclei in terms of cytoarchitecture and connectivity such as the vocal control system can lead to different phenotypes considering sex differences and adult plasticity for learning. Testosterone and its metabolites bind to ARs of the vocal control system causing important organizational effects during the ontogeny of vocal production learning; thus, it is unknown how vocal production learning can take place in the absence of ARs. Some hummingbird species may possess the ability to transiently express AR during the early stages of development and lose it during their adult life; a steppingstone to investigate this would be to map the AR in the brain of juveniles. Alternatively, these hummingbird species undergo the ontogeny of vocal production learning solely under the influence of estrogens; to investigate this, it is necessary to map the expression of estrogen receptors (ER) in the brain of adults. Furthermore, the capacity of estrogens to induce the expression of genes related to neuronal growth remains to be uncovered.

Another dissimilarity in the anatomy of the putative vocal control system of all hummingbird species investigated in this thesis is the possible absence of a nucleus in the striatum analogous to Area X of songbirds, the VASt, known to be important for the ontogeny of song learning (e.g.: Brainard and Doupe 2000). The VASt was identified in males of rufous-breasted hermit and sombre hummingbird by the differential expression of the immediate early gene EGR-1 (Jarvis et al., 2000). Nevertheless, none of the two studies using further markers that show differential expression in other nuclei of the vocal control system of hummingbirds delimited VASt. The first study investigated the AR expression in males Amazilia hummingbird (*Amazilia amazilia*) and Anna’s hummingbird  (Gahr, 2000) and the second, the expression of two subunits of glutamate receptors, NR2A and mGluR2 in male sombre hummingbird (Wada et al., 2004). Considering the need to verify the presence of VASt using further markers, I can speculate that if VASt is absent in most hummingbird species, the connectivity of the anterior pathway differs from what is known for vocal learners, therefore, memorization of learned vocalizations is probability independent of the striatal-thalamic or recursive loop in the anterior pathway.

In songbirds, the recursive loop is made of Area X sending projections to the thalamic nucleus DLM that send projections to LMAN in the forebrain which, in turn, sends projections back to Area X (reviewed in Zeigler and Marler 2012). In juvenile zebra finches, lesions of Area X resulted in atypical songs consisting of rambling series of unusually long and variable notes (Scharff & Nottebohm, 1991). In adult zebra finches, neurotoxic lesions of Area X caused a long-term increase in song tempo and changes in syllable sequencing, particularly profound repetition that resembles human stuttering (Kubikova et al., 2014). Thus, the recursive loop seems important not only for the ontogeny of vocal production learning but also for the maintenance of temporal aspects of the learned song even in adults that are close-ended learners.

Interestingly, the VAN (LMAN-like) of the long-tailed hermit (*Phaethornis superciliosus*) showed lower EGR-1 expression relative to the surrounding nidopallium in the posterior part (dorsocaudal) and higher EGR-1 expression in the anterior part. The expression of EGR-1 in Anna’s hummingbird (Chakraborty et al., 2015), NR2A and mGluR2 in sombre hummingbird showed also a similar dual pattern in VAN that was unique to hummingbirds (Wada et al., 2004). These findings suggest that the posterior and anterior parts of VAN are different in their receptor’s distribution and maybe in their function; one may have afferent and the other efferent connections. Given the importance of the recursive loop to vocal-production learning, hummingbirds that lack VASt may have an alternative to this loop. In these hummingbirds, the recursive loop is perhaps made of VAN sending a direct projection to the thalamus which, in turn, sends projections to another area of the VAN. The reduction of the recursive loop from three to two nuclei was maybe a solution to make this loop more compact and yet maintain its functionality. Connectivity studies are key to confirming the presence of a recursive loop in hummingbirds.

Previous studies have shown that some hummingbird species such as Anna's hummingbird possess a putative vocal control system that is more similar to that of songbirds than to that of other hummingbird species studied in this thesis (Gahr, 2000). This could indicate that not only singing but also the vocal control system is heterogeneous in hummingbirds (**Chapter 1**). This heterogeneity may vary in at least three stages: (*i*) a rudimentary system in hummingbird species that lost singing similar to the system of non-singing females in some songbirds, for example, zebra finch; (*ii*) a system with fewer nuclei and with differences in their cytochemistry in hummingbird species in which song may be only partially learned; and (*iii*) a complete system similar to some songbirds in species with complex songs. This variability is in line with the vocal learning continuum hypothesis which suggests that vocal production learning evolved in a stepwise rather than a dichotomic manner (Arriaga & Jarvis, 2013; Petkov & Jarvis, 2012). According to the continuum hypothesis, there are limited vocal learners, for example, monkeys that can learn to contract their diaphragm to mimic human coughing (Perlman & Clark, 2015), and moderate vocal learners, for example, mice that can learn some elements of their ultrasonic vocalizations (Arriaga & Jarvis, 2013; Jarvis, 2019). This process has supposedly happened in several orders, and I propose that a similar process may have happened within the hummingbird family that includes taxa up to 20 million years apart (McGuire et al., 2014). The development of a vocal motor pathway in hummingbirds may have had intermediate stages in which the motor vocal pathway was initially composed of fewer nuclei and lacking androgen receptors.

Furthermore, the ontogeny of vocal production learning also seems to differ from that of songbirds. In most songbirds, the sensory period declines after around 100 days post-hatch, and the sensory exposure required for song memorization can be relatively short (reviewed in Brainard and Doupe 2002). For example, nightingales (*Luscinia megarhynchos*) can learn up to 60 songs that they have heard only once a day for 20 days whereas zebra finches can learn well if exposed to a tutor song less than a minute per day (reviewed in Brainard and Doupe 2002). Recent studies of the vocal leaning ontogeny in Costa's hummingbird (*Calypte costae*) demonstrated that males have a relatively long sensitive phase that begins before day 35 days post-hatch and continues until after 115 days depending on relatively long sensory exposure (more than ~ 4,000 songs over 20 hours of tutoring); learning happens if the bird is exposed to both the playback of the song and the presence of a conspecific (Johnson & Clark, 2020, 2022). This long exposure is needed to learn a whistled song which was believed to be innate because of its relative simplicity compared to the spectrally variable song of the sister species Anna's hummingbird (*Calypte anna*) (Williams & Houtman, 2008). Investigations of the cytochemistry and connectivity of the vocal control system comparing Costa's hummingbirds to Anna's hummingbird and other distantly related species such as the black jacobin have the potential to disentangle phylogenetic constraints and functional adaptations of the vocal control system in hummingbirds.

Despite its independent evolution, the vocal control system showed many convergences in the distant avian groups of vocal learners which were suggested to be driven by more than 50 common genes involved in the enrichment of the motor control and neural connectivity functions (Pfenning et al., 2014). These convergent specializations for a long period reinforced the hypothesis that there is a limited way in which learning can be coordinated in the forebrain. However, the study of this system in several hummingbird species provides hints that this hypothesis may originate from comparative studies done on the same few model species. For example, studies including hummingbirds often use Anna's hummingbird as model species and in this thesis, Anna's hummingbird was proven to possess many differences from phylogenetic distant species such as the black jacobin. In this regard, it would be illuminating to extend comparative studies on the neuroanatomy of the vocal control system to phylogenetic distant species that better represent the large diversity of life history characteristics of hummingbirds; this approach has the potential to unveil alternative ways to achieve variability of learning.

## Testosterone insensitivity and implications for vocal production learning

Although the presence of the androgen receptor (AR) in the vocal control system is ubiquitous in both sexes of all other avian vocal learners investigated to date with different degrees of sex differentiation in their vocal behavior (Frankl-Vilches & Gahr, 2018; Gahr, 2020b), in hummingbirds, it seems that ARs in the forebrain is rather an exception (**Chapter 4**). Hummingbirds also show a relatively low AR expression in the testis and synrix (**Chapter 4**). This absence offers further support to the outcome of the testosterone manipulation; high levels of testosterone kept implanted birds aggressive but did not affect their vocal behavior (**Chapter 3**). It is important to bear in mind that the results of **Chapter 3** may be restricted to a specific life stage and those of **Chapter 4** to a few individuals therefore the interpretations are given in a speculative tone.

There is substantial evidence that shows the detrimental effects of prolonged high levels of circulating testosterone on lifetime fitness such as exposure to predators, increased risk of injury and loss of mass and fat reserves, therefore, many species evolved mechanisms to avoid these costs of circulating testosterone in the nonbreeding season (reviewed in Wingfield et al. 2001). In comparison to other birds, hummingbirds are intrinsically on the upper limit of aggressiveness and metabolism, thus, a minor energy loss can be crucial for their survival (Ruschi, 1973; Suarez, 1992). It is possible that to avoid the detrimental effects of testosterone, most hummingbird species might have evolved an even more restrictive mechanism than tilts according to the life stage; they may have become almost insensitive by having little or almost no androgen receptors in most of their tissues, in particular, in their forebrains. The evaluation of mass loss driven by testosterone implants comparatively in testosterone sensitive (e.g.: Anna’s hummingbird) and less sensitive species (e.g.: black jacobin) could shed light on this possibility.

In most of the songbirds species investigated to date, testosterone affects song rate but its effect on other parameters such as song duration, frequency and stereotypy varies among species (reviewed in Gahr 2020b). There are a few exceptions such as male blue tit (*Parus caeruleus*) that shows a mismatch between the peaks of song rate and testosterone plasma level; their song rate peaks during nest building whereas testosterone peaks a few weeks later, during female egg-laying and mate guarding (Caro et al., 2005). Although a weak correlation between testosterone and the duration of the dawn chorus was observed (Foerster et al., 2002), as expected, in blue tits, experimentally elevated testosterone did not influence singing (Kunc et al., 2006), but, interestingly, nor aggression or territorial defense (Foerster & Kempenaers, 2005). Hence, it is possible to speculate that when song is not subjected to mate choice, none of its parameters is affected by testosterone; this may be the case for blue tits and most hummingbirds. Eventually, song may serve other functions that affect undirectedly sexual selection such as territorial defense; thus, it is the quality of territory rather than that of song that counts. Playbacks of song to conspecifics of the opposite sex followed by the evaluation of the behavioral response whether affiliative or agonistic can give hints on the role of song in mate choice in these species which song is testosterone insensitive.

Hummingbirds seem to be the very first group of avian vocal learners that lacks AR in their putative vocal control system which makes them, in consequence, potentially testosterone insensitive. Testosterone has important organizational and activational effects on the brain during the process of vocal production learning, however, the implications of its insensitivity in hummingbirds remain unknown. Thus, I speculate that this condition can be explained by one of the following scenarios: hummingbird species that lack AR in their vocal control system are (*i*) non-vocal learners, their song is innate and their putative vocal control system is involved in functions other than the vocal production learning; (*ii*) they are closed-ended vocal learners, there is a transient up-regulation of AR only during the ontogeny of the vocal control system when vocal production learning takes place or (*iii*) they are open-ended vocal learners, vocal production learning happens via a mechanism independent of androgen regulation. To clarify which is the possible scenario, a further study with more focus on the ontogeny of vocal production learning in the black jacobin is suggested.

## Conclusion

In this thesis, I shed light on the principles of vocal communication that contextualize the evolutionary pressure of vocal-production learning in a unique group of birds such as hummingbirds. Additionally, this thesis provides insights into how hummingbirds' unique adaptations for hovering flight and extreme metabolism, might have shaped further adaptations related to their vocal communication, expanding from their vocal organ to their brain, not only in their morphology but also in their endocrinological regulation. Having in mind that the considerations about vocal-production learning in hummingbirds are restricted to speculations because this thesis lack an experimental paradigm that directly approaches ontogeny such as the classic investigation of song development on acoustic isolation or neurobiology such as the lesion of a certain nuclei of the vocal control system, I do believe that in the future such studies can, nevertheless, benefit from the knowledge on the vocal communication of understudied model systems provided in this thesis. About two decades ago, studies showed that hummingbirds have a vocal control system, the neuroanatomical specialization to learn songs, analogous to other avian vocal learners (songbirds and parrots) and since then, most of the following studies searched for similarities among these groups. However, investigations in this regard are limited to a few model systems that do not reflect the diversity of life history within each group. This diversity could implicate differences in their communication and perhaps in their learning process. The findings of this thesis indicate that not only their vocal communication seems diverse and heterogeneous but also their learning process may have evolved under distinct pressures and followed different evolutionary pathways within the hummingbird family. A similar scenario could be revealed by investigating some understudied groups of songbirds and parrots. I hope that this thesis will encourage the investigation of further understudied model systems that have the potential to unveil alternative mechanisms in which the learning process occurs. Hummingbirds will remain to be a reminder that although guided by the principles of parsimony evolution can also lead to innovation.

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