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Evolution of High-Frequency Vocalizations in Hummingbirds

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Evolution of High-Frequency Vocalizations in Hummingbirds

by

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Under the Direction of Laura Carruth, PhD

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ABSTRACT

While most birds sing and hear between 2–6 kHz, four Andean hummingbirds

vocalize between 9–16 kHz. This phenomenon provides an opportunity to investigate the

evolution of vocal signals, the factors that selected for these traits, and the adaptations that

enable their use. I hypothesized that some hummingbirds have evolved high-frequency

(HF) vocalizations to adapt to their habitat acoustics, facilitating communication.

I conducted behavioral observations to elucidate the function of HF signals and

found that hummingbirds use these vocalizations in territorial contexts. In one species,

males also use HF song to court females, and there are dialects across populations. These

findings suggest that HF vocalizations are used in conspecific communication and that, in

some cases, sexual selection exerted pressure for the evolution and diversification of HF

signals. Then, I evaluated ecological factors driving the evolution of HF vocalizations in

hummingbirds. I found that these vocalizations are broadcast at a noise-free frequency

range in the acoustic environment, likely avoiding masking by ambient noise. Moreover, HF

vocalizations attenuate and degrade at short distances, suggesting that they are likely

short-range communication signals. These results support the hypothesis that

hummingbirds adapted to vocalize at high frequencies to prevent signal masking during

conspecific communication. Finally, I investigated neural responses to HF vocalizations in

hummingbirds. I studied behavioral and brain responses to the playback of the HF song in

the Ecuadorian Hillstar, showing that these hummingbirds hear conspecific HF songs. This

is the first evidence that birds can hear HF sounds and suggest that other hummingbirds

producing HF calls likely hear these sounds. Finally, I investigated sex differences in the

activation of the brain's Social Behavior Network in response to the HF song, a territorial signal for males and a courtship song for females.

Altogether, my research shows that some hummingbirds evolved extraordinary vocal and hearing capabilities to avoid signal masking in their habitats when communicating with conspecifics. Studying HF vocalizations in hummingbirds unveiled the presence of auditory adaptations for communication amid challenging environmental conditions. It also opens new avenues to study adaptations for vocal production and sensory processing of an evolutionarily novel signal.

INDEX WORDS: Vocal communication, Birds, Hummingbirds, Auditory processing, Dialects, Animal communication

Evolution of High-frequency Vocalizations in Hummingbirds

by

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DEDICATION

Indigenous communities in the Ecuadorian Andes are always grateful to the mountains calling them Taitas (fathers) and mamas (mothers), for the mountains provide the harvest and water. Taita Chimborazo is the highest summit in Ecuador and the most salient point on Earth as measured from the center of the planet; we, Ecuadorians, like to boast about it. I will be forever grateful for all I have learned at the foothills of this mountain.

Becoming a scientist has been a dream that started early in my life. It could have not been achievable without the support of my family, who have always believed in me, even at times when I did not believe in myself. I dedicate this work to my father who showed me the love for books and breaking things apart to learn how they work. To my mother, who kept a big sign in the dining room for years to remind me every day of my dream: "PhD in neuroscience", it said. I also dedicate this work to my loving and unconditional siblings, whom I admire for their hard work and resilience. My husband Carlos Antonio is the best partner, friend, and colleague that I can have. I am so grateful for his life and for having him in mine. Carlos, I love and admire you. Thanks for inspiring me every day to be the best version of myself in every aspect of my life. Thanks for sharing this great discovery and helping me take it to new heights. My life is always better and happier because of my dogs, Dana and Ginger. Their love and company have been enriching and healing.

Finally, I dedicate this dissertation to Walter Wilczynski and Laura Carruth, my PhD advisors, who gave me the opportunity to join their lab despite my limited prior experience in research. Thank you for seeing the potential in me and in this project. Your life, teachings, and advice inspire and guide me every day.

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This project started long before I joined graduate school. Thanks to Dr. Carlos A. Rodriguez-Saltos for showing me the Hillstar and letting be part of this great discovery. In the early days, many friends and family volunteered as field assistants: Martin Santacruz, Jorge Castillo, Alex Duque, Fernando Duque, Anaiz Mendoza, Francesca Angioliani, Yaku Romoleroux.

Finally, I would like to thank my committee members for their advice and support throughout my graduate years and during the difficult times after Walt's passing.

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1   VOCAL COMMUNICATION IN HUMMINGBIRDS

Hummingbirds, like other birds, exhibit complex social behaviors and use vocal communication to mediate their conspecific social interactions. Although vocal communication is common among animals (Bradbury & Vehrencamp, 2011), the ability to learn vocalizations has evolved independently in just a few groups of birds and mammals (Janik & Slater, 1997; Jarvis, 2004; Nowicki & Searcy, 2014). Hummingbirds (Apodiformes: Trochilidae) are among the birds that exhibit vocal learning, together with songbirds (order Passeriformes) and parrots (order Psittaciformes) (Baptista & Schuchmann, 1990; Bradbury & Balsby, 2016; Jarvis, 2004; Salinas-Melgoza & Wright, 2012). Despite these characteristics, research on vocal communication in hummingbirds has not been as extensive as in songbirds. Here, I aim to summarize research in vocal communication in hummingbirds by addressing different aspects of their vocal abilities, including vocal repertoire, learning and song variation, and the neural and vocal structures that facilitate these behaviors.

1.1   Vocal repertoires in a hummingbird

Hummingbirds exhibit diverse vocalizations that serve different functions in their social interactions, all of which constitute the animal's vocal repertoire (Ferreira et al., 2006). These vocalizations vary in structure, complexity, and spectral content, and are produced in distinct social contexts, mainly for territorial defense, courtship, parent-offspring interactions, foraging, and announcing a potential predator (Bradbury & Vehrencamp, 2011; Ferreira et al., 2006; Ficken et al., 2002; Ortiz-Crespo, 2003; Pytte & Ficken, 1994).

Here, I present the vocal repertoire of the Ecuadorian Hillstar (*Oreotrochilus chimborazo*) (**Figure 1**), an Andean hummingbird specialized to live at high altitudes (Carpenter, 1976; Fjeldså & Krabbe, 1990), to exemplify the types of vocalizations that hummingbirds exhibit. The repertoire of this hummingbird has not been described in detail before, but some of these vocalizations are well known by locals and often used as playback to attract these hummingbirds for observation.

Territorial signals help animals defend their territories and resources from intruders (Bradbury & Vehrencamp, 2011; T. A. Hunter, 2008). These signals can vary in complexity, from single-note vocalizations or trills produced at rapid succession to more elaborate songs with multiple frequency-modulated elements (Ferreira et al., 2006; Ficken et al., 2002). Territorial calls are mostly used to prevent aggressive encounters; however, if aggression escalates, some hummingbirds increase the rate at which they produce their calls, often aggressively chasing the intruder away from their territory (Ferreira et al., 2006; Wolf, 1969). Some species may also have signals that have a dual function, serving as territorial signals for males and advertisement for females (Catchpole & Slater, 2008).

The Ecuadorian Hillstar has a distinctive loud vocalization that is uttered while chasing away an intruder from their territory. This chasing call is the most aggressive vocal signal in the species, and both males and females produce it (**Figure 1A**). The duration of the vocalization can extend for several continuous repetitions while the aggressive chase lasts. Sometimes, these calls are produced as a territorial signal while perching to prevent potential intruders from landing in an individual's territory.

During breeding season, hummingbirds produce characteristic courtship sounds to attract females, usually in association with complex visual displays (Clark & Feo, 2010; Feo

& Clark, 2010; Hogan & Stoddard, 2018; Pytte & Ficken, 1994). **Figure 1B** shows a spectrogram of the high-frequency courtship song of the Ecuadorian Hillstar, a vocalization only produced by males. Ecuadorian Hillstar males hold separate foraging territories from the females, so while the males are in their own territory, these signals can also be produced as a territorial signal for neighboring males. In contrast, contact calls are often produced by both, males and females while foraging or when the female approaches the nest (**Figure 1C**). Nestlings are highly sensitive to their mother's contact calls, quickly responding with high-pitch vocalizations of their own while begging for food (**Figure 1D**).

Singing in female birds has been largely ignored. In recent years, however, we are learning more about the vocal capabilities of females, and the use of female vocalizations in social interactions (Catchpole & Slater, 2008; Riebel, 2003; Wilkins et al., 2020), including examples of songs produced by female hummingbirds (Clark, Rankin, et al., 2018; Ficken et al., 2000). Blue-throated hummingbird (*Lampornis clemenciae*) females exhibit complex songs that differentiate from those of males in structure and frequency content (Ficken et al., 2000). Females produce these vocalizations in close proximity to males suggesting that these signals are used to mediate social interactions with male conspecifics. Also, female Costa's Hummingbirds (*Calypte costae*) have been reported to produce vocalizations that resemble those of males in a territorial context (Clark, Rankin, et al., 2018). In the case of the Ecuadorian Hillstar, females produce the same vocalizations as males, except for the high-frequency courtship song (see **Fig. 1A–C**).
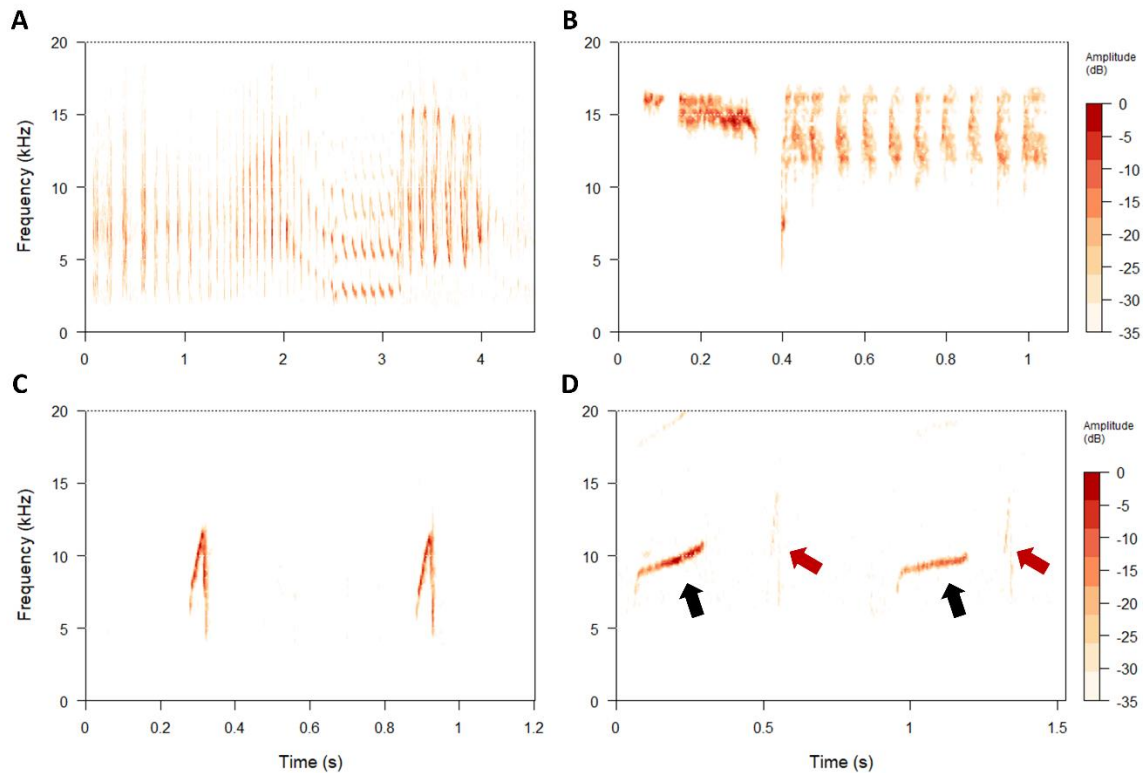
Figure 1. Vocal repertoire of the Ecuadorian Hillstar
Spectrograms showing the four most common vocalizations in the Ecuadorian Hillstar. Unless otherwise specified, these vocalizations are produced by both males and females. **A)** aggressive chasing calls; **B)** HF courtship song produced only by males; **C)** contact calls; and **D)** nestling/juvenile begging calls (black arrows) accompanied by traces of a female contact call in response (red arrows).

1.2   Vocal learning and dialects

Hummingbirds are among the few vertebrate groups in which vocal learning has evolved (Nowicki & Searcy, 2014). Baptista and Schuchmann (1990) observed that individuals within a group of Anna's hummingbirds (*Calypte anna*) shared common features in their songs, but these vocalizations differed from those of conspecifics in other groups. Consequently, three hummingbirds raised together developed more stereotypical songs in with multiple shared song elements, while raising a hummingbird in isolation resulted in a less stereotyped song than those found in the wild (Baptista & Schuchmann,

1990). These results confirmed that the Anna's hummingbirds, and likely other hummingbird species, learn their songs.

More recently, Johnson and Clark (2020) described the ontogeny of vocal learning in the Costa's hummingbird (*C. costae*). Here, they found that sensitive developmental periods for song learning in this hummingbird are similar to those found in songbirds (K. E. Johnson & Clark, 2020). Moreover, exposure to a conspecific male was necessary for song learning, emphasizing the role of social interactions for vocal learning in hummingbirds, which do not exhibit paternal care. As a result, juveniles learn vocalizations from neighboring males once the juveniles are outside the nest exploring their surroundings. Contrasting with species that exhibit critical periods for vocal learning early in development (F. Johnson et al., 2002; K. E. Johnson & Clark, 2020), some animals, known as open-ended learners, learn their vocalizations as adults (Marler et al., 1994; Mountjoy & Lemon, 1995). For instance, Long-billed Hermits (*Phaethornis longirostris*) are known for learning and switching song types even as adults while lekking with conspecifics (Araya-Salas & Wright, 2013).

Vocal learning allows for song variation, and these song variants or dialects can be found across populations or groups of individuals of the same species. Dialects are present in humans, whales, dolphins, birds, and frogs (Burridge, 2017; Capranica et al., 1973; Ford, 2011; Janik, 2000; Marler & Tamura, 1962). Although, they are more common among vocal learners, dialects can also be observed in vocal non-learners.

Hummingbirds also exhibit variation in their song, and this feature helped to elucidate the presence of vocal learning in some species (Araya-Salas & Wright, 2013; Baptista & Schuchmann, 1990; Gaunt et al., 1994; Snow, 1968). Moreover, the coevolution

of vocalizations and visual signals among individuals of highly social species such as the Long-billed Hermit exemplifies the complex learning that some hummingbirds undertake throughout their lives (Araya-Salas et al., 2019). Altogether, vocal learning and the presence of dialects in hummingbirds point out to a diverse use of vocal signals mediating hummingbird social interactions.

1.3   Other acoustic signals

Some species of hummingbirds not only use vocalizations to communicate with conspecifics, they also produce sonations, which are sounds resulting from beating the tail and wing feathers (Bostwick & Prum, 2003; Clark, 2011; Clark & Feo, 2010; T. A. Hunter, 2008). Hummingbirds can produce sonations while courting females with acrobatic courtship displays (Feo & Clark, 2010; Pytte & Ficken, 1994). Among those hummingbirds that produce sonations, each species has its distinctive courtship display and sounds associated with it. Furthermore, their feathers are optimized to produce sounds under the specific conditions that the species' acrobatic displays render (Clark, McGuire, et al., 2018). In many species, vocal sounds closely resemble tail and wing sounds suggesting that males learned to imitate sonations that they already produced during courtship displays and were attractive to conspecific females (Clark, McGuire, et al., 2018; Clark & Feo, 2010). Nonetheless, female preference or perception of either sonations or other courtship sounds has not been tested in hummingbirds thus far.

1.4   The hummingbird brain – auditory processing

Vocal communication and particularly, vocal learning require the presence of specialized interconnected brain nuclei that facilitate song control and auditory perception (Vicario & Nottebohm, 1988). In 2000, two studies independently confirmed the presence

of song system nuclei in the hummingbird brain homologous to those in the brains of

songbirds. Using a comparative approach that included vocal learners and non-learners,

Gahr (2000) employed estrogen receptors to map these nuclei in the hummingbird brain

(Gahr, 2000). Later that year, Jarvis and collaborators (2000) also showed the presence of

these nuclei by inducing the expression of the immediate-early gene *zenk*, in the song

system in response to playback of conspecific vocalizations and during singing bouts (Jarvis

et al., 2000). Both studies confirmed that the hummingbird brain exhibits specialized

regions involved in song learning and production (**Figure 2**).
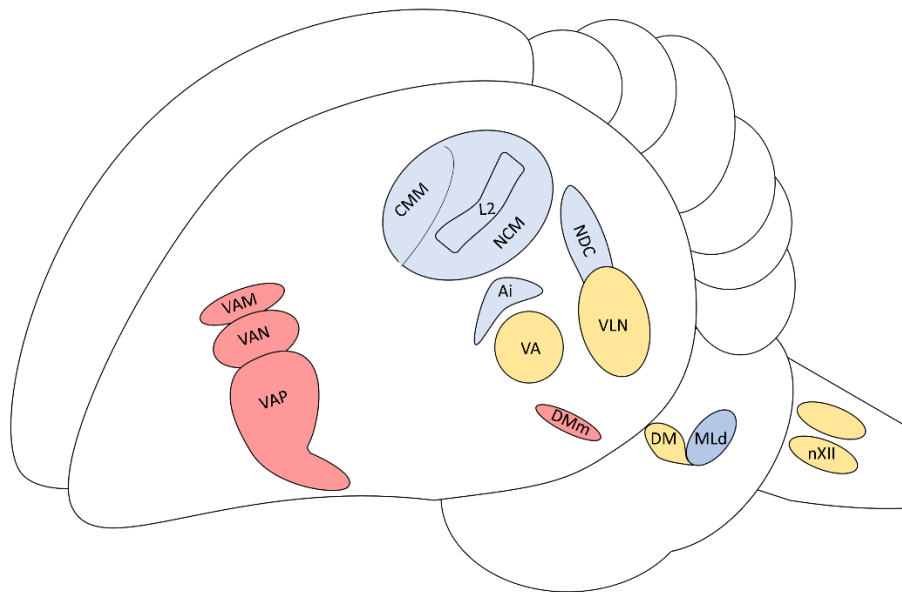
Figure 2. Schematic of the hummingbird brain.
Several regions involved in the song control system are shown here, including striatal regions (red), those involved in vocal control (yellow), and areas for auditory processing (light blue). Based on *(Jarvis et al., 2000)* for anatomical regions and *(Reiner et al., 2004)* for updated nomenclature.

Although song control and auditory nuclei have been identified in the brains of hummingbirds, it has been harder to obtain detailed characterizations of their auditory sensitivity. The Blue-throated hummingbird (*L. clemenciae*) produces a complex song with low- and high-frequency notes (Pytte et al., 2004). Measurements of auditory sensitivity using Auditory Brainstem Responses (ABRs) showed that these hummingbirds hear best at low frequencies, matching only the low-frequency components of the song (Pytte et al., 2004). Similar sensitivity was observed in the Ruby-throated hummingbird (*Archilochus colubris*), which produces a song with a fundamental frequency of 3 kHz and hears best at the same frequency (Lohr & Dooling, 2004). It is possible that some hummingbirds have a hearing range restricted to lower frequencies to match their vocal production or because the high-frequency notes in their songs do not have biological relevance for the species (Dooling, 1982, 2004). In contrast, some species of hummingbirds produce vocalizations entirely in the high-frequency range (Clark & Feo, 2010; Duque et al., 2018; C. R. Olson et al., 2018), beyond what most birds produce and hear (2-6 kHz) (Dooling, 1982, 2004). The production of high-pitched vocalizations in hummingbirds suggests that at least some species have evolved to hear high-frequency sounds, although this hypothesis is yet to be tested.

To accommodate for high-frequency hearing, hummingbirds could have expanded their hearing range, maintaining sensitivity to low- and high-frequency sounds (**Figure 3A**). This may be the case of species exhibiting a vocal repertoire that spans a wide range of frequencies, like that of the Ecuadorian Hillstar. Other species may have shifted their auditory sensitivity toward the high-frequency range, losing some sensitivity to low-frequency sounds (**Figure 3B**). This scenario could be possible in species whose entire

vocal repertoire is restricted to higher frequencies and do not face strong environmental

pressures to maintain sensitivity to low-frequency sounds.
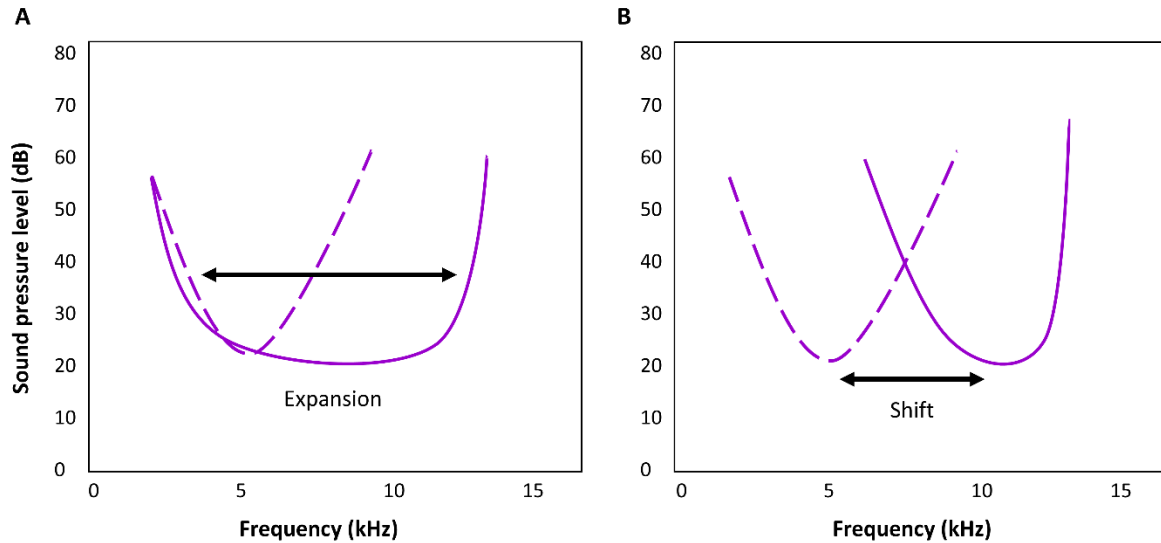
Figure 3. Hypothesis of auditory sensitivity for HF sounds in hummingbirds.
Schematic of auditory curves depicting two possible scenarios of how some species of hummingbirds may have tuned their auditory sensitivity to HF sounds. HF hearing could be attained by **A)** expanding their hearing range, or by **B)** shifting their sensitivity toward HF sounds. Dotted line depicts a hypothetical ancestral state of restricted auditory sensitivity, while solid lines show how hearing may have changed to facilitate detection of HF sounds.

1.5   The vocal organ

Birds have both a larynx and a syrinx, however the syrinx is the avian vocal organ.

For most birds the syrinx is located in the thorax (Düring et al., 2013) (cite), however the

hummingbird syrinx is positioned outside of the thorax (Monte et al., 2020b; Riede & Olson,

2020). It is still unknown why hummingbirds have evolved this feature, but their enlarged

hearts, which occupies a big part of the chest cavity, may be the main reason (Monte et al.,

2020b). Although the hummingbird syrinx shows convergence with the vocal organ in

songbirds, including its bipartite structure, there are subtle differences which may account

in part for the diversity of vocalizations and the frequencies that hummingbirds can

produce. For instance, small differences in the position of the syrinx and its morphology have been described among species of hummingbirds in the Bee clade that exhibit complex songs compared to closely related species that produce simple calls and have lost the ability to sing (Riede & Olson, 2020). Likewise, a recent description of the syrinx of the Black Jacobin (*Florisuga fusca*)(Monte et al., 2020b), showed differences in the directionality of fibers in some syringeal muscles compared to their counterparts in the syrinx of songbirds or other hummingbirds (Düring et al., 2013; Riede & Olson, 2020). Subtle differences like the position of the syrinx and tracheal length, as well as changes in the intrinsic muscles of the syrinx may be involved in the production of extraordinary high-frequency vocalizations, which have not been described in other avian species. Comparative studies on the morphological and physiological properties of the syrinx from hummingbirds producing high-frequency vocalizations and from closely related species that sing low-frequency sounds are much needed.

1.6   Other factors that influence vocal communication

Physical constraints such as body size can also influence vocal communication, either facilitating or restricting the vocal range of animals (Ryan & Brenowitz, 1985). Across multiple taxa, an acoustic allometry has been observed, so that smaller animals produce vocalizations at higher frequencies than their larger counterparts (Bradbury & Vehrencamp, 2011; Gingras et al., 2013). This phenomenon has been extensively documented among multiple avian groups (Goller & Riede, 2013; Ryan & Brenowitz, 1985). However, no systematic study has been conducted to determine whether this acoustic allometry is present among hummingbirds.

Furthermore, hummingbirds are unique to the Americas, but they are distributed across diverse habitats in the continent, ranging from the rain forest to the high-altitude grasslands (Winkler et al., 2015). Environmental features can impact vocal communication by influencing sound transmission. The Acoustic Adaptation Hypothesis (AAH) proposes that animals adapt to environmental conditions to maximize signal transmission and facilitate communication. Consequently, animals living in closed, forested habitats generally vocalize at lower frequencies than those living in open grassy environments, optimizing their vocalizations for transmission in densely vegetated habitats (Morton, 1975; Wiley & Richards, 1978). In addition to vegetation structure, other environmental features, such as temperature, humidity, and wind, can also affect transmission of a signal (Catchpole & Slater, 2008; Snell-Rood, 2012), all of them exerting pressure on the vocalizations that an animal produces in a particular habitat. Since it was initially proposed, the AAH has amounted extensive support (Boncoraglio & Saino, 2007; Ey & Fischer, 2009; M. L. Hunter & Krebs, 1979; Nottebohm, 1969; Ryan, Cocroft, et al., 1990) with some exceptions (Boncoraglio & Saino, 2007; Penna & Solís, 1998; Röhr & Juncá, 2013).

Ambient noise is also an important factor affecting vocal signals in a habitat, so that animals adjust different features in their vocalizations to facilitate communication in a noisy environment (Brenowitz, 1982; Brumm & Naguib, 2009). This phenomenon, known as the Lombard Effect, can include changes in amplitude, frequency range, rate at which the animal vocalizes, or a combination of any of the three (Cynx et al., 1998; Potash, 1972; Slabbekoorn & den Boer-Visser, 2006; Walters et al., 2019). Moreover, animals sharing the same habitat tend to vocalize at frequencies in which they will find less noise interference, effectively partitioning the acoustic environment to avoid signal masking (Duellman &

Pyles, 1983; Luther, 2009; Stanley et al., 2016). Despite the diversity of environments that

hummingbirds inhabit, no studies have been conducted to characterize the influence of

physical and environmental constraints in the vocal production of these birds.

1.7   Dissertation goals

In this dissertation I aim to understand how HF vocalizations have evolved in

hummingbirds and what their role is in communication. HF vocal signals have not been

described in other bird species, and they challenge our understanding of vocal production

and auditory processing in birds. Furthermore, the use of these signals in some species of

hummingbirds while being absent in others offers a unique opportunity to investigate the

different pressures that influence the evolution of a vocal signal and the evolutionary

innovations that enable its use.

In chapter 2, I describe in detail the vocalizations of four species of Andean

hummingbirds, and the social context in which these vocalizations are commonly

produced. Detailed accounts of behaviors associated with these vocalizations are key to

understanding the function of these signals in different species of hummingbirds. In

addition, in chapter 3 I examine the variation in the HF song of a hummingbird, adding to

our understanding about the complex use of HF vocalizations in communication in some

species.

Physical and ecological constraints exert pressure on the evolution of vocal signals.

So far, hummingbirds with documented HF vocalizations have different body size and are

found in diverse habitats such as the rain forest in the Amazon, the montane cloud forest of

the Andes, or the high-altitude grasslands. It is still unknown whether HF vocalizations

have evolved in some species in response to unique selection pressures in their

environment or whether these hummingbirds face similar challenges despite their distinct

habitats. In chapter 4, I study the role of body size as a physical constraint that may

influence the production of HF vocalizations in some species of hummingbirds, while

others do not produce these sounds. Moreover, I study ambient noise as a potential

ecological factor driving the evolution of HF vocal signals that avoid masking in the

environment. Additionally, I explore the role of habitat type, as a broad variable affecting

the transmission of HF sounds. Experiments of signal transmission provide information

about the distance that a signal can travel in a particular environment before being

completely attenuated and degraded. These results paired with behavioral observations

offer insights into the function and use of these vocalizations.

Furthermore, one of the most pressing questions associated with the discovery of

HF vocalizations in hummingbirds is whether these animals can hear these sounds. If so,

HF hearing in hummingbirds may require peripheral and central adaptations that enable

auditory processing. In chapter 5, I investigate whether a hummingbird that produces the

HF song with the highest fundamental frequency documented in a bird vocalization, can

also hear these sounds. I investigate behavioral and neural responses to the playback of HF

song to determine whether this signal is in fact used for communication with conspecifics

as behavioral observations suggest. Additionally, vocal signals can serve more than one

function in a species. For instance, an advertisement call aimed at females can also be

broadcast as a territorial signal toward competing males. Therefore, I also characterized

the neural responses of the brain's social behavior network (SBN) in male and female

Ecuadorian Hillstars in response to the HF song. This is of particular interest in this species

because males and females exhibit high levels of aggression against conspecifics of both sexes.

Altogether, this dissertation provides insights into the ecological pressures that have influenced the evolution of HF vocalizations in hummingbirds as well as the function and the neural processing of these remarkable signals.

2   HIGH-FREQUENCY VOCALIZATIONS AND THEIR FUNCTION IN HUMMINGBIRDS

Hummingbirds never fail to impress us with their unique features. A recent report in *Current Biology* (C. R. Olson et al., 2018) showed that the Black Jacobin (*Florisuga fusca*), a Brazilian hummingbird in the Topazes clade, produces high-frequency (HF) vocalizations outside the known hearing range of birds. Here, we report that four hummingbird species in the Andean clade (McGuire et al., 2007, 2014) also exhibit HF vocalizations: the Ecuadorian Hillstar (*Oreotrochilus chimborazo*), with the highest fundamental frequency (mean F_0 = 13.4 kHz), the Buff-tailed Coronet (*Boissonneaua flavescens*), Speckled Hummingbird (*Adelomyia melanogenys*), and Violet-tailed Sylph (*Aglaiocercus coelestis*). The presence of HF vocalizations in hummingbirds belonging to different lineages poses the question of whether HF vocalizations in this group of birds have been, so far, overlooked. These Andean species are closely related but live in two different habitats.

2.1   Methods

2.1.1   *Vocalizations*

We collected vocalizations for the four species of hummingbirds **(Figure 4)** during the months of May - June and December - March, in 2015 and 2016 in Ecuador. To record HF vocalizations from *O. chimborazo,* we first identified preferred perches of individuals defending patches of *Chuquiraga jussieui*, a flowering shrub preferred by *O. chimbrazo* as a source of nectar. For recordings, we used a TASCAM DR-40 recorder (TEAC America Inc., CA, USA) at a sampling rate of 96,0000 Hz and a mono parabolic microphone capable of responding to frequencies above 20 kHz, although the best frequency response is between 200 - 20,000 Hz (Wildtronics LLC, OH, USA). Once a perch was identified, we placed the

recorder and parabolic microphone in close vicinity of the perch (1-4 meters

approximately) and recorded indefinitely until we got samples of high-frequency

vocalizations. We repeated this procedure at several patches of *C. jussieui.*at three different

locations in the high-altitude grasslands: the slopes of Rucu Pichincha volcano (-0.169411, -

78.553702), Apagua (-0.969519, -78.929356), and the slopes of Chimborazo volcano (-

1.432457, -78.797489) **(Figure 5)**.
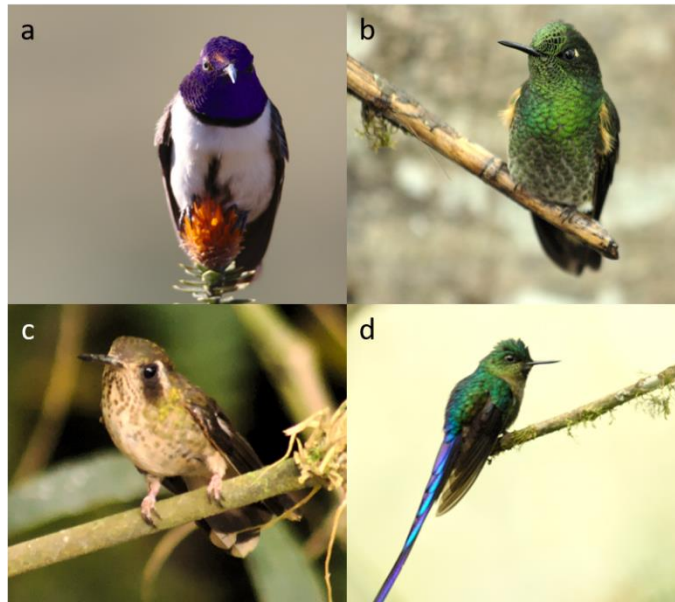
Figure 4. Andean hummingbirds that produce HF vocalizations.
Pictures of **a)** Ecuadorian Hillstar (*O. chimborazo*) living in the high-altitude grasslands, and **b)** Buff-tailed Coronet (*B. flavescens*), **c)** Speckled Hummingbird (*A. melanogenys*), and **d)** Violet-tailed Sylph (*A. coelestis*) living in the cloud forest. Photo credits: All photos by Carlos A. Rodriguez-Saltos.

For *B. flavescens, A. melanogenys,* and *A. coelestis,* we collected recordings in the

cloud forest, at three different locations in the Tandayapa Valley: Tandayapa (0.005160, -

78.678059), Bellavista (-0.001406, -78.685397), and Quinde Luna (0.038916, -78.694676)

(Figure 5). We identified individuals close to feeders and their favorite perches. We placed

the recorder and parabolic microphone near the perches (1-4 m.) and recorded indefinitely

until we collected samples of HF vocalizations.
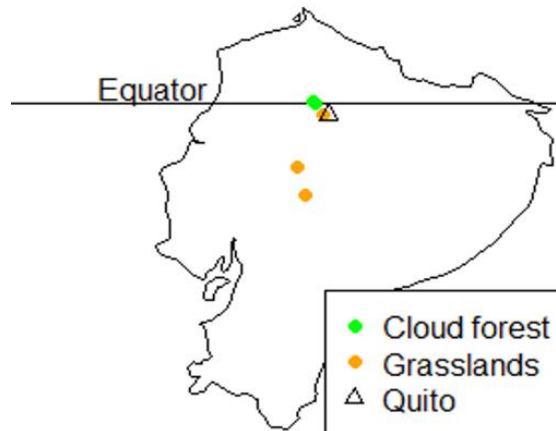
Figure 5. Location of field sites.
Map of Ecuador showing the locations where this study was conducted. Locations in the
cloud forest are shown by one green dot, because they are concentrated in the Tandayapa valley, on
the Northwestern slopes of the Ecuadorian Andes. Quito is the capital city of Ecuador.

2.1.2   Sound analysis

HF vocalizations were analyzed using the *seewave* (Sueur et al., 2008) package in R

(R Core Team, 2013). Recordings were band-pass filtered to delete ambient noise and

artifacts (7–30 kHz for *O. chimborazo* and *B. flavescens*; 9–30 kHz for *A. coelestis* and *A.*

melanogenys). Spectrograms were generated using a window size of 512 samples. **Figure 6**

shows a sample of an unfiltered recording of a HF vocalization.

We collected measures of fundamental frequency (F_0), and dominant frequency. We

report F_0 for those recordings with high enough quality to contain harmonics. For all

recordings, we report mean dominant frequency, which corresponds to the F_0 in recordings

containing harmonics. We analyzed a total of 136 recordings of HF vocalizations, from a

total of 36 individuals from all four species (*O. chimborazo,* n = 10; *B. flavescens,* n = 9; *A. melanogenys,* n = 8; *A. coelestis,* n = 9).
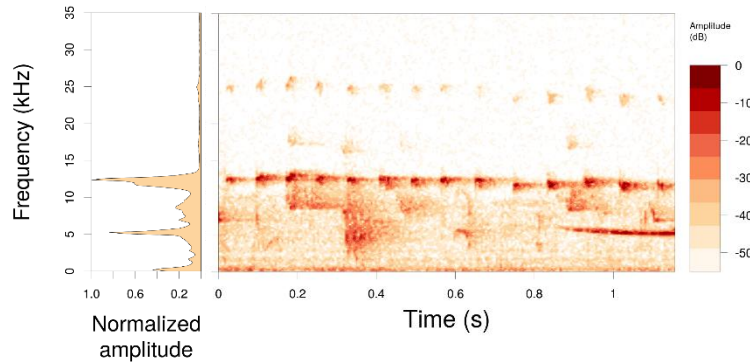
Figure 6. Unfiltered recording of HF vocalization
Power spectrum and spectrogram of high-frequency vocalization of *A. melanogenys* shown in Figure 1C without a bandpass filter. The spectrogram shows other species vocalizing concurrently at lower frequencies.

2.1.3   Courtship display

During the Summers 2018 and 2019, we observed the social interactions of males and females particularly at the females' territories at Chimborazo during the breeding season. Normally, such interactions involve aggressive behaviors of the females directed towards the males. Upon detecting a male intrusion into the shared female territory, or that males are perching at preferred perching sites for females, the latter swiftly chase away males while producing aggressive chasing calls. Nonetheless, during the breeding season, males search and approach females perching, and sing the HF song to them. We identified 7 couples of Ecuadorian Hillstars engaged in courtship behaviors, that is courtship song and hovering and chase-like dance. Additionally, we also observed one more couple in the slopes of Cotopaxi volcano, in a males' territory but close enough to a female territory and nesting site.

2.2   Results

2.2.1   Description of HF vocalizations

O. chimborazo, living in high-altitude grasslands, vocalized at high F_0 (13.4 kHz). These vocalizations were complex and composed of three different phrases. The first two were shorter, rich in frequency modulation, and within a narrow frequency range (13.3–16.4 kHz), while the third phrase was composed of several fast trills between with energy concentrated between 10–16 kHz, short intervals, and one note dropping to 7 kHz (**Figure 7A**). The first harmonic of several elements in the song were in the ultrasonic range (~28 kHz). Hillstars produced this vocalization while perched atop *Chuquiraga jussieui* flowers. To our knowledge, this is the first report of HF vocalizations in this species and the highest fundamental frequency in a bird vocalization known to this date.

B. flavescens, *A. melanogenys*, and *A. coelestis* live in the cloud forest and also vocalize at high F_0 (9.701 kHz, 11.697 kHz, and 11.848 kHz, respectively) (**Figure 7B – D**). These vocalizations were simple, composed of one syllable with little frequency modulation. The hummingbirds produced these calls while perched. *B. flavescens* and *A. melanogenys* vocalized repeatedly throughout the day, and each bout could last for several minutes. Considering the rate at which these hummingbirds vocalized, these calls may serve as territorial advertisements; however, no systematic study has been conducted to confirm their behavioral relevance or responses induced in conspecifics.
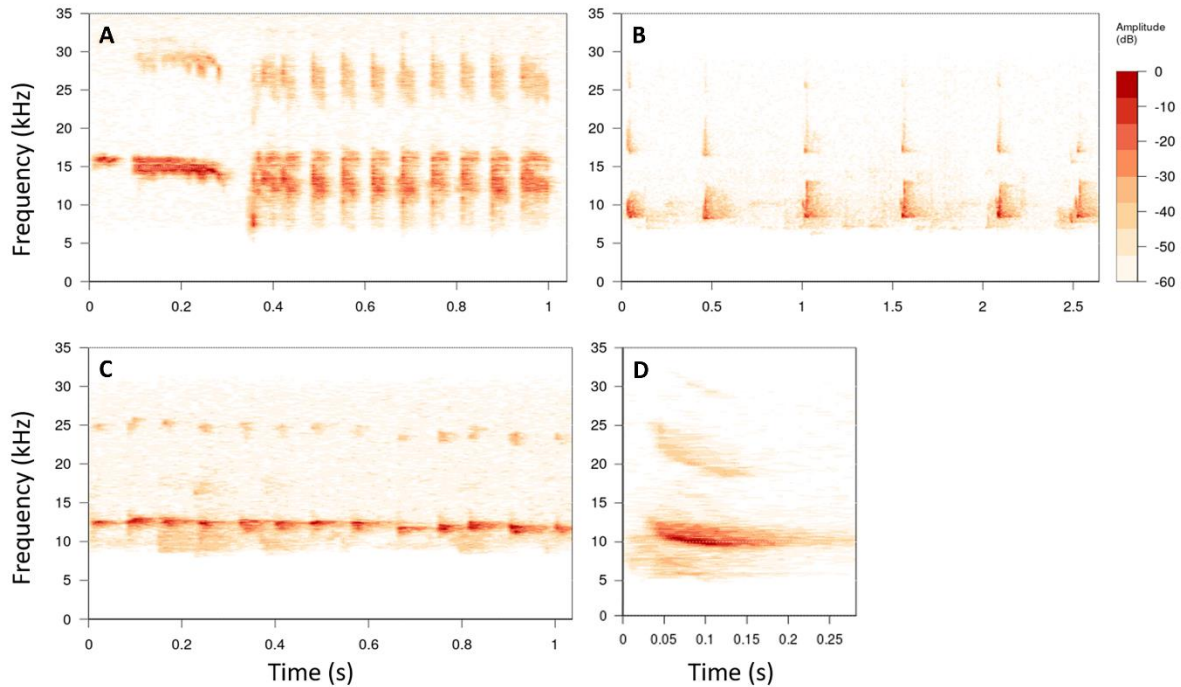
Figure 7. Spectrograms of HF vocalizations.
Each spectrogram shows vocalizations with fundamental frequency and one harmonic. **A)** *O. chimborazo* (F_0=13.4 kHz, SEM=0.470; Dominant freq.=12.316 kHz, SEM = 0.185), **B)** *B. flavescens* (F_0=9.701 kHz, SEM=0.041; Dominant freq.=10.041 kHz, SEM=0.191), **C)** *A. melanogenys* (F_0=11.697 kHz, SEM=0.336; Dominant freq.=11.431 kHz, SEM=0.202), **D)** *A. coelestis* (F_0=11.848 kHz, SEM=0.470; Dominant freq.=11.530 kHz, SEM=0.191).

Among the four species that produce HF vocalizations, *B. flavescens* shows the greatest variation among the four species; we have observed variation within and between individuals. Vocalizations in the other three species show less variation in dominant frequency, which rarely falls below 10 kHz. Variation within *O. chimborazo* may be due to differences between the subspecies *O. c. jamesonii* and *O. c. chimborazo* **(Figure 8)**.
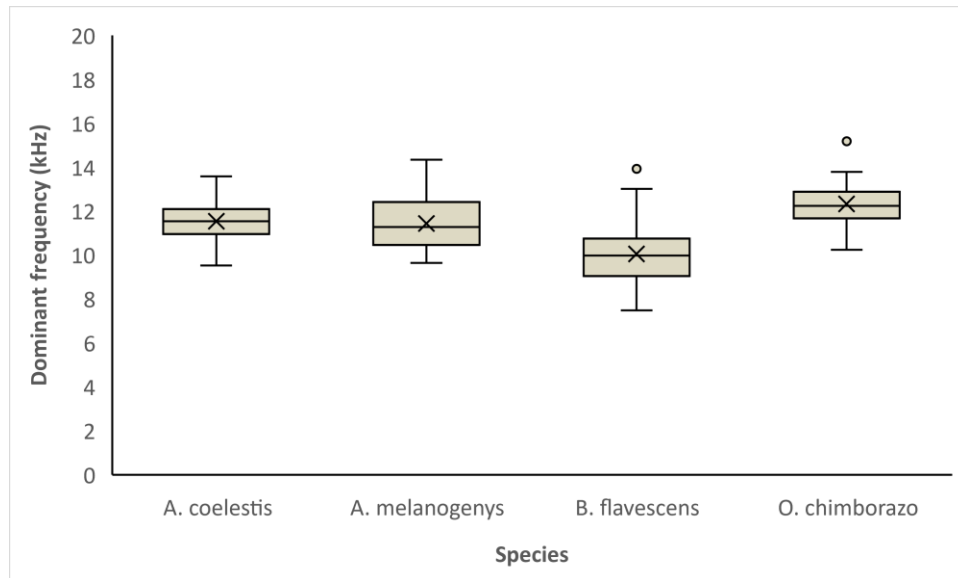
Figure 8. Variation in the mean dominant frequency of HF vocalizations
Dominant frequency of *A. coelestis* (11.530 kHz, ± 0.191; mean ± SEM), *A. melanogenys* (11.431 kHz, ± 0.202), *B. flavescens* (10.041 kHz, ± 0.191) and *O. chimborazo* (12.316 kHz, ± 0.185).

2.2.2 Courtship displays in the Ecuadorian Hillstar (O. chimborazo)

O. chimborazo produces the most complex HF vocalization among the species of hummingbirds producing these extraordinary signals (Duque et al., 2018). This stereotyped song, which only males produce, consists of introductory motifs followed by trills (**Figure 7A**). Males change their behavior depending on the social context in which they produce the song. If a male is vocalizing in its own territory, it usually perches at a preferred high branch while patrolling the site, suggesting that in this context the HF song is used as a territorial signal (Duque et al., 2018). In addition, we have observed that when a male visits a female's territory, he sings the HF song while displaying iridescent feathers from his hood to the female. While singing, the throat inflates generating a wave-like motion on the iridescent feathers (**Figure 9**). If the female is interested, both hummingbirds engage in a dance-like chase, in which the female briefly chases the male

and vice versa. This behavior suggests that the Ecuadorian Hillstar uses its HF song as a

courtship signal, in addition to its previously suspected territorial function.

Figure 9. Ecuadorian Hillstar male singing its HF song
Pictures show how **A)** feathers on the cheeks flare while singing, and **B)** the throat inflates eliciting waves of iridescent feathers moving along the purple hood. Photo credits: Fernanda G. Duque.

2.3   Discussion

These results show that high-frequency vocalizations are more widespread in

hummingbirds than previously thought. Furthermore, the radical difference in structure of

high-frequency vocalizations among these four species suggests that they are exposed to

additional selection pressures in their respective habitats. Studies in hummingbirds

producing high-frequency vocalizations like the ones reported here will be key to elucidate

auditory adaptations that allow the use of these vocalizations in conspecific

communication. Similar studies have been successfully carried out in some anurans (Arch et al., 2009; Feng et al., 2006).

So far, there is no evidence that hummingbirds can hear frequencies in the range of the vocalizations reported here; although, it would be very surprising that these species produce conspicuous calls that they cannot hear. A study on the Blue-throated Hummingbird (*Lampornis clemenciae*), which exhibits high-frequency and ultrasonic components in its song, failed to demonstrate that these birds could hear these frequencies (Pytte et al., 2004). Interestingly, the best frequency sensitivity in this hummingbird matched some of the low-frequency components of the song, suggesting that vocal production is still consistent with hearing in *L. clemenciae*. Additional studies are also necessary to understand the biological relevance of high-frequency vocalizations in hummingbirds. Andean hummingbirds provide a rich model to study the evolution of high-frequency vocalizations in hummingbirds because they share a recent evolutionary history, coinciding with the geologically recent uplift of the Andes (McGuire et al., 2014). This condition is ideal for comparative studies in search of the ecological factors explaining the use of high-frequency vocalizations as communication signals in hummingbirds.

Statement of Publication: The information, text, and figures in this chapter have already been published as part of two manuscripts. All information pertaining to the initial description of HF vocalizations in four species of Andean hummingbirds was part of a manuscript published in 2018 in the journal *Current Biology* (Duque et al., 2018) (DOI: 10.1016/j.cub.2018.07.058)*.* All information regarding the use of HF vocalizations in courtship in the Ecuadorian Hillstar was published in a manuscript in 2020 in the journal

Science Advances (Duque et al., 2020) (DOI: 10.1126/sciadv.abb9393)*.* In both articles, I am the first and corresponding author. I was involved in the conceptualization, methodology, data collection and analysis in both manuscripts. I also wrote the initial drafts and led the revision of new versions of the manuscripts until their publication.

3   DIALECTS IN THE HIGH-FREQUENCY SONG OF THE ECUADORIAN HILLSTAR

As a critical component of the social life in many animals, vocal communication offers fertile grounds to investigate the evolution and diversification of acoustic social signals. Vocal signals are particularly interesting because they can diversify in a relatively short evolutionary time, providing opportunities to study the selection pressures and mechanisms that lead to signal diversity (Edwards et al., 2005). Moreover, the diversification of vocal signals in a species can potentially contribute to the generation of premating barriers via sexual selection on local songs (Irwin et al., 2001, 2008; Marler & Tamura, 1962; Nottebohm, 1969).

Song variants, often referred to as dialects (Marler & Tamura, 1962), can be produced due to different factors or a combination of factors. Random genetic variation, also known as genetic drift, can produce variation in behavioral phenotypes, including acoustic signals (Baker et al., 2006; García et al., 2018). Variation can also arise due to specific ecological pressures in different geographic regions that favor some song features over others (Morton, 1975; Nottebohm, 1975; Ryan, Cocroft, et al., 1990). Finally, sexual selection, via female preference, can favor some song characteristics in different populations, resulting in the establishment of song variants (Capranica et al., 1973; Catchpole, 1987; Nowicki & Searcy, 2004; Ryan, Fox, et al., 1990).

Geographic variation of song can occur at micro and macro levels. Microgeographic variation in songs involves song variants existing in small areas, called neighborhoods, within a population (Catchpole & Slater, 2008; Price, 2008). In this context, individuals from one neighborhood can interact with those of nearby neighborhoods, and sometimes even switch neighborhoods as adults (Araya-Salas et al., 2019; Baptista & Schuchmann,

1990; González & Ornelas, 2009, 2014). On the other hand, macrogeographic variation of

song is found across populations of the same species. Usually, these populations span

larger distances and may encounter geographic barriers that separate one population from

another (Catchpole & Slater, 2008; Price, 2008; Wright et al., 2005). Geographic variation of

song is often associated with learning of vocal production (Baptista & Schuchmann, 1990;

Gaunt et al., 1994; Marler & Tamura, 1962), and is more common among animals exhibiting

this ability. Nonetheless, it has also been observed in diverse vertebrate groups such as

anurans, and mice, which do not exhibit vocal learning (Campbell et al., 2010; Capranica et

al., 1973).

Hummingbirds constitute one of three groups of birds in which vocal learning has

evolved (Nowicki & Searcy, 2004). Song variation among neighborhoods of the same

species were key to identify this ability in hummingbirds (Baptista & Schuchmann, 1990).

The Ecuadorian Hillstar (*Oreotrochilus chimborazo*) is a hummingbird species in which

males produce a complex high-frequency (HF) courtship song, with introductory notes and

a series of trills (Duque et al., 2018). Males vocalize while hovering in front of the females,

displaying and swaying their tail feathers. Singing the HF song produces a wave-like

movement of the head and throat feathers that enhances the iridescent colors of the male's

hood plumage (Duque et al., 2020).

There are two Ecuadorian Hillstar subspecies, *O. c. jamesonii* (Jardine, 1849), widely

distributed along most of the high-altitude grasslands or paramos of Ecuador and the most

southern paramos in Colombia (Rodríguez Saltos & Bonaccorso, 2016; Woods et al., 1998).

The subspecies *O. c. chimborazo* (Delattre & Bourcier, 1846), in contrast, is restricted to the

foothills of Chimborazo volcano and its surrounding areas. A turquoise patch on the male

gorget is distinctive in the subspecies *chimborazo* and absent in *jamesonii* males.

Ecological niche modeling has shown the presence of a potential contact zone

between some populations of the subspecies *jamesonii* in the North and *chimborazo*

(Bonaccorso et al., 2021). Meanwhile in the South, models show the existence of geographic

barriers isolating the *jamesonii* populations in Cajas. In addition, analysis of mitochondrial

DNA did not yield any significant genetic differences among populations of the two

subspecies (Rodríguez Saltos & Bonaccorso, 2016). However, a recent analysis of

microsatellite loci revealed greater population structure in the species than previously

thought (Bonaccorso et al., 2021), highlighting the distinction between the subspecies

jamesonii and *chimborazo* and unveiling differences between populations of the subspecies

jamesonii in the northern and southern ranges of the distribution.

In this study, we investigated the variation in the HF song of the Ecuadorian Hillstar

to determine whether there are dialects in the courtship signal of this hummingbird. We

predicted that there will be dialects characteristic of each subspecies. Given the ability of

hummingbirds to learn vocalizations, it is also possible that there are distinctive song

variants across populations of the species regardless of subspecies.

3.1   Methods

3.1.1   Field sites

We recorded vocalizations along the full distribution range of the species from

northern Ecuador to the most southern location, following sites reported (Rodríguez Saltos

& Bonaccorso, 2016) where genetic sampling had been conducted in the past. In this

species, males and females hold separate territories (Carpenter, 1976; Duque et al., 2020),

and females tend to live at lower elevations near creeks, while males forage at higher

elevations. Therefore, female territories are mostly located between 3600 and 3900 meters

above sea level (m.a.s.l.) (11811 to 12795 ft), and male Ecuadorian Hillstars can be found at

higher elevations, approximately at 4200 m.a.s.l. (13779 ft) (F.G. Duque, Personal

observations). *Chuquiraga jussieui* flowers are the main source of nectar in this species

(Smith, 1969). Therefore, at each field site, we searched for patches of *Chuquiraga j.* flowers

at those elevations, focusing mostly on male territories. Within each field site, we moved to

different locations to record from multiple individuals. **Table 1** shows field sites where we

sampled vocalizations and the final sample size for each site. In the most northern

population, El Angel in Carchi, we only collected one poor-quality recording; thus, we did

not include this vocalization in our analysis. In Culebrillas (Parque Nacional Sangay), we

only recorded six HF vocalizations in total from two different males. We confirmed that

both males produced the HF song more than once, suggesting that the recorded songs are

stereotypical of this population. Thus, we included these recordings despite the small

sample size.

Table 1. Sample size per field site where we recorded HF vocalizations from Ecuadorian Hillstar males.
*This recording was not included in any analysis.

Field site	N
El Angel - Carchi	1*
Cotacachi	37
Antisana	9
Apagua	113
Cotopaxi	10
Chimborazo	22
Culebrillas - P.N. Sangay	6
Soldados - Cajas	69
TOTAL	**266**

3.1.2 Vocalizations

We recorded vocalizations of the Ecuadorian Hillstar throughout the full distribution range of the species in the months of May to July and November to January from 2015 to 2019. We placed either a TASCAM DR-40 recorder (TEAC American Inc., CA, USA) or a SM4 recorder (Wildlife acoustics Inc., MA, USA) among *Chuquiraga j.* bushes, from which the hummingbirds feed. Recorders were set up at a 96000 kHz sampling rate. We recorded at high peaks of activity (06h00 to 07h30; 10h30 to 12h00; 16h30 to 18h00) based on our observations of the species' behavior. HF vocalizations are produced only by males; thus, even HF songs recorded in a females' territory were traced back to a male visiting the site.

3.1.3 Sample selection

Recordings were visually inspected using *Audacity 2.3.3* (Audacity Team, 2019), to select HF vocalizations that followed two criteria: 1) there was no ambient noise interfering with the notes in the song, and 2) each vocalization had at least introductory notes and some trills. Sometimes, male Ecuadorian Hillstars produced only the introductory notes of the HF song, or they stopped after a few trills. This was common at the beginning of a singing bout or when the singing hummingbird was distracted by an intruder. Then, the territorial male stopped singing the HF song and instead produced chasing calls from its perch or actively chased away the intruder. Consequently, we did not include recordings in which males only produced introductory notes in our sample vocalizations. After selecting vocalizations, we applied a bandpass filter between 7-17 kHz, and normalized recordings using *Audacity 2.3.3* (Audacity Team, 2019) (See **Table 1** for sample size in each field site).

3.1.4 Acoustic analysis

3.1.4.1 Song variants, song elements, and duration

In this study, we used single-vocalization recordings. While selecting our samples, we used visual inspection to group together vocalizations that share a common structure despite the population where they were recorded. We also manually counted the number of trills and the total number of elements or notes in the song.

Recordings were analyzed using customized code in *R* (R Core Team, 2020)**.** Since the total number of elements and total number of trills are correlated, we only present results on the total number of elements of the song (but see **Table 2** for summary data on trills). We conducted a Kruskal Wallis analysis and pairwise comparisons with Bonferroni correction to determine whether the total number of elements in the HF song of each population differs from those of other populations. Furthermore, we used the function *specan* in the package *warbleR* (Araya-Salas & Smith-Vidaurre, 2017) to obtain several acoustic parameters in each vocalization including duration of each single-vocalization recording. Then, we conducted a Kruskal Wallis analysis and pairwise comparisons with Bonferroni correction to evaluate differences in song duration among variants of the HF song.

3.1.4.2 Trajectories of dominant frequency (kHz)

To obtain the trajectories of dominant frequency in each recording, we wrote additional customized code in *R* (R Core Team, 2020). Using the packages *tuneR* (Ligges et al., 2018)*,* and *seeWave* (Sueur et al., 2008)*,* we obtained the dominant frequency at different time points throughout each song. Briefly, we set up a 12% amplitude threshold for detection, 512 window size, bandpass filter of 7 to 20 kHz, and a maximum number of

detections of 20 per recording. Then, we plotted these trajectories using *ggplot2 version 3.3.0* (Wickham, 2016).

3.1.4.3   Principal Coordinate Analysis (PCoA)

In order to evaluate dissimilarities in the HF song of the Ecuadorian Hillstar among several populations, we conducted a Principal Coordinates Analysis (PCoA) (Araya-Salas et al., 2019). We first created a spectral correlation matrix, comparing spectrograms of all vocalizations in our sample against one another (n = 266 recordings) (**Table 1**) using the *batch correlator* in *Raven Pro 1.6.1* (Center for Conservation Bioacoustics, 2019). Using *R,* we transformed the correlation matrix into a distance matrix by subtracting all values from 1 (Araya-Salas et al., 2019). We then measured the dissimilarities among HF song using the *cmdscale* function to obtain coordinates for each vocalization in our sample. Finally, we plotted coordinates using *gglplot 2 v.3.3.0* (Wickham, 2016)*.* We also conducted this analysis in a subset of recordings (n = 79 recordings) in which we only analyzed dissimilarities among the introductory motifs of the HF song.

3.2   Results

3.2.1   Variants of the HF song

After collecting recordings of the HF song of the Ecuadorian Hillstar along the Ecuadorian Andes, we found four song variants or dialects in this species: *Jamesonii north*, which includes populations of the subspecies *O. c. jamesonii* located north of Chimborazo volcano and its surroundings. The second group, *chimborazo,* corresponds to the subspecies *O. c. chimborazo*, located on the outskirts of the volcano. South and east of Chimborazo, there are other populations of the subspecies *O. c. jamesonii*, as in the North. However, unlike their northern counterparts, these populations do not share a common

song variant between them or with the populations in the North. Thus, we classified them

as separate dialects, based on the location where the samples were collected: *Culebrillas* for

the populations in Parque Nacional Sangay, and *Cajas* for the most southern population.

Figure 10 shows sample vocalizations from populations along the distribution range in the

Ecuadorian Andes.
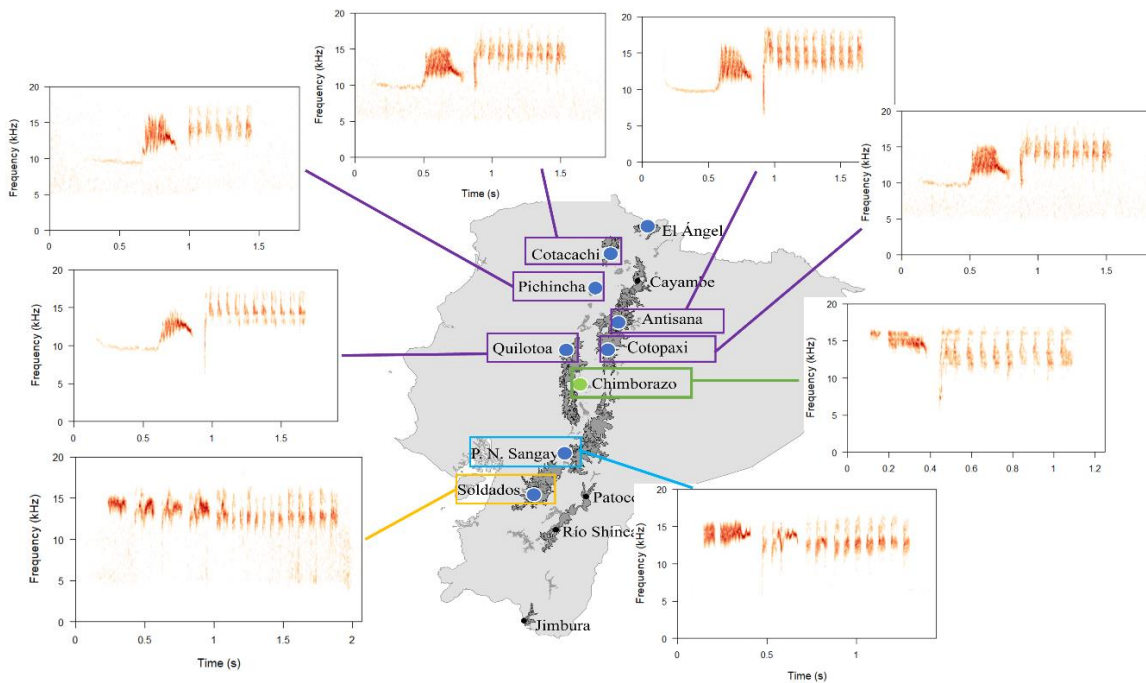
Figure 10. Sample HF vocalizations at each field site.
Map of Ecuador showing field sites where HF vocalizations of the Ecuadorian Hillstar were
recorded. Blue dots show populations of the subspecies O. c. jamesonii and the green dot shows the
population of the subspecies O. c. chimborazo. Populations that share a variant of the HF song are
grouped by colored boxes: purple, Jamesonii North; green, Chimborazo; blue teal, Culebrillas (P.N.
Sangay); yellow, Cajas (Soldados).

The HF song of the Ecuadorian Hillstar consists of two parts: introductory notes and

trills (**Figure 11**). There are substantial differences in the introductory motifs of the HF

song among the variants. The *Jamesonii north* dialect features an introductory whistle

starting at 10 kHz and a frequency-modulated element at higher frequencies; so far, this is

the only dialect with an introductory whistle. In contrast, the *Chimborazo* and *Culebrillas*

dialects show two frequency-modulated elements at the beginning, produced at higher

frequencies than the introductory motif in the northern dialect. In both cases, the first

element is shorter than the second. Although the introductory notes of *Chimborazo* and

Culebrillas are not equal, they do resemble one another more than any of the introductory

notes in other dialects. Meanwhile, the most southern variant, *Cajas,* exhibits four

frequency-modulated elements in the introduction. Thus, this is the dialect with the highest

number of introductory elements in the HF song compared to the other three, which

exhibit only two. In addition, there were also differences in the total number of trills

produced in each song, so that each dialect has a different maximum number of trills

(**Table 2**). However, there was a lot of variation in the number of trills produced from

rendition to rendition by most individuals recorded regardless of location.
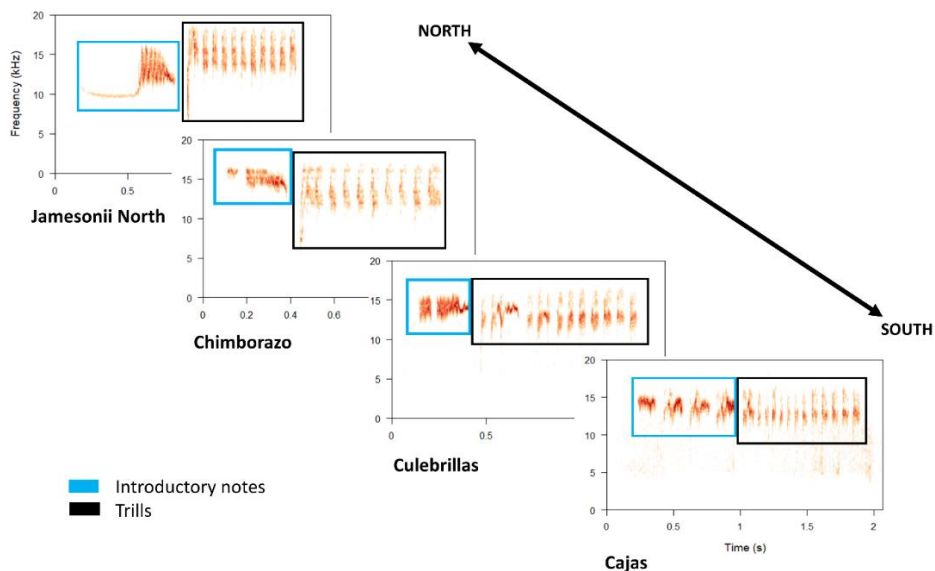
Figure 11. Variants of the HF song.
Dialects are arranged by geographic distribution of sampled populations along the
Ecuadorian Andes. Squares show the introductory notes (blue) and trills (black) in each variant.

Table 2. Summary of features in the dialects of the HF song of the Ecuadorian Hillstar.

Dialect	Number of elements		Number of trills		Duration (s)	
	Median	Max	Median	Max	Median	Max
Jamesonii North	13	15	11	13	1.6464	3.0842
Chimborazo	13	14	11	12	0.9945	1.1269
Culebrillas	17	18	15	16	1.2591	1.3645
Cajas	18	24	14	20	1.8365	2.3896

3.2.2   Differences in song elements and song duration

We found statistically significant differences in the total number of elements among the song variants ($X^2_{(3)}$ = 104.46, p < 0.001; Kruskal-Wallis test). The dialects *Jamesonii north* and *Chimborazo* have lower number of elements in the song compared to *Cullebrillas* (p = 0.017) and *Cajas* (p < 0.001) in the South. There was no statistically significant difference between *Jamesonii north* and *Chimborazo* (p = 0.098) or between the southern dialects (p = 0.069) despite a trend in the latter (**Figure 12A**). **Table 2** shows the median and maximum number of elements in each variant of the HF song.

Likewise, the analysis of song duration also showed statistically significant differences among the four dialects ($X^2_{(3)}$ = 107.78, p < 0.001; Kruskal-Wallis Test). We found that the *Jamesonii north* song was consistently longer than that from *Chimborazo* and *Culebrillas* despite having equal or lower number of elements, respectively (p<0.001, for both comparisons) (**Figure 12B**). This condition is likely due to the long introductory whistle in the dialect of the North compared to the shorter introductory elements in the other two variants. The *Cajas* dialect, which exhibits the highest number of introductory elements and trills, is also the longest (p < 0.001 for all comparisons) (See **Table 2** for median and maximum duration of song variants).
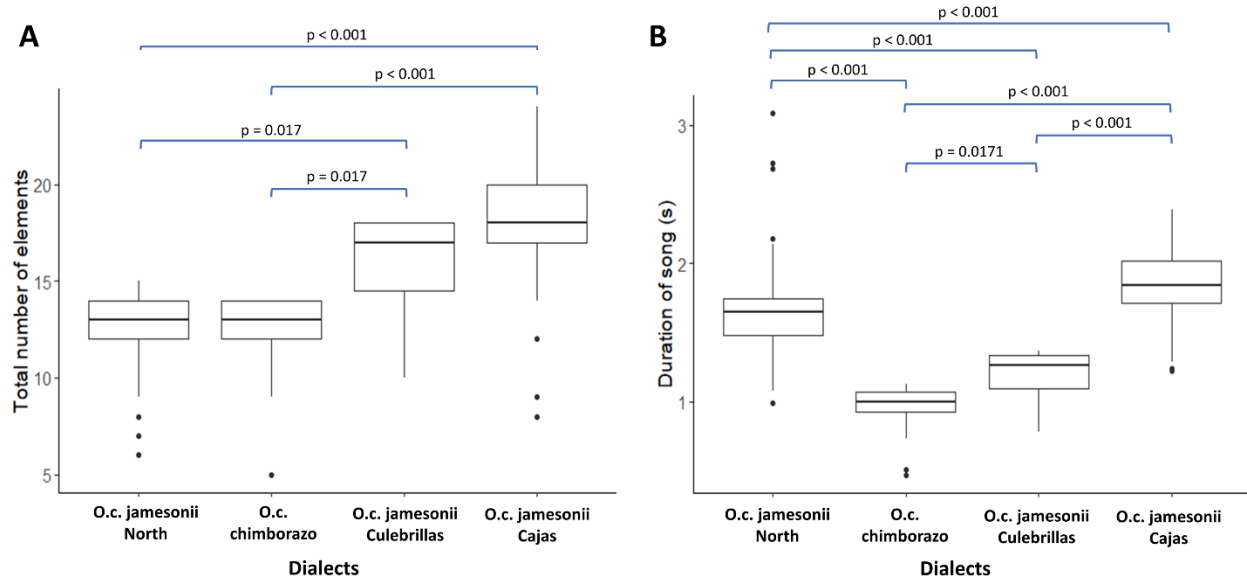
Figure 12. Number of elements and song duration in the HF song dialects.
Variation in **A)** the total number of elements, and in **B)** song duration among the four variants of the HF song. Only statistically significant comparisons are shown here.

3.2.3   Diverse trajectories of dominant frequency

We found that each dialect of the HF song in the Ecuadorian Hillstar follows a unique trajectory of dominant frequency (kHz) (**Figure 13**). Individuals singing the *Jamesonii north* dialect start vocalizing at a lower frequency that corresponds to the introductory whistle, and then gradually emphasize higher frequencies as the song progresses. Toward the end of the song, the dominant frequency is lower compared to the peak frequency in the middle of the song, generating an inverted U-shaped trajectory for this variant. In contrast, the three other dialects start with a similar dominant frequency at the beginning, although higher than that in the northern dialect. After a few notes, however, each dialect follows its own unique trajectory.
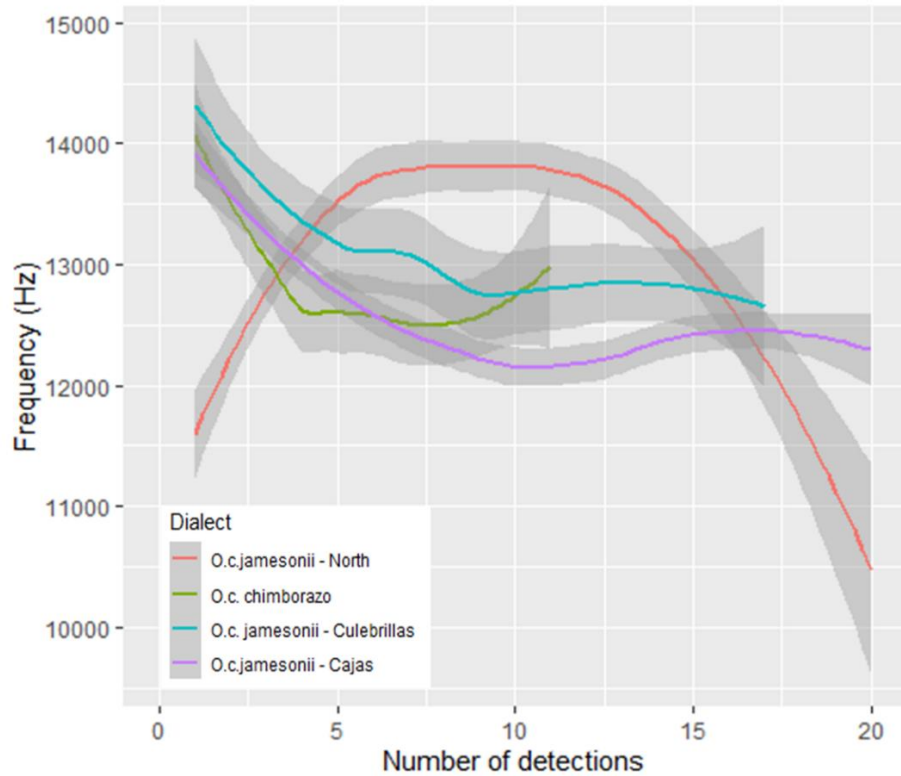
Figure 13. Trajectories of dominant frequency of each dialect.
Number of detections refers to the total sampled points in each song variant to obtain its corresponding dominant frequency trajectory; this may vary depending on the number of elements in the song.

3.2.4 Dissimilarities among variants of the HF song

We used Principal Coordinates Analysis (PCoA) to identify dissimilarities among the full songs and the introductory notes in the HF song of the Ecuadorian Hillstar. The full song analysis showed that songs from populations corresponding to the dialect *Jamesonii north* cluster together demonstrating that they are different from the other three dialects (**Figure 14A**). These results also show that the dialect of *Jamesonii north* is the most different variant of the HF song compared to the other three, which share more spectral features.

The same analysis conducted only on the introductory notes of the songs showed that dissimilarities between the northern dialect and the other three variants are more accentuated. Although there is less differentiation among the dialects *Chimborazo, Culebrillas*, and *Cajas* compared to *Jamesonii north*, the first two group closer together compared to songs from *Cajas* (**Figure 14B**). This result emphasizes the fact that the introductory notes in the *Chimborazo* and *Culebrillas* dialects resemble each other more closely than to those in other dialects of the HF song.
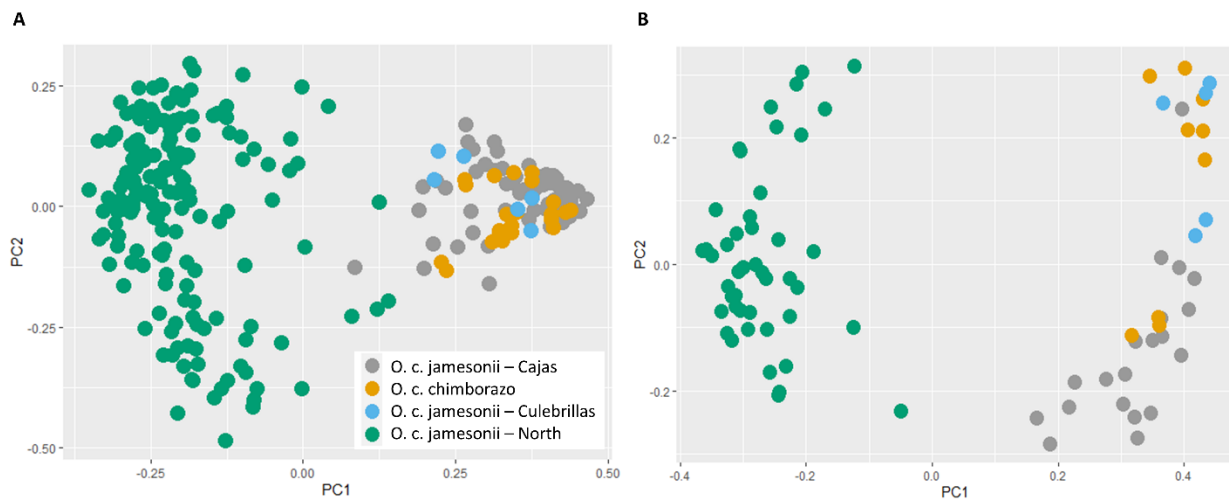
Figure 14. Principal Coordinate analysis of dialects of HF song.
Analysis conducted on **A)** the full HF song (n = 266 recordings), and **B)** the introductory notes of a subset of recordings (n = 79 recordings).

3.3 Discussion

Altogether, our results showed that there are four dialects in the HF song of the Ecuadorian Hillstar. The most common dialect, *Jamesonii north*, comprises most of the populations of the subspecies *O. c. jamesonii*, while the southern populations of the same subspecies, *Culebrillas* and *Cajas*, have their own variants of the song. Meanwhile, the subspecies *O. c. chimborazo* exhibits its own dialect of the HF song. We observed significant

differences in the total number of elements of the song as well as differences in note

structure, especially in the introductory notes. We also found differences in the trajectories

of the dominant frequencies, so that individuals broadcasting a specific dialect emphasize

different frequencies throughout the song compared to other males singing other variants.

This result is particularly interesting because all variants of the HF song are produced

within the same frequency range, suggesting that there are strong ecological pressures to

produce these signals at high frequencies (Bradbury & Vehrencamp, 2011; Duque et al.,

2018, 2020). Thus, differences in the dominant frequencies that males in each population

emphasize paired with other structural differences in the song can be key for

discrimination of local vs foreign dialects.

Studies of song discrimination in birds have shown that females commonly prefer

the local dialect over foreign signals (Danner et al., 2011; Maney et al., 2003; O'Loghlen &

Rothstein, 2003). These results suggest that dialects can be effective at creating premating

isolating barriers via sexual selection. Meanwhile, males respond more aggressively to

playback of their own dialect or a variant from neighboring populations than to the

playback of a song from distant populations (Ratcliffe & Grant, 1985; Tomback et al., 1983;

Wright & Dorin, 2001). Altogether, evidence points to fine-tuned discrimination in vocal

signals in birds.

In the case of the Ecuadorian Hillstar, HF song is used as both, a courtship and

territorial signal (Duque et al., 2020) which can be used by females of different populations

to identify and prefer males broadcasting the local dialect. The lack of fragmentation in

suitable habitat for populations of the subspecies *jamesonii* distributed North West of

Chimborazo, and the subspecies *chimborazo* (Bonaccorso et al., 2021) rules out the

possibility that genetic structure among these populations has arisen from geographic isolation. In this study, we sampled the songs of *jamesonii* males from a site close to the potential contact zone (Apagua) and confirmed that males produce the *Jamesonii north* dialect. Although both, the *Jamesonii north* and *Chimborazo* dialects have the same number of elements, the *Jamesonii north* song is longer due to the introductory whistle that is absent in the *Chimborazo* dialect. The structure of the introductory notes and the trajectories of dominant frequency are significantly different between the two variants. Additionally, a genetic hybrid collected at Culebrillas that exhibited normal *jamesonii* plumage (Bonaccorso et al., 2021) suggests that there is some migration between populations of the subspecies *jamesonii* that exhibit the *Culebrillas* dialect and the subspecies *chimborazo*. The evolution of dialects between these two populations in which migration may still occur emphasizes the potential role of female preference for local variants of the song in maintaining barriers between the two subspecies. Furthermore, the production of HF song enhances the visual effect of the iridescent hood plumage in males during courtship (Duque et al., 2020), probably highlighting differences in plumage coloration among males of the two subspecies. Altogether, sexual selection and female preference for courtship visual and acoustic signals may be responsible for the divergence of the two subspecies and the maintenance of isolating barriers at the contact zone.

Studies evaluating female preference for dialects of the HF song and visual components of the courtship display are necessary across populations and at the contact zone. Such behavioral studies will help determine whether dialects are necessary and sufficient for mate discrimination in the Ecuadorian Hillstar (Kozak & Uetz, 2016; Ryan, 1990; Taylor et al., 2011). Past behavioral studies in the field showed that these

hummingbirds can hear the HF song, but males do not exhibit aggressive responses to the playback (Duque et al., 2020). The lack of bold responses to playback in the Hillstar, like those often observed in other birds (Ratcliffe & Grant, 1985; Tomback et al., 1983), suggests that in this species, acoustic signals need to be paired with a visual stimulus or presented in the appropriate social context to elicit a strong response. The need for conserving energy amid extreme environmental conditions may be a contributing factor, so that hummingbirds only respond robustly to imminent and clearly identifiable competitors.

We also found two dialects in the southern populations of the subspecies *jamesonii*. Acoustic signals can diversify at faster rates than other traits (Price, 2008), and this seems to be the case in the southern populations of the Ecuadorian Hillstar. Recent microsatellite analysis and niche modelling suggest that Hillstars at Cajas and Sangay (*Culebrillas*) have a common genetic structure and are diverging in geographic isolation from their counterparts in the North (Bonaccorso et al., 2021). However, we found two distinct dialects in the southern *jamesonii* populations, which are indistinguishable in the genetic microsatellite analysis. The *Culebrillas* and *Cajas* dialects exhibit higher number of elements in the song, compared to the other two variants. Moreover, the dialect from *Cajas* is the longest considering duration and total number of elements in the song, which features four introductory elements and up to 20 trills. Therefore, in southern populations in which microsatellite analysis fail to identify population structure, dialects have already emerged.

Geographical distance across populations may also play a role in song divergence in the Ecuadorian Hillstar. **Fig. 10** and **11** show the geographic arrangement of the HF song variants showing how the structure in some song elements are similar between pairs of

song variants that are geographically closer. For instance, trills in *Jamesonii north* are more similar to those found in *chimborazo* than to trills in the two southern dialects. Likewise, the structure of trills in the two southern dialects resemble each other more than those of the northern counterparts. Furthermore, the introductory notes in the *Chimborazo* and *Culebrillas* dialects are more similar than those in the other dialects as shown by acoustic dissimilarities (**Fig. 14B**). Finally, the *Jamesonii north* and *Cajas* dialects, the most northern and southern dialects respectively, are the most different in structure, suggesting that geographic distance is also involved in song divergence. Our study did not include vocalizations from Carchi, the most northern population in the distribution of the species. We confirmed that males produce HF songs but were unable to collect recordings of these vocalizations. Ecological niche modeling suggests that this population is isolated from other populations of the subspecies *jamesonii* in the North (Bonaccorso et al., 2021). It remains to be determined whether individuals in Carchi have evolved their own variant of the HF song or share the *Jamesonii north* dialect with other northern populations. Additional studies of migratory patterns in this species are necessary to establish whether habitat fragmentation shown in the models represents a significant isolating barrier.

While geographic distance may be involved in song divergence in the Ecuadorian Hillstar, it is unlikely that differences in elevation or other local environmental factors may be promoting the emergence of dialects in this species. Separate territories for males and females at different elevations suggest that there is altitudinal migration in this species. Dialects have been found in species distributed along an altitudinal gradient (Nottebohm, 1969). However, this pattern is exhibited provided that the altitudinal range is wide enough to encompass different habitats; this is not the case of the Ecuadorian Hillstar.

Furthermore, most of our sample vocalizations were collected at roughly the same elevation in all field sites (~4,2000 m.a.s.l). Male vocalizations collected in females' territories at lower elevations (~3,600 – 3,9000 m.a.s.l.) do not differ from those recorded from individuals in the same populations at higher elevations.

Overall, our analysis of four of the dialects of the HF courtship song of the Ecuadorian Hillstar shows that dialects are evolving faster than the genetic structure of the species. Although, it is unknown whether female preference for local song variants is contributing to the genetic divergence of the species, some conditions suggest that sexual selection is indeed playing a role. Moreover, population structure in this species have emerged in recent times (Bonaccorso et al., 2021), offering an interesting scenario to study the evolution of courtship acoustic signals and female preference in the divergence of a species.

4   ECOLOGICAL FACTORS INFLUENCING THE EVOLUTION OF HIGH-FREQUENCY

VOCALIZATIONS IN HUMMINGBIRDS

Vocal communication constitutes an essential aspect of social interactions across

multiple species (Bradbury & Vehrencamp, 2011). Senders produce vocalizations that

carry information for an intended receiver, and the quality of these signals influences the

behavioral response in that receiver (Naguib & Wiley, 2001; Penna et al., 2017; Pohl et al.,

2009; Sprau et al., 2010; Vignal et al., 2005). Senders are faced with several challenges in

their environment when communicating with conspecifics. Factors such as ambient noise,

vegetation structure, temperature, and humidity affect signal transmission (Boncoraglio &

Saino, 2007; Slabbekoorn, 2004). These environmental factors can limit the reach of a

signal or degrade its structure, thus hindering communication.

Attenuation and reverberation occur during the transmission of a sound signal in

the environment. Attenuation refers to the decrease in sound levels as the sound travels

further away from its source (Catchpole & Slater, 2008; Slabbekoorn, 2004). This natural

phenomenon can be accentuated by features in the environment, originating what is

known as excess attenuation. Reverberation refers to the degradation of a sound as a result

of scattering and echo as sound waves bounce off their surroundings (Catchpole & Slater,

2008; Slabbekoorn, 2004). Degradation resulting from reverberation is more pronounced

in rapidly modulated sounds, such as trills or in sounds with a wide frequency range

(Brumm & Naguib, 2009). In contrast, reverberation can have an enhancing effect in pure

tones with a narrow-frequency range and in long song elements, facilitating their

transmission (Nemeth et al., 2006; Slabbekoorn et al., 2002).

Hence, habitat, including vegetation coverage, contributes selection pressures that shape the evolution of song features to maximize signal transmission and facilitate communication, which is known as the Acoustic Adaptation Hypothesis (AAH) (M. L. Hunter & Krebs, 1979; Morton, 1975; Wiley & Richards, 1978). According to the AAH, it is expected that animals vocalize at lower frequencies in closed habitats such as forests because low-frequency sounds are less attenuated and degraded in these conditions (Barker, 2008; Mathevon et al., 2008). Meanwhile, open habitats are more permissive of signals with higher frequencies, frequency-modulated elements, wider frequency bands, and shorter inter-element intervals in the structure of a song. This effect results from less reflection and absorption of sound in lower vegetation compared to forested environments (Badyaev & Leaf, 1997; Brumm & Naguib, 2009; Morton, 1975).

Multiple studies have confirmed the predictions of the AAH to varying degrees. In the Neotropics, the songs of species living in open habitats are generally produced at higher frequencies compared to songs from species in closed habitats (Morton, 1975). Similar patterns have been observed between subspecies (Ryan, Cocroft, et al., 1990), and even conspecifics living in open vs. closed environments (M. L. Hunter & Krebs, 1979; Nottebohm, 1969). A meta-analysis conducted by Boncoraglio and Saino (2007) showed that habitat structure better predicts the mean dominant frequency of song but not any other acoustic feature. Consequently, the songs of species living in closed habitats have, on average, lower dominant frequencies than the songs of species living in open habitats. In contrast, other studies have found mixed results with regards to these predictions, so that not all vocalizations are adapted to maximize transmission as the AAH proposes (Boncoraglio & Saino, 2007; Penna & Solís, 1998; Röhr & Juncá, 2013). Therefore, habitat

structure affects sound transmission, influencing the evolution of vocal signals, but other

ecological factors can effectively outweigh this effect (Daniel & Blumstein, 1998; Kime et al.,

2000; Penna & Solís, 1998; Slabbekoorn, 2004).

Ambient noise has been widely studied for its effects on sound signals, revealing

that animals adapt their vocalizations to prevent signal masking by background noise in

their environment (Brenowitz, 1982; Brumm & Naguib, 2009; Walters et al., 2019). The

severity of signal masking is associated with the amount of overlap between the frequency

content in an acoustic signal and that of the environmental noise (Brumm & Naguib, 2009).

To deal with this challenge, animals adapt their vocalizations by singing louder, shifting the

frequency of their calls or increasing the rate at which they vocalize (Cynx et al., 1998;

Potash, 1972; Slabbekoorn & den Boer-Visser, 2006; Walters et al., 2019). These strategies

allow an individual to increase the probability that their signal reaches its intended

receiver. Additionally, climate factors also affect the transmission of acoustic signals

(Boncoraglio & Saino, 2007; Snell-Rood, 2012). Wind and thermals, for instance, attenuate

vocalizations while having little effect on spectral features (Boncoraglio & Saino, 2007;

Brumm & Naguib, 2009). High levels of humidity also contribute to more sound absorption

in a frequency-dependent manner, so that this effect is more pronounced in high-frequency

sounds (Snell-Rood, 2012). High humidity also correlates with a decrease in song

bandwidth in some birds (Snell-Rood, 2012).

In addition, morphological constraints for vocal production can also limit the effect

of habitat on vocal signals in a species-specific manner (Blumstein & Turner, 2005; Goutte

et al., 2018; Ryan & Brenowitz, 1985), sometimes exerting stronger pressure on the

evolution of song features than those imposed by habitat structure (Billings, 2018;

Derryberry et al., 2018). Body mass for instance, has been negatively correlated with

fundamental frequency of vocal production (Francis et al., 2011; Ryan & Brenowitz, 1985).

This pattern results from a physical constraint in small animals to produce low-frequency

sounds (Bradbury & Vehrencamp, 2011; Goller & Riede, 2013). Larger animals can

potentially produce low- and high-frequency sounds. However, the poor transmission

properties of HF sounds in some habitats makes it less likely that a large animal vocalizes

at high frequencies unless there is a strong pressure to do so (Bradbury & Vehrencamp,

2011).

    Recently, high-frequency (HF) vocalizations produced by four species of Andean

hummingbirds were described (Duque et al., 2018). These vocalizations are unusual

because their frequency content is beyond the expected vocal and hearing range of most

birds, which tops at 8 kHz (Dooling, 2004). Thus, we define here a HF vocalization as a call

or song with a fundamental frequency above this limit (8 kHz). The presence of these

vocalizations in such small birds poses the question of whether the species that produce HF

vocalizations, do so because they are smaller than most other hummingbirds. However, the

species of Andean hummingbirds producing HF sounds vary substantially in size spanning

from 4.2 g to 8.2 g (Dunning Jr, 2007).

    One species, the Ecuadorian Hillstar *(Oreotrochilus chimborazo)* (Delattre &

Bourcier, 1846) lives in a high-altitude open habitat, characterized by grasslands, strong

winds, low temperatures, and rapidly changing weather conditions throughout the day.

This species produces a song with the highest fundamental frequencies known in any bird

(Duque et al., 2018). Contrastingly, the other three species producing HF vocalizations, the

Buff-tailed Coronet *(Boissonneaua flavescens)* (Loddiges, 1832)*, the* Speckled Hummingbird

(Adelomyia melanogenys) (Fraser, 1840), and the Violet-tailed Sylph *(Aglaiocercus coelestis)*

(Gould, 1861) live in the cloud forest. This forested montane habitat is characterized by

dense vegetation and high humidity, which does not favor the transmission of HF sounds

over long distances (Morton, 1975). Interestingly, the species in the cloud forest produce

calls at lower dominant frequencies and within a more restricted range than the

frequencies in the song of *O. chimborazo* in the grasslands (7-16 kHz) (**Figure 7**).

       These four species of hummingbirds vocalize while perching on preferred branches

and guarding their territories, suggesting that they use these HF signals for territorial

defense (Duque et al., 2018). Moreover, *O. chimborazo* males also produce their HF song

while courting females (Duque et al., 2020). These findings suggest that these

hummingbirds have evolved HF vocalizations to avoid background noise, facilitating signal

transmission and communication with conspecifics (Brenowitz, 1982; Brumm & Zollinger,

2011; Slabbekoorn & den Boer-Visser, 2006; Walters et al., 2019).

       In this study, we investigated the role of body size in vocal production in

hummingbirds to determine whether an acoustic allometry exists in hummingbirds and

whether the species producing HF vocalizations are exceptions to this pattern. We also

studied the profile of ambient noise in the high-altitude grasslands and cloud forest to test

the hypothesis that some species of hummingbirds vocalize at high frequencies to avoid

signal masking in their noisy environment. Finally, we investigated the transmission of HF

vocalizations of *O. chimborazo, B. flavescens,* and *A. melanogenys* in the cloud forest and the

grasslands to determine how far these signals can travel before being completely

attenuated and degraded. We looked at measures of sound attenuation, spectral and

temporal degradation to build a complete profile for signal transmission of these

vocalizations. We hypothesized that each vocalization is adapted for transmission in its native environment. Therefore, we predicted that HF vocalizations will exhibit less attenuation and degradation, thus, reaching further distances in their native habitat compared to transmission in a foreign environment. Alternatively, any of these signals may transmit better in both habitats compared to the others due to loosened constraints in one of the habitats (Ryan, Cocroft, et al., 1990).

4.1   Methods

4.1.1   Hummingbird species and their body mass

To evaluate the potential role of body size in the production of high-frequency sounds in hummingbirds, we studied body mass and vocal production in hummingbird species found in Ecuador (n = 136 species). This sample includes species from all clades in the hummingbird phylogeny (McGuire et al., 2007, 2014).

We obtained the body masses for the species in our sample from the *Handbook of Avian Body Masses* (Dunning Jr, 2007). We determined the average body mass of each species following one of two parameters: 1) if weight (grams) was available for males and females, including sample sizes for each sex, we followed the following formula:

$$Species\ weight\ (gr) = \frac{[(n_{males} * weight_{males}) + (n_{females} * weight_{females})]}{total\ N\_species}$$

2) if no reference to sex and/or sample size were available, but minimum and maximum body mass was provided, we averaged those to obtain a single number for the

species. We discarded all the species for which there was no body mass available (final n =

118 species).

4.1.2   *Vocalizations of hummingbirds and acoustic analysis*

To obtain vocalizations from the species in this study, we collected recordings from

the online database Xeno-Canto (*Xeno-Canto :: Sharing Bird Sounds from around the World*,

n.d.). Using the function *querxc* from the package *warbleR* (Araya-Salas & Smith-Vidaurre,

2017), we downloaded A-rated vocalizations from the species of hummingbirds that occur

in Ecuador (n = 625 recordings in total). To this sample, we added our own recordings of

HF vocalizations from four species of Andean hummingbirds that occur in Ecuador, namely

Adelomyia melanogenys, Aglaiocercus coelestis, Boissonneaua flavescens, and *Oreotrochilus*

chimborazo (Duque et al., 2018)*.*

To analyze these vocalizations, we first applied a bandpass filter (1 to 17 kHz) to all

recordings, using the software *Audacity v. 2.3.3* (Audacity Team, 2019); all the remaining

analysis was conducted in *R* (R Core Team, 2020)*.* Next, we uploaded recordings using the

package *tuneR* (Ligges et al., 2018) and wrote customized code to obtain measures of

fundamental and dominant frequency (kHz) from all recordings, using the package *seewave*

(Sueur et al., 2008)*.* Then, we built a dataset including minimum, maximum, and average

fundamental and dominant frequency for each species of hummingbird in the study.

Finally, we used *ggplot2 v. 3.3.0* (Wickham, 2016) to build a plot showing the frequency

range of vocal production of hummingbirds along the full range of body masses in our

sample.

4.1.3   Relationship between body mass and frequency of vocal production

To determine whether body mass can predict the frequency of vocal production in hummingbirds, we used a phylogenetic generalized linear model. Dr. Jimmy McGuire (UC Berkeley) kindly provided the phylogenetic tree for hummingbirds (McGuire et al., 2014). Using the package *phytools* (Revell, 2012), we pruned the phylogeny according to the species in our dataset. To obtain the phylogenetic regression, we used the *pgls* function with maximum likelihood in *R* (R Core Team, 2020).

4.1.4   Recordings of ambient noise

We collected samples of ambient noise from 2015 to 2017 in the same locations in the grasslands and cloud forest where we recorded high-frequency vocalizations of four species of hummingbirds: *A. melanogenys, A. coelestis , B. flavescens,* and *O. chimborazo* (**Figure 2**). We placed recorders atop perches of *Chuquiraga j.* in the grasslands (1.5 meters above the ground) to simulate the conditions during which *O. chimborazo* produced high-frequency vocalizations. For recordings of ambient noise in the cloud forest, we placed recorders on perches in trees along trails and close to hummingbird feeders (1.5–2 m. above the ground) to obtain a portrait of the conditions of environmental noise to which these species were exposed in their habitat. Samples were collected at different times of the day from 06h00 to 18h20 for several days at each location.

4.1.5   Sound analysis of ambient noise recordings

To analyze ambient noise in each habitat, we analyzed the environmental noise that the species in this study encounter prior to vocalizing. Therefore, we visually evaluated each recording using Audacity to delete any trace of high-frequency vocalizations from the species in the study; all other sounds were preserved. Then, using *seewave* (Sueur et al.,

2008) in R, we built a spectral profile of ambient noise in each habitat by evaluating the

power spectrum (amplitude as a function of frequency (kHz)) of 10-second time bins in

each recording. This allowed us to generate a precise profile of the environmental noise in

the cloud forest and grasslands. We later averaged these results to obtain a final profile of

ambient noise. We analyzed a total of 40 recordings of ambient noise samples in the cloud

forest; in the grasslands, 46 recordings were analyzed.

4.1.6   *Playback design for signal transmission experiments*

We collected recordings of HF vocalizations from *A. melanogenys* and *B. flavescens* in

the cloud forest and from *O. chimborazo* in the high-altitude grasslands in Ecuador during

Summer 2017 (Duque et al., 2018). We identified preferred perches and placed a TASCAM

DR-40 recorder (TEAC American Inc., CA, USA) and a parabolic microphone (Wildtronics

LLC, OH, USA) 1 m away from the perch. The selected vocalizations of each species have a

distinctive dominant frequency (*A. melanogenys* = 12.38 kHz; *B. flavescens* = 8.44 kHz; and

O. chimborazo = 14.44 kHz) (**Figure 15**). First, we applied a bandpass filter (7–20 kHz) to

the selected recordings to remove ambient noise. For the cloud-forest species, playbacks

consisted of a series of 5 bouts of calls, each bout separated by 5-second intervals, which

together made up one block. For the grassland species, a block consisted of single

vocalizations repeated ten times at 0.5-second intervals. Blocks were then repeated several

times at random intervals to build 5-minute sound files for each species using *R* (R Core

Team, 2013). We used these files as playbacks for our experiments.

Each final playback stimulus was normalized using *Audacity version 2.3.2* (Audacity

Team, 2019)*.* We measured baseline sound levels at 1 m in a sound attenuating chamber in

the laboratory using a Sound Pressure Level (SPL) meter Type 2235 (Brüel & Kjaer,

Denmark) with Fast - A weighted configuration. Twenty measurements were obtained for

each playback showing comparable sound levels across playbacks.
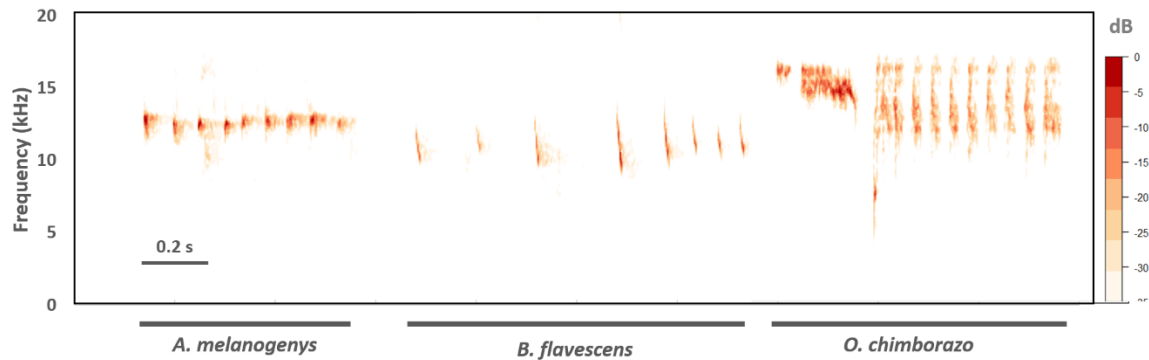
Figure 15. Spectrograms of HF vocalizations to study signal transmission
Vocalizations by *A. melanogenys* and *B. flavescens,* native to the cloud forest, and *O. chimborazo*, native to the high-altitude grasslands. These vocalizations differ in structure and frequency content.

4.1.7   *Study sites for signal transmission*

In order to study the transmission of HF signals, we broadcast playbacks of HF

vocalizations of two species of hummingbirds from the cloud forest and the one from the

high-altitude grasslands in Ecuador in June 2018. Both, *A. melanogenys* and *B. flavescens* are

native to the cloud forest, a tropical moist broadleaf forest (D. M. Olson et al., 2001),

consisting of dense vegetation and rich in animal diversity. These characteristics result in a

noisy environment in which many species can vocalize at the same time. In contrast, *O.

chimborazo*, a high-altitude specialist, lives in the high-altitude grasslands or paramo, an

open habitat dominated by small bushes and grass above the tree line (Peyre et al., 2018).

In this habitat, most of the noise is caused by strong winds (Duque et al., 2018).

In the cloud forest, we conducted signal transmission experiments at Siempre Verde

Natural Preserve in the Intag Valley (00.37558, -78.41856). We selected a specific location

inside the preserve, where we confirmed the presence of *A. melanogenys* and *B. flavescens*.

In the grasslands, we recorded the transmission of HF signals on the slopes of Mt.

Chimborazo, where *O. chimborazo* naturally occurs (-01.52209, -78.83674).

At each field site, we collected general measurements of ambient temperature and

relative humidity during the days of the transmission experiments using a Kestrel D2 DROP

device (Kestrel Instruments, PA, USA) (**Figure 16**).
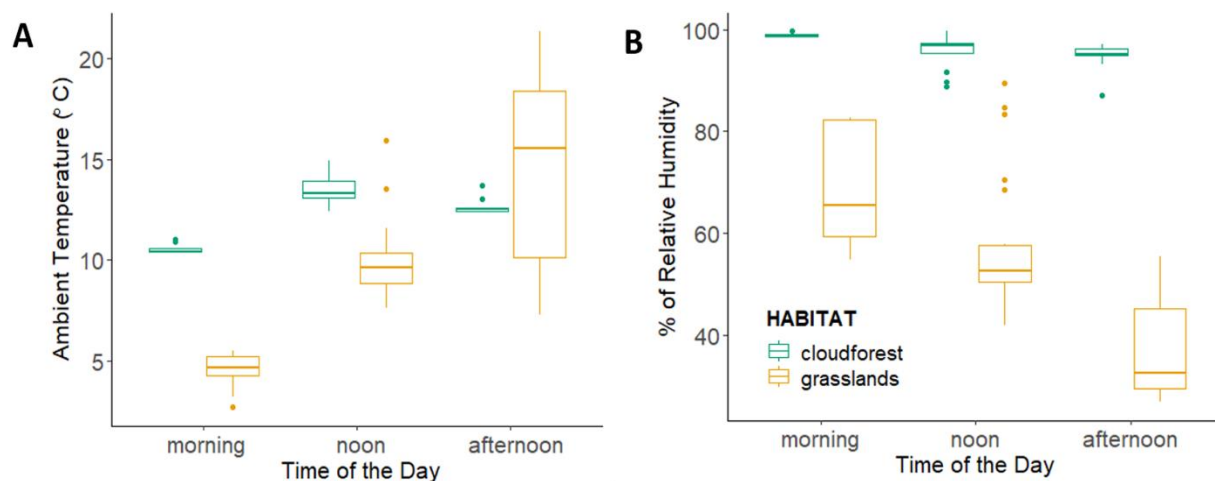
Figure 16. Weather in each habitat.
We show variation in **A)** ambient temperature and **B)** Percentage of relative humidity in the cloud forest and the high-altitude grasslands during the times when we conducted the signal transmission experiments.

4.1.8   *Recordings of signal transmission*

We evaluated the transmission of the HF vocal signals of the three species in both,

the cloud forest and the high-altitude grasslands. A playback speaker (Harman, CT, USA)

was positioned 1.5 m above the ground to simulate natural conditions under which the

hummingbirds vocalize in their habitat (Duque et al., 2018). We conducted experiments

three times per day (morning: 6h00 – 7h30; noon: 11h00 – 12h30; afternoon: 17h00 –

18h15), following natural patterns of peak vocal production for the three species based on

our observations in the field. These times also coincide with peaks of general vocal activity

of birds (Luther & Wiley, 2009), presenting high levels of ambient noise and one of the

most challenging conditions for signal transmission in each habitat (Brumm & Naguib,

2009).

We recorded playback along a transect at 1, 5, 10, 20, and 40 m from the portable

speaker. Multiple playback recordings at each distance were collected at each time of the

day (morning, noon, afternoon) in both habitats. In the cloud forest, we used a trail

traversing the mountain where we placed the speaker and microphone. The cloud forest

presents steep slopes so that on one side of the trail one can find the base of a tree, while on

the other side, one can see the canopy. In the grasslands, we conducted the experiment

along horizontal patches of *Chuquiraga jussieui* plants where we had observed

hummingbirds perching and vocalizing. In both habitats, we confirmed the presence of the

native hummingbird species prior to selecting the specific locations for the transmission

experiments. We made recordings using the built-in microphone of a TASCAM DR-40

recorder (TEAC America Inc., CA, USA) (flat frequency response between 120 Hz – 40 kHz

+1/-3 dB) at a 96000 Hz sampling rate placed at the same height as the speaker (1.5 m

above the ground). **Figure 17** shows samples of transmission recordings for each species

collected at every distance in both habitats.
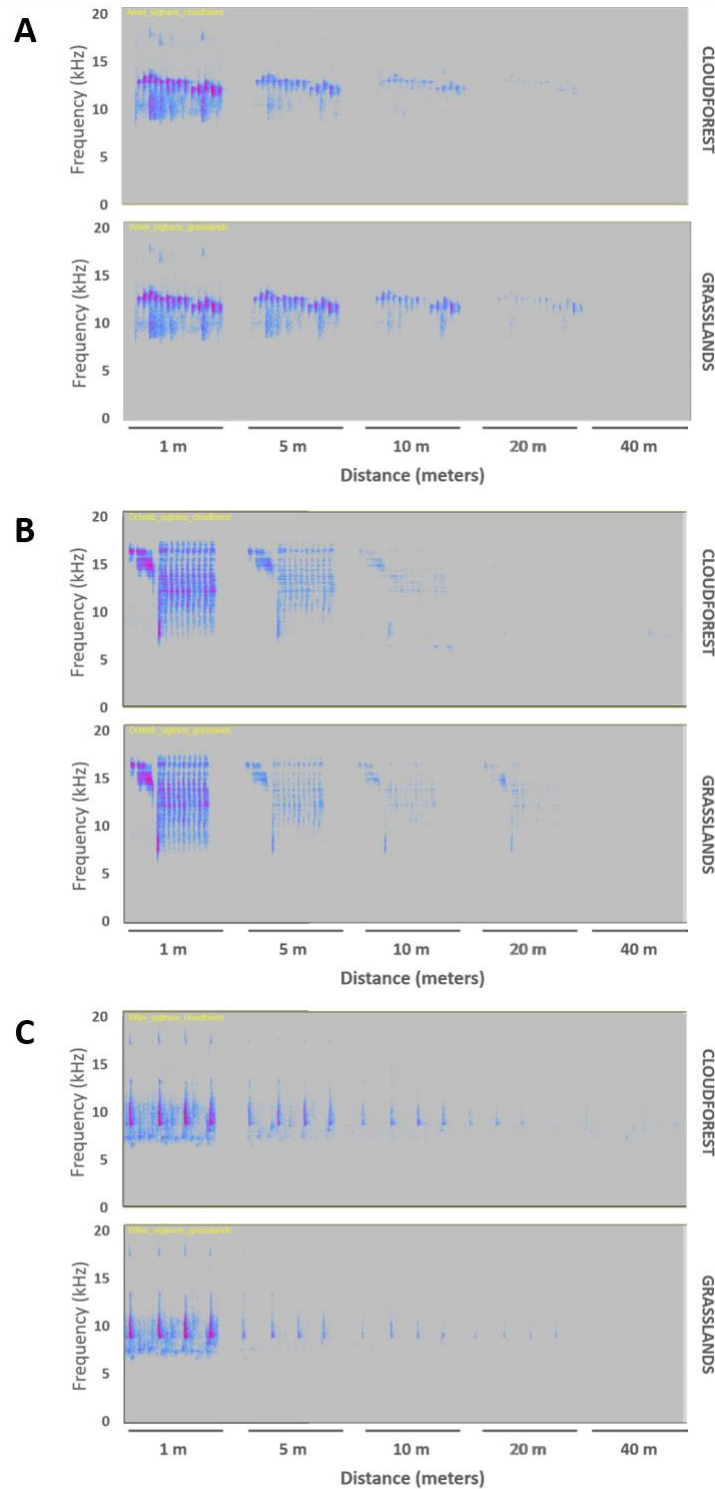
Figure 17. Samples of attenuated and degraded high-frequency vocalizations across distance.
Spectrograms of sample recordings at each distance in the cloud forest and the grasslands for **A)** *Adelomyia melanogenys*, and **B)** *Boissonneaua flavescens*, both native to the cloud forest, and **C)** *Oreotrochilus chimborazo*, native to the high-altitude grasslands.

4.1.9   Acoustic analysis of transmission recordings

To select our samples for acoustic analysis, we visually inspected spectrograms of experimental recordings using *Audacity version 2.3.2* (Audacity Team, 2019). This allowed us to identify files with traces of the recordings even if the sound levels were hardly distinguishable from ambient noise, which was common at longer distances. We sorted the recordings collected during our transmission experiment in the field based on species and applied different bandpass filters to remove as much background noise as possible. For *A. melanogenys,* the bandpass filter was 10–15 kHz, while for both *B. flavescens* and *O. chimborazo* it was 7–20 kHz. Playback recordings that contained vocalizations from other birds were removed from our sample. This situation was more common in the cloud forest than in the grasslands, which is reflected in the different sample sizes for each habitat. Then, we extracted single vocalizations from the recordings of *O. chimborazo* (grasslands (GL), n = 660; cloud forest (CF), n = 190) and single-bout samples for the two cloud-forest species with simpler calls (*A. melanogenys,* GL, n = 474 and CF, n = 216; and *B. flavescens,* GL, n = 119 and CF, n = 81).

To study attenuation and spectral degradation, we compared our experimental data to a library of 25 files of reference vocalizations for each species. These reference vocalizations were not attenuated or degraded because they were extracted from the original playback file. Each library reflects the conditions of the experimental data in that it is composed by single-vocalization files for *O. chimborazo*, and single-bout files for *A. melanogenys* and *B. flavescens.*

To evaluate attenuation, we measured the maximum relative amplitude at the dominant frequency in the reference library using the *tuneR* (Ligges et al., 2018) and

seewave (Sueur et al., 2008) packages in *R* (R Core Team, 2013). The average of the

maximum amplitude at the dominant frequency for each library was established as the

normalization value for the experimental data of each species. Then, we measured the

maximum relative amplitude at the dominant frequency in each recording of our

experimental dataset and normalized them to the corresponding normalization value. This

transformation allowed us to make comparisons across species and habitats. The high

levels of noise in both habitats prevented us from using a SPL meter to evaluate

attenuation. Often the SPL readings reflected overall levels of ambient noise rather than

sound levels of the signals we were broadcasting.

To evaluate degradation of spectral content, we compared the recordings of signal

transmission of each species in both habitat to their libraries. To make these comparisons,

we used the spectral correlation function in *Raven Pro* software (Center for Conservation

Bioacoustics, 2014). A spectral correlation of 1 shows that the spectral content in a file is

identical to that of the species library, therefore showing that there is no degradation. In

contrast, lower correlations imply increased degradation in the data.

Finally, we analyzed temporal degradation, which presents as smearing or loss of

definition in the temporal patterns of a sound and is common at longer distances (Brumm

& Naguib, 2009; Ryan, Cocroft, et al., 1990). Thus, the loss of definition in temporal

structure makes it harder for a sound to be distinguished from ambient noise. We only

analyzed the song from *O. chimborazo* because it is the only HF vocalization in this study

with multiple song elements (**Figure 15**). First, we randomly selected transmission

recordings from the grasslands at each distance to approximate to the sample size in the

cloud forest. Recordings at 40 m were excluded from the analysis because no traces of the

HF song were found at this distance in any habitat. Each recording was manually labeled

using *Audacity* (Audacity Team, 2019) to identify each element in the song and the inter-

syllable intervals. The final number of samples were n = 193 for the grasslands, and n = 139

for the cloud forest.

For the analysis of temporal degradation, we wrote customized *R* code to measure

the ratio of the amplitudes (dV) of the syllables (S) and the inter-syllable interval (ISI)

using the following formula:

$$dV = 1 - \frac{RMS\ ISI}{RMS\ S}$$

where RM ISI is the root-mean-square amplitude of the inter-syllable interval, and

RM S is the root-mean-square of each syllable, as described in (Ryan & Sullivan, 1989). This

analysis allowed us to obtain measures of temporal degradation over distance in both

habitats. A lower dV value was indicative of increased temporal degradation.

4.1.10 Statistical analysis for signal transmission

Prior to conducting the statistical analysis, we graphed the data for attenuation,

spectral degradation, and temporal degradation using the *ggplot2 v.3.3.0* package

(Wickham et al., 2019) in *R* (R Core Team, 2013). Upon visual inspection, we confirmed the

presence of outliers in the dataset for attenuation that exceeded the normalization value.

This was possible because the normalization value for each species was calculated as the

average of the maximum amplitude in the library of reference vocalizations. Therefore, we

changed the value of these seven data points to 1. We did not remove or transform any

other outlier in the data.

For the statistical analysis of attenuation and spectral degradation, we conducted three-way ANOVAs that included species, habitat, and distance as independent variables. Our data did not meet the assumptions of normality and homoscedasticity. However, an analysis of the mean values in each group compared to their corresponding median values showed that the means were representative of the distribution of our data as shown in **Appendix A.1** for attenuation data, and **Appendix A.2** for spectral degradation data. Mean values at 40 m were further from the median because there were a few recordings from the cloud-forest species that still showed traces of HF vocalizations. Tukey HSD post hoc tests were used to assess the statistical significance of pairwise comparisons.

To evaluate temporal degradation in the song of *O. chimborazo*, we conducted a two-way ANOVA including habitat and distance as independent variables, and a Tukey HSD post hoc analysis (Distribution plots are shown in **Appendix A.3**). All statistical analyses were conducted in *R* (R Core Team, 2013).

4.2   Results

4.2.1   Analysis of body mass and vocal production in hummingbirds

After calculating the average body weight for each species, we calculated the average weight of hummingbirds in our sample size. **Table 3** shows summary statistics for body weight in our sample.

Table 3. Summary statistics on hummingbird body weight in the study.
N = 118 species of hummingbirds that occur in Ecuador

Minimum Weight (g)	1.9
1st Quartile	4.213
Median Weight	5.25
Mean Weight	5.647
3rd Quartile	6.793
Maximum Weight	20.2
n = 118 hummingbird species	

Hummingbirds that have been documented to produce HF vocalizations exhibit diverse body masses, spanning from 3 to 8.2 g; some of these species also exhibit wide frequency ranges for vocal production. **Figure 18** shows the vocal range of hummingbirds across weight. The wide vocal range of some hummingbirds (red arrows in **Fig. 18**) appears to be independent from body size suggesting that body mass may not be a good predictor of vocal production in hummingbirds.
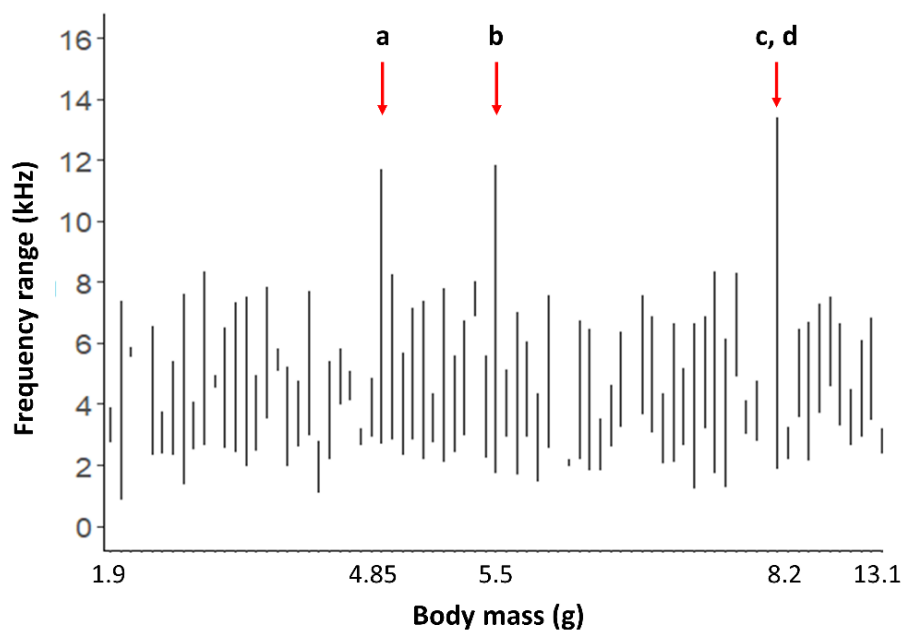
Figure 18. Frequency range of vocal production in hummingbirds by weight
Red arrows show the weight for four species of Andean hummingbirds that produce HF vocalizations: **a)** A. melanogenys, **b)** A. coelestis, **c)** B. flavescens, and **d)** O. chimborazo.

The phylogenetic regression showed that there is no correlation between body mass and minimum fundamental frequency in vocal production (R^2 = -0.0088, p = 0.9877) (**Figure 19A**). Similar analysis was conducted, using maximum and average fundamental frequency with the same results. Our analysis on dominant frequency, that is, the frequency that hummingbirds emphasize throughout their song, yielded similar results to those of fundamental frequency (R^2 = 0.0041, p = 0.2279) (**Figure 19B**).
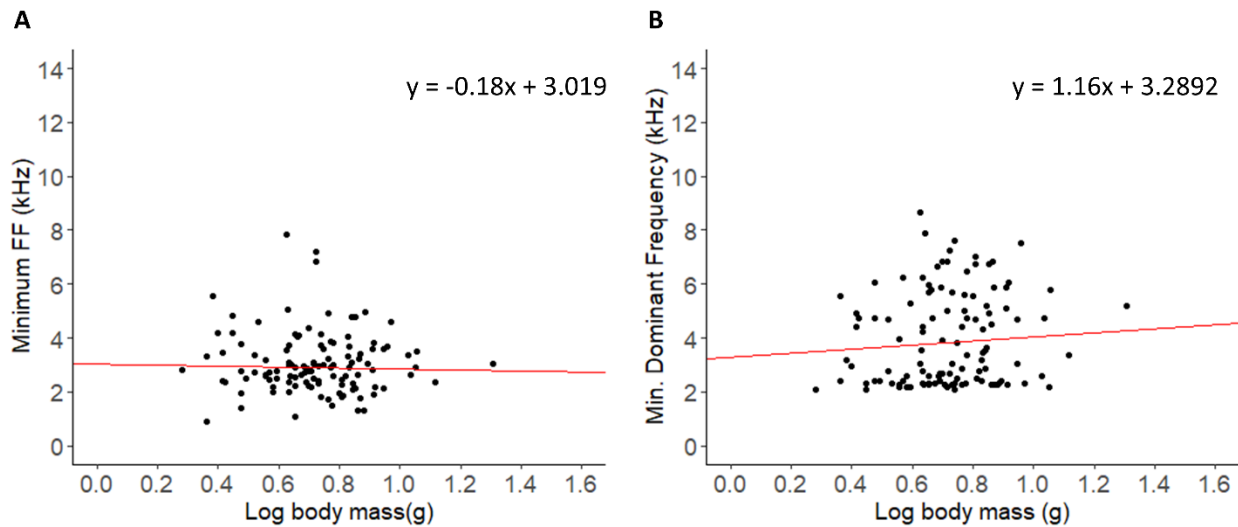
Figure 19. Body mass does not predict frequency of vocal production in hummingbirds
Phylogenetic regression evaluating the relationship between body mass (g) and **A)** minimum Fundamental Frequency (FF) (R2 = -0.0088, p = 0.9877), and **B)** Minimum Dominant Frequency (R^2 = 0.0041, p = 0.2279).

Based on our analysis, we concluded that body mass does not have an inverse relationship with frequency of vocal production in hummingbirds. Thus, body size is not a good predictor of vocal capabilities in this group of birds. It is likely that other selection pressures exert stronger influence in the evolution of vocal signals in hummingbirds and HF vocalizations in some species.

4.2.2   *Analysis of ambient noise in each habitat*

We compared the dominant frequency of each hummingbird vocalization to the frequency content of the ambient noise in each habitat. Our characterization of habitat acoustics in the grasslands showed that environmental noise was concentrated below 5 kHz (**Figure 20A**) and was produced mainly by wind. In contrast, most of the noise in the cloud forest came from insects (12.5 – 15 kHz) and other birds (3 – 9 kHz) (**Figure 20B**). Our comparison showed that the three species of hummingbirds that we studied in the cloud forest broadcasted their calls in a relatively noise-free frequency band in their habitat. Meanwhile in the grasslands, the Ecuadorian Hillstar had little acoustic competition. The acoustic environment in the grasslands may have allowed this species to evolve vocalizations with higher frequencies because high-frequency noise, such as that produced by insects in the cloud forest, is absent in this habitat.
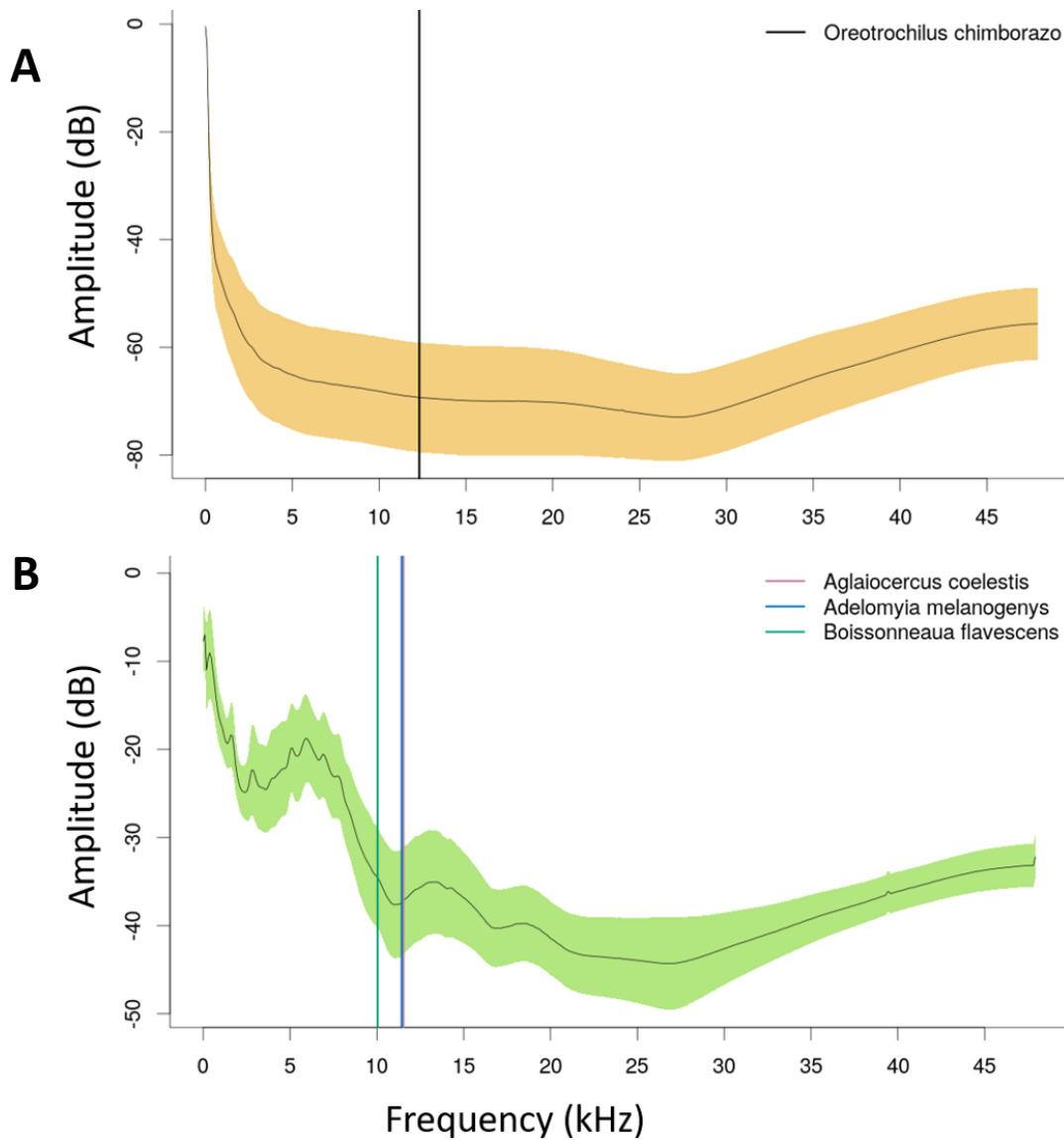
Figure 20. Ambient noise in habitats where HF vocalizations are produced.
Power spectra of ambient noise in **A)** the grasslands and **B)** the cloud forest. Shaded area shows standard deviation of the average amplitude for each frequency (kHz). Each vertical line represents the dominant frequency of high-frequency vocalizations in each species.

4.2.3 Analysis of signal transmission in each habitat

4.2.3.1 Attenuation

Sound levels steeply decreased at short distances from the source (**Figure 14**). A

three-way ANOVA showed significant main effects of species and distance on sound levels,

but no main effect of habitat (*species*, $F_{(2, 1710)}$ = 568.884, p < 0.001; *distance*, $F_{(4, 1710)}$ =

2911.993, p < 0.001; *habitat*, $F_{(1, 1710)}$ = 0.648, p = 0.4211). All interactions were significant

(*Species*Habitat*, $F_{(2, 1710)}$ = 44.751, p < 0.001; *Species*Distance*, $F_{(8, 1710)}$ = 240.151, p <

0.001; *Habitat*Distance*, $F_{(4, 1710)}$ = 2.891, p = 0.0212; *Species*Habitat*Distance*, $F_{(8, 1710)}$ =

25.650, p < 0.001). Below, we will discuss statistically significant differences in attenuation

based on pairwise comparisons resulting from analysis of the interaction among species,

habitat, and distance.

We found significant differences in attenuation between the cloud forest and the

grasslands only at 1 m, for the three vocalizations (p < 0.01 for all comparisons) (**Figure

21**). No other relevant differences in attenuation were found between habitats for any of

the HF vocalizations. **Table 4** summarizes the results of the study including attenuation of

HF vocalizations in both habitats. **Appendix B.1** shows the normalized amplitude values

for HF vocalizations as they attenuated over distance in each habitat.

In the cloud forest (**Figure 21A**), the HF calls of *B. flavescens* attenuated less in

general than the vocalizations of the other two species. At 1 m, the three vocalizations

already exhibited different sound levels from each other (p < 0.001). At 5 m, the calls of *B.

flavescens* still had higher sound levels than those in the calls of *A. melanogenys* and *O.

chimborazo* (p < 0.001 for both comparisons), while the calls of the other two species were

similar. At further distances, there were no differences in the attenuation among the three

HF vocalizations. Likewise, in the grasslands (**Figure 21B**) the calls of *B. flavescens*

attenuated significantly less at 1 m than the vocalizations of the other species (p < 0.001 for

both comparisons), which were not different from each other. Similar results were

obtained at 5 and 10 m, at which the vocalizations of *B. flavescens* attenuated significantly

less than those of the other two hummingbirds (p < 0.01 for all comparisons). No

differences in attenuation were found at 20 and 40 m.
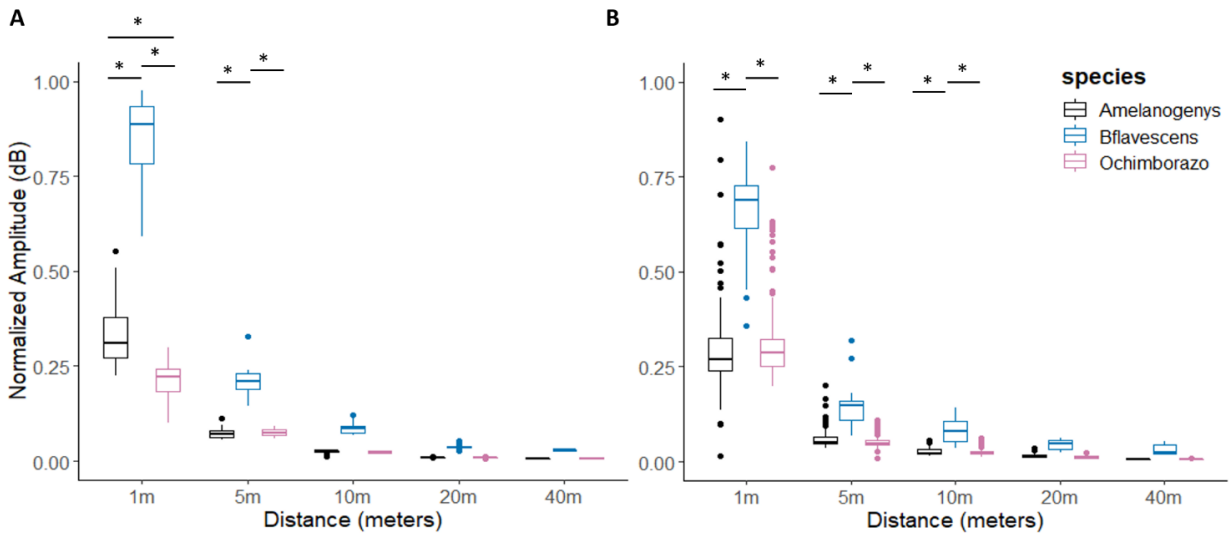
Figure 21. Attenuation of HF vocalizations in each habitat.
Normalized amplitude of high-frequency vocalizations over distance in **A)** the cloud forest, and **B)** the grasslands. Amplitude values (dB) are normalized to the average maximum amplitude in the template from the original playback. * (p < 0.05, three-way ANOVA, Tukey's HSD post hoc).

4.2.3.2 Spectral degradation

The three-way ANOVA showed main effects of habitat, species, and distance

(*habitat*, $F_{(1, 1614)}$ = 353.87, p < 0.001; species, ($F_{(2, 1614)}$ = 412.43, p < 0.001; distance, $F_{(4, 1614)}$ = 1856.84, p < 0.001), and statistically significant interactions (habitat*species, $F_{(2, 1614)}$ = 17.12, p < 0.001; habitat*distance, $F_{(4, 1614)}$ = 45.68, p < 0.001; species*distance, $F_{(8, 1614)}$ = 18.77, p < 0.001; habitat*species*distance, $F_{(8, 1614)}$ = 11.36, p < 0.001). Below we present

results of pairwise comparisons based on the interaction among habitat, species, and

distance.

Overall, the calls of the three species of hummingbirds underwent different patterns of spectral degradation, and the calls of *B. flavescens* showed the least degradation, followed by *A. melanogenys*, and *O. chimborazo* ($p < 0.001$ for all comparisons) (**Table 4**). The HF vocalizations of the three species degraded more in the cloud forest than in the grasslands ($p < 0.001$). Values of spectral correlation for each group are shown in **Appendix B.2**. Below, we present differences in spectral degradation first by species and then by habitat.

Figure 22 shows results of spectral degradation for each species in both habitats. The HF call of *A. melanogenys* showed no difference in spectral degradation at 1 m between habitats, but at 5 m and beyond this vocalization degraded more in the cloud forest than in the grasslands ($p < 0.001$) (**Figure 22A**). Spectral degradation in the grasslands is significant after 20 m ($p < 0.001$), contrasting with the cloud forest, where degradation of the HF calls consistently accentuated with distance ($p < 0.001$ for all comparisons). Vocalizations from *B. flavescens* showed a significant difference in spectral degradation between habitats starting at 10 m ($p < 0.001$) (**Figure 22B**). After that, the vocalization of *B. flavescens* degraded more in its native habitat than in the grasslands, following the same pattern as *A. melanogenys*. Similarly, the HF song of *O. chimborazo* also degraded more in the cloud forest than in the grasslands (**Figure 22C**). However, significant differences in degradation between habitats were found only at 1 and 20 m ($p < 0.001$). At all other distances, degradation increased constantly with distance in both habitats ($p < 0.001$ for all comparisons). Altogether, these results demonstrate that HF vocalizations of *O. chimborazo* underwent significant spectral degradation at shorter distances in both habitats compared to the HF calls of the cloud-forest species. The HF song of *O. chimborazo* also degraded

more in the foreign environment than in its own habitat, while the opposite was observed in the vocalizations of the other two species.

We then compared the spectral degradation of HF calls for all three species in each habitat. In the cloud forest (**Figure 23A**), at 1 m, the three vocalizations had different levels of degradation ($p < 0.001$ for all comparisons). The call of *B. flavescens* showed the least degradation, while the song from *O. chimborazo* was the most degraded at 1 m. At 5 m, only the calls of *A. melanogenys* and *B. flavescens* were different ($p < 0.01$), while at 10 m, all three vocalizations showed similar degradation. At 20 m, the calls of *A. melanogenys* and *B. flavescens* showed similar degradation but significantly less than the song of *O. chimborazo* ($p < 0.001$ for both comparisons). Finally, at 40 m, the three vocalizations underwent substantial but comparable spectral degradation.

In contrast, in the high-altitude grasslands (**Figure 23B**) vocalizations of *A. melanogenys* and *B. flavescens* showed similar degradation at 1 m, but different than that in the song of *O. chimborazo* ($p < 0.001$ for both comparisons). Only at 5 m, the vocalization of *B. flavescens* showed better transmission in the grasslands than the call of *A. melanogenys* ($p < 0.001$), while at all other distances, both calls degraded similarly and less than the song of the grasslands species ($p < 0.001$ for all comparisons). Surprisingly, at 40 m the calls native to the cloud forest exhibited great variability in degradation but overall degraded less than the song of *O. chimborazo*, which was undetectable in recordings at this distance ($p < 0.001$ for both comparisons).
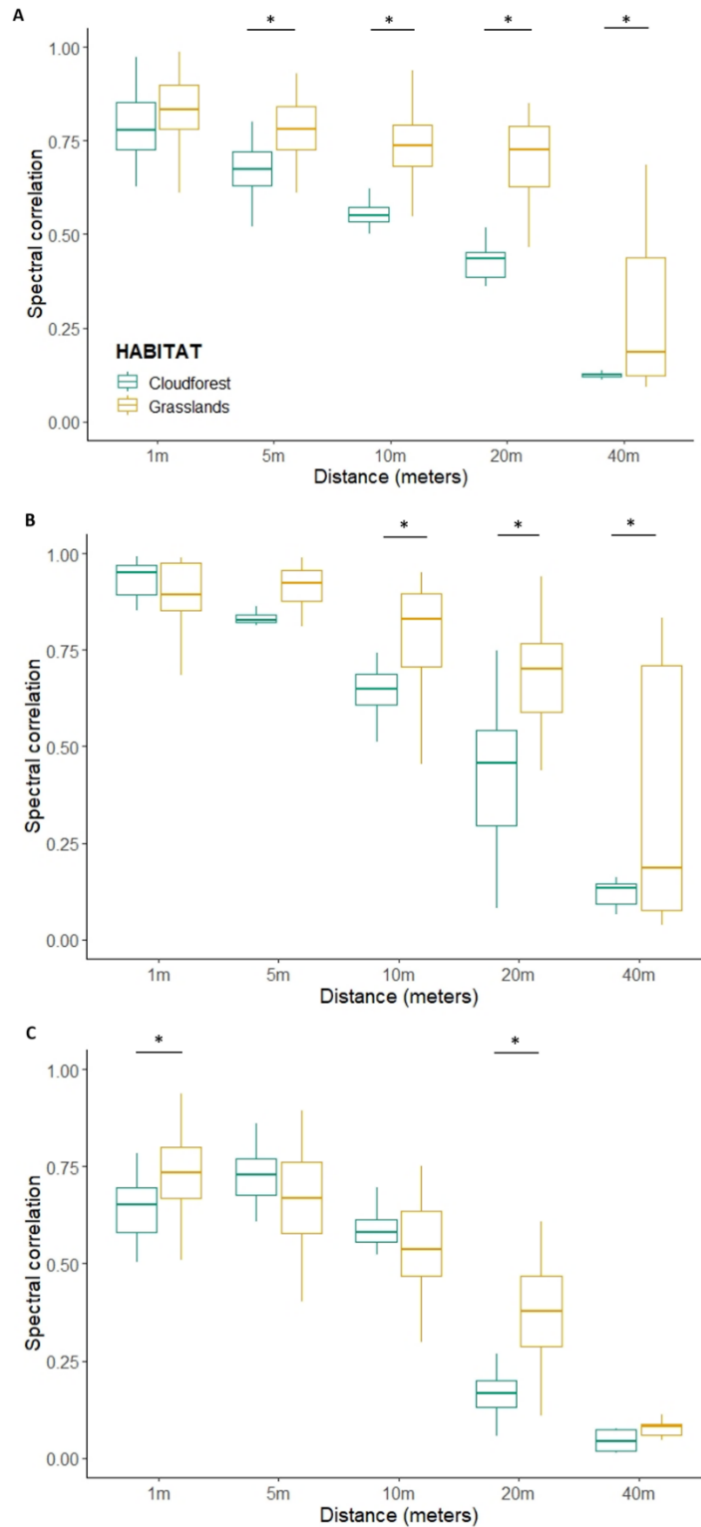
Figure 22. Spectral degradation of HF vocalizations of each species.
Graphs showing spectral correlations across distance and habitats for **A)** A. melanogenys,
B) B. flavescens, and **C)** O. chimborazo. * (p < 0.05, three-way ANOVA, Tukey's HSD post hoc test).
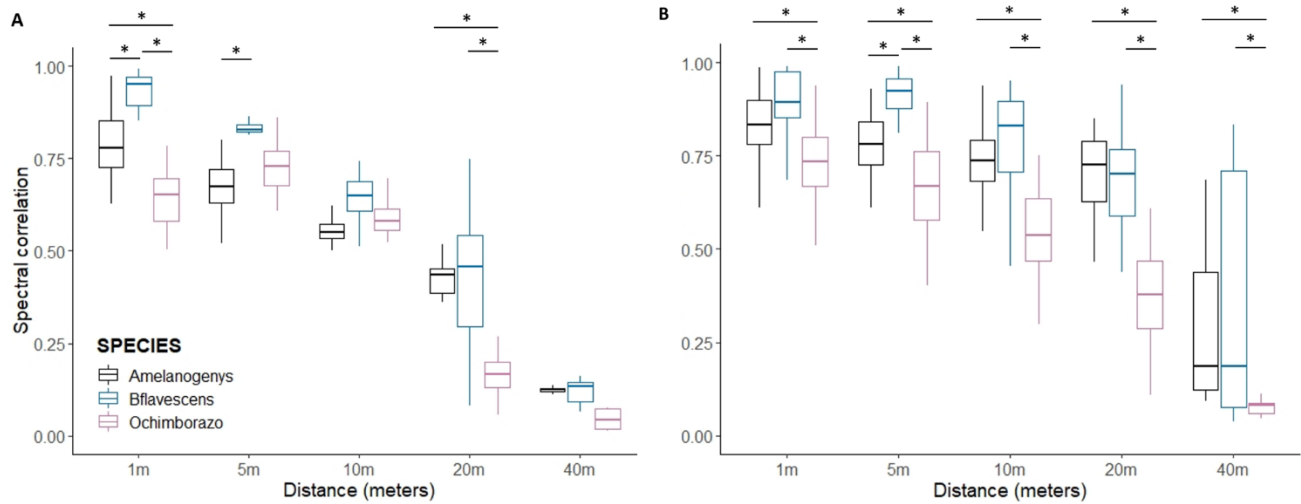
Figure 23. Spectral degradation of HF vocalizations in each habitat.
Spectral correlations for HF vocalizations of the three species recorded in **A)** the cloud forest, and **B)** the grasslands. * ($p < 0.05$, three-way ANOVA, Tukey's HSD post hoc test).

4.2.3.3 Temporal degradation

We analyzed changes in temporal features of the HF song of *O. chimborazo*, the most complex among the three vocalizations. The two-way ANOVA showed that there was no effect of habitat, but there was a strong effect of distance in the temporal degradation of the song (*habitat,* $F_{(1, 324)} = 1.231$, $p = 0.268$; *distance,* $F_{(3, 324)} = 1047.41$, $p < 0.001$). There was also a significant interaction between habitat and distance ($F_{(3, 324)} = 27.606$, $p < 0.001$).

Figure 24 highlights that temporal degradation of the HF song of *O. chimborazo* was more accentuated in the cloud forest than in the grasslands at 10 and at 20 m ($p < 0.001$ for both comparisons) (**Table 4**). Analysis by habitat showed that temporal degradation in the HF song of *O. chimborazo* constantly increased with distance in both habitats starting at 1 m ($p < 0.001$, for all comparisons). (See **Appendix B.3** for values of temporal degradation).
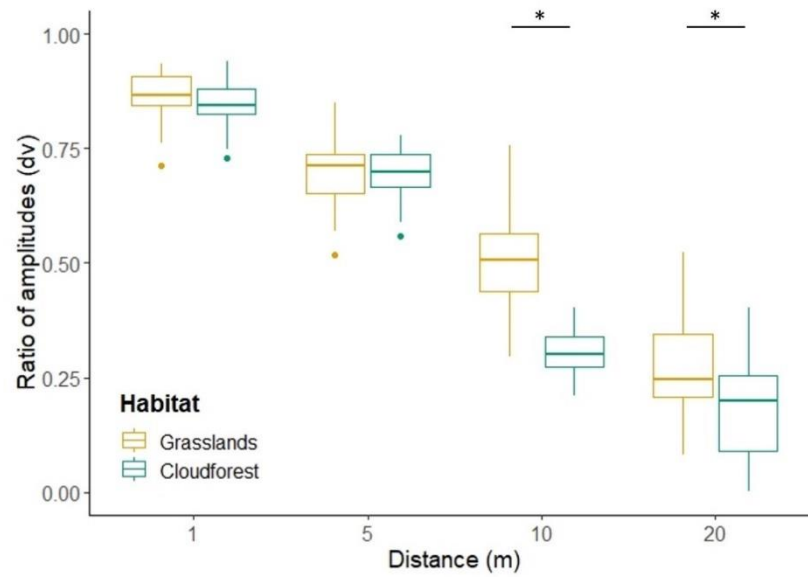
Figure 24. Temporal degradation of the HF song of O. chimborazo
The HF song of *O. chimborazo* did not exhibit differences in temporal degradation at 1 and 5 meters across habitats. Differences were significant across habitats at 10 and 20 meters. * ($p < 0.001$, two-way ANOVA, Tukey's HSD post hoc test).

Table 4. Summary results of attenuation and degradation of HF vocalizations
Statistically significant pairwise comparisons by species (AM, *A. melanogenys*; BF, *B. flavescens*; OC, *O. chimborazo*) and by habitat (CF, *Cloud forest*; GL, *Grasslands*) are shown (* p < 0.05; ** p < 0.01; *** p < 0.001, Tukey's HSD post hoc test).

A) ATTENUATION

Species	Comparison	1	5	10	20	40
		Distance (m)				
A. melanogenys	GL – CF	**				
B. flavescens	GL – CF	***				
O. chimborazo	GL – CF	***				

Habitat	Comparison	1	5	10	20	40
Cloud forest	AM – BF	***	***			
	AM – OC	***				
	BF - OC	***	***			

	Comparison	1	5	10	20	40
Grasslands	AM - BF	***	***	*		
	AM - OC					
	BF - OC	***	***	**		

B) SPECTRAL DEGRADATION

Species	Comparison	1	5	10	20	40
		Distance (m)				
A. melanogenys	GL - CF		***	***	***	***
B. flavescens	GL - CF			*	***	***
O. chimborazo	GL - CF	***			***	

Habitat	Comparison	1	5	10	20	40
Cloud forest	AM - BF	***	**			
	AM - OC	***			***	
	BF - OC	***			***	

	Comparison	1	5	10	20	40
Grasslands	AM - BF		***			
	AM - OC	***	***	***	***	***
	BF - OC	***	***	***	***	***

C) TEMPORAL DEGRADATION

Species	Comparison	1	5	10	20
		Distance (m)			
O. chimborazo	GL - CF			***	***

4.3 Discussion

In this study, we evaluated potential physical constraints such as body mass and ecological factors that may be involved in the evolution of HF vocalizations in some species of Andean hummingbirds. Our analysis of body mass and vocal production in hummingbirds showed that body mass is not a good predictor of fundamental or dominant frequency in the vocalizations of this group of birds. Vocal production in hummingbirds, unlike that of other animals, does not follow an acoustic allometry in which smaller individuals produce vocalizations at higher frequencies compared to larger individuals. In fact, the species producing HF vocalizations exhibit a wide range of body masses, from 4.6 g in *A. melanogenys* to 8.2 g in *O. chimborazo* (Dunning Jr, 2007), which produces the HF song with the highest fundamental frequency. Previous studies investigating acoustic allometries in birds have only included one or few species of hummingbirds, which in comparison to larger birds, fit the predicted pattern. However, no study had investigated whether this allometry exists among hummingbirds. In other birds, body size is positively correlated with the length of the syringeal labia, showing that body mass is a good predictor of the fundamental frequency in vocal production for these animals (Goller & Riede, 2013). However, no such data is available for hummingbirds.

Our results on body mass and vocal production suggest that morphological adaptations in the vocal organ rather than body size may better explain the presence of HF vocalizations in some hummingbirds. Recent reports describing the vocal organ in hummingbirds (Monte et al., 2020b; Riede & Olson, 2020) show variation in the position of the syrinx and its musculature across species. This variation may be key for enabling the production of HF vocalizations in some species of hummingbirds while restricting it in

others. Alternatively, the hummingbird syrinx may be naturally well suited for producing

HF sounds, but the ecological pressures that most hummingbirds encounter in their habitat

select for vocal signals at lower frequencies. If this is the case, then species producing HF

vocalization may have encountered a particular combination of pressures that have

selected for the use of HF vocalizations in some contexts. In addition, it remains to be

determined whether hummingbirds produce vocalizations at frequencies lower than those

expected for their body size, and if so, which mechanisms they employ to achieve this

feature.

We also tested the hypothesis that HF vocalizations have evolved in part to avoid

signal masking in noisy habitats. Ambient noise affects the transmission of acoustic signals.

Therefore, animals adapt their vocalizations to overcome masking by background noise in

their environment (Pytte et al., 2003). Our research showed that the vocalizations of *B.

flavescens* and *A. melanogenys* avoid noisy frequency bands in their habitats (Duque et al.,

2018), just like other species of birds living in noisy environments (Brenowitz, 1982; Ryan

& Brenowitz, 1985; Slabbekoorn & Peet, 2003; Walters et al., 2019). Likewise, *O.

chimborazo* females hold territories near water streams, which produce noise at higher

frequencies than wind, potentially contributing to sound masking (Duque et al., 2020).

Therefore, just like its counterparts in the cloud forest, *O. chimborazo* males may have

evolved a HF song to avoid signal masking while courting females in their territories.

Once we evaluated the role of body mass and ambient noise as physical and

ecological constraints influencing the evolution of HF vocal signals, we wanted to

determine how well these vocalizations transmit in their native environments. We tested

the hypothesis that HF vocal signals in hummingbirds are adapted for transmission in their

habitat. We assessed attenuation, spectral and temporal degradation in these vocalizations

in the cloud forest and grasslands, where HF vocalizations are produced by two and one

species of hummingbirds, respectively.

First, we compared the pattern of attenuation in each HF vocalization in both

habitats. Excess attenuation, which may vary depending on the vegetation composition of

the habitat, were expected to be different between the cloud forest – a closed habitat – and

the grasslands – an open habitat (Boncoraglio & Saino, 2007; Morton, 1975). We also

expected that differences in ambient temperature, relative humidity, precipitation, and

wind would only accentuate differences more. The profile of attenuation of HF signals

differed significantly between habitats only at 1 m. This was a surprising result because

differences and excess attenuation at 1 m was not expected in either habitat. At this

distance, the vocalizations of the three species attenuated less in the native habitat than in

the foreign environment, respectively. At 5 m, HF vocalizations of the three species showed

a steep decline in sound levels (**Fig. 21**). Excessive attenuation in the HF vocalizations of

these hummingbirds suggests that these species may be using HF vocal signals for short-

range communication with conspecifics (Mathevon et al., 2008). In contrast, studies of

signal transmission in songbirds that vocalize at lower frequencies, have shown that their

vocalizations can reach longer distances before being undetectable in their habitat

(Mathevon et al., 2008; Mouterde et al., 2014).

While there is little information about territory size in the three species in this

study, it may help explain the use of HF vocalizations as short-range communication

signals. Resource availability and pressure from intrusion influence territory size in

hummingbirds (Norton et al., 1982). In the grasslands, territories with larger patches and

more abundant flowers of *Chuquiraga jussieui,* the main source of nectar for *O. chimborazo*, host higher numbers of individuals (Ortiz-Crespo & Bleiweiss, 1982). Consequently, we have observed that territory size in these sites tends to be smaller compared to other sites where *C. jussieui* is scarce and sparsely distributed. Thus, despite their aggressiveness, males hold preferred perches delimiting small, adjacent territories. Males visit their preferred perches constantly, producing the HF song atop them. We have observed *B. flavescens* exhibiting similar patrolling behavior in a small defined area. Broadcasting HF vocalizations from preferred perches emphasizes the territorial role of these vocalizations. Given that territories can be small, transmission of HF vocalizations within a short distance may suffice for these species. In addition, *O. chimborazo* also produces its HF song as part of its courtship display to females, usually at less than 1 m from the receiver (Duque et al., 2020). Other species of hummingbirds, like the Blue-throated hummingbird (*Lampornis clemenciae*) also have specific vocalizations for short-range communication (Ficken et al., 2002).

We analyzed the degradation of spectral content in HF vocalizations over distance in each habitat. Our analysis showed that overall, these vocalizations degrade more in the cloud forest than in the grasslands. This finding supports predictions of increased spectral degradation of HF sounds as they transmit in closed environments (Mathevon et al., 2008; Morton, 1975). Overall, the calls of *A. melanogenys* and *B. flavescens,* native to the cloud forest, are not particularly affected by habitat composition at short distances. At 40 m, the calls of the two species were completely degraded in the cloud forest but still distinguishable in several recordings in the grasslands. In contrast, the song of *O. chimborazo* increasingly degraded with distance in both habitats, although less so in its

native grasslands. This result emphasizes that in general, an open habitat with low vegetation favors the transmission of HF sounds (Morton, 1975).

The loss of spectral content over distance affects communication because important sound features can be distorted, making it harder for conspecifics to discriminate the signal and respond accordingly at longer distances (Slabbekoorn, 2004). Interestingly, the calls of *B. flavescens,* which show the least degradation among the three vocalizations, also has the lowest fundamental frequency (9.7 kHz) compared to the other two vocalizations (Duque et al., 2018). This feature paired with the simple structure of the call may favor the transmission of the *B. flavescens* call, making it less susceptible to degradation in both habitats. The AAH states that habitat structure may shape not only the frequency range but also the structure of vocalizations, favoring tonal signals with narrow frequency bandwidth in closed habitats (Morton, 1975), such as the cloud forest.

Wind and weather conditions may be attributed for rapid changes in sound levels of the three vocalizations, which varied substantially as close as 1m in the grasslands (**Fig. 21B**). Our data on ambient temperature and relative humidity (**Fig. 16**) suggest substantial differences between the cloud forest and high-altitude grasslands during the transmission experiments. In the cloud forest, temperature and humidity were more stable than in the grasslands, where these features fluctuated drastically throughout the day. These differences likely affected the transmission of HF vocalizations differently in each habitat. These weather conditions may exert selective pressures on song structure. For instance, frequent wind gusts, typically found in open habitats, may favor the evolution of frequency-modulated elements produced in a rapid sequence. This call structure allows for the transmission of information in very short windows of time (Catchpole & Slater, 2008),

which may be the case of *O. chimborazo* in the high-altitude grasslands. In the cloud forest, constant high humidity promotes absorption of HF sounds and may favor narrow frequency bandwidths in vocalizations (Snell-Rood, 2012).

Overall, the combination of multiple selection pressures including habitat structure, ambient noise and weather may be exerting their forces differently on each species. It is unlikely to find HF vocalizations that resemble the song of *O. chimborazo* in a habitat like the cloud forest, where environmental conditions often degrade these signals (Badyaev & Leaf, 1997). Thus, while the cloud-forest species may be pressured to evolve simple HF calls in a narrow frequency range, pressures in the high-altitude grasslands may have favored a more complex vocalization produced at higher frequencies.

Finally, we assessed temporal degradation in the song of *O. chimborazo,* measuring changes in the amplitude of each element of the song relative to that of the noise in the inter-syllable intervals (Ryan & Sullivan, 1989). If ambient noise masks the signal, it is less likely that the receiver will discriminate the temporal structure of the song and respond to it appropriately (Kuczynski et al., 2010). We found that the HF song of *O. chimborazo* degrades more in the cloud forest than in its native habitat at 10 m and further distances, adding to the evidence that this vocalization is better adapted for transmission in its native habitat.

Limitations in the current study did not allow us to dissect the contributions of different environmental factors such as ambient temperature, humidity or wind speed to the transmission of the HF signals in each habitat. Here, we focused on obtaining a general profile for transmission of HF vocal signals in two strikingly different environments. Further research is necessary to understand the contributions of each environmental

component to the evolution of HF signals in these habitats. In addition, vocal signals

broadcast at different elevations from the ground can transmit differently (Kime et al.,

2000). In this study, we simulated typical conditions in which hummingbirds sing at mid

elevation perches within the forest. However, we have occasionally observed individuals

vocalizing from the canopy, at the edge of a steep decline in the mountain, which is most

consistent with conditions in the forest edge.

Sexual selection has also proved to be a strong selection pressure in the evolution of

vocal signals (Porter & Smith, 2020). This pressure is relevant for understanding the

evolution of the HF song in *O. chimborazo,* which produces this signal as part of its

courtship display (Duque et al., 2020). Sexual selection adds to factors like habitat, climate,

and phylogenetic constraints to exert pressures that shape the evolution of vocal signals.

In summary, our results show that HF vocalizations are not the result of an acoustic

allometry in hummingbirds, rather it is likely that evolutionary innovations in the vocal

organ are responsible for this ability. Moreover, the frequency content of HF vocalizations

from hummingbirds living in the cloud forest avoid noisy frequency bands in their habitat.

It is likely that *O. chimborazo*, like its counterparts in the cloud forest, also produces HF

vocalizations to avoid noise interference while broadcasting to females, which live in

territories near creeks where noise can reach higher frequencies.

Furthermore, our transmission experiments show that HF vocalizations are best

suited for short-range communication, as they attenuate and degrade substantially at short

distances. This is consistent with the use of HF song as a courtship signal in *O. chimborazo*

(Duque et al., 2020), and observations of patrolling behavior in this species and *B.*

flavescens. In addition, HF calls of hummingbirds from the cloud forest transmit better in

both habitats than the song of the grasslands species. The latter undergoes significant

spectral and temporal degradation, although more so in the closed habitat. In our

experiment, cloud-forest vocalizations performed slightly better in the grasslands than in

their habitat. This phenomenon may result from relaxation of constraints in the open

habitat for transmission of HF sounds (Ryan, Cocroft, et al., 1990). Constraints in the cloud

forest may have selected for simple single-note calls produced in a narrow HF range, to

reduce degradation and to favor transmission in a closed and noisy environment. In

contrast, the grasslands may have facilitated the evolution of a complex song, consisting of

frequency-modulated elements and several trills with a broad range of high frequencies

(Brumm & Naguib, 2009; Naguib, 2003; Ryan, Cocroft, et al., 1990). Altogether, this study

increases our understanding of how habitat influenced the evolution of HF vocal signals in

hummingbirds. More research is needed to identify the individual contributions of biotic

and abiotic environmental components in each habitat to the evolution of these

vocalizations.

Statement of publication: The information, text, and figures in this chapter have already

been published as part of two manuscripts. All information pertaining to the

characterization of ambient noise in both habitats was part of a manuscript published in

2018 in the journal *Current Biology* (Duque et al., 2018) (DOI:10.1016/j.cub.2018.07.058).

All information regarding the transmission of HF vocalizations in their habitat was part of a

manuscript published in 2021 in the *Biological Journal of the Linnean Society* (Duque et al.,

2021) (DOI:10.1093/biolinnean/blaa180). In both articles, I am the first and

corresponding author. I was involved in the conceptualization, methodology, data

collection and analysis in both manuscripts. I also wrote the initial draft and led the

revision of new versions of the manuscripts until their publication.

5   BEHAVIORAL AND NEURAL RESPONSES OF HUMMINGBIRDS TO HF SONG

Vocal communication is a fundamental component of diverse social contexts such as aggression, territoriality, courtship, and parental care (Bradbury & Vehrencamp, 2011). For vocal signals to be effective, the intended receiver should be able to detect and discriminate each signal (Ryan, Fox, et al., 1990; Wiley, 2013). Therefore, the auditory sensitivity of the intended receiver often coevolves with the vocal production of the sender of the signal (Wiley, 2013). The Ecuadorian Hillstar (*Oreotrochilus chimborazo*) is a hummingbird that lives in Andean high-altitude grasslands. It produces a high-frequency (HF) song with a fundamental frequency of 13.4 kHz, the highest in any bird vocalization known to date (Duque et al., 2018). The frequency content of this song is also far beyond the recorded hearing range in most birds (2–8 kHz) (Dooling, 2004). It has never been shown that hummingbirds or any other bird, except for some species of owls (Dyson et al., 1998), can hear sounds in this frequency range. While owls use specialized ears for hunting their prey, the Ecuadorian Hillstar seems to use its HF song for conspecific communication.

The males in this species produce their HF song in territorial contexts when patrolling their territories and to court females during the breeding season. These behavioral patterns of vocal production suggest that *O. chimborazo* can hear the HF songs produced by conspecifics. Therefore, we wanted to test whether *O. chimborazo* has evolved HF hearing consistent with the production of its HF song. Next, we wanted to investigate how the regions involved in modulating social behavior in the brains of males and females respond to the HF song which has a different function depending on the intended receiver.

Studies with songbirds have shown that individuals respond to the playback of conspecific song by vocalizing, approaching the speaker, or producing aggressive displays

(Luther & Wiley, 2009; McGregor et al., 1992; Naguib et al., 2002; Nelson & Soha, 2004;

Nielsen & Vehrencamp, 1995). These are often territorial responses directed at the

simulated intrusion of the playback. It has also been observed that protein expression of

the immediate-early gene *zenk* (also known as *zif268*, EGR-1, NGFI-A, *krox24*) is a

consistent marker for neuronal activation in response to salient stimuli in the secondary

regions of the avian auditory forebrain, the Caudal Medial Mesopallium (CMM) and the

Caudal Medial Nidopallium (NCM) (Jarvis et al., 2000; Mello et al., 1992; Mello & Clayton,

1994).

Furthermore, exposure to conspecific song also activates other brain regions

involved in the modulation of social behaviors (Heimovics & Riters, 2007; Maney et al.,

2008; Sewall & Davies, 2017). These regions form a highly interconnected network known

as the Social Behavior Network (SBN) and interact with other areas in the reward system of

the brain (Goodson, 2005; Newman, 1999). Thus, regions in the SBN are important targets

for understanding the neural mechanisms that modulate male-male and male-female social

interactions including responses to social vocal signals.

The sexually dimorphic Paraventricular Nucleus (PVN), and Supraoptic Nucleus

(SON) are involved in the regulation of the stress response and aggressive behavior in

males; activation of the Preoptic Area (POA) has also been associated with courtship and

copulatory behavior in males and parental behavior in females (O'Connell & Hofmann,

2011). In males, the Bed Nucleus of the Stria Terminalis (BST) activates selectively in

response to positive social stimuli while other positive stimuli that do not include a social

component fail to elicit a response (Goodson et al., 2009). In contrast, in females the BSTm

modulates precopulatory behavior and nest building as well as parental care. In addition,

the mammalian amygdala and its avian homolog, the Nucleus Taenia of the Amygdala

(TnA) have been associated with mediating responses to emotionally-relevant social

stimuli including vocal signals, sexual and appetitive behavior, and pair bonding (Cheng et

al., 1999; Curtis & Wang, 2003; Fujii et al., 2016; Svec et al., 2009). In contrast, a negative

correlation between activation of the Lateral Septum (LS) and the expression of aggressive

behavior and territoriality has been observed in mammals and birds (Goodson, 2005;

Sewall & Davies, 2017).

All these regions are sensitive to the modulatory effects of gonadal steroid

hormones and neuropeptides such as arginine vasotocin (AVT) and its mammalian

homolog arginine vasopressin (AVP) (Burmeister et al., 2001; Dunham & Wilczynski, 2014;

Goodson et al., 2009; Maney et al., 2008; Newman, 1999). Moreover, the production of a

particular social behavior results from a distinctive pattern of activity within the SBN,

rather than by the activation of a single nucleus (Newman, 1999). This explains how a

highly conserved network of brain nuclei, the SBN, can give rise to a great variety of

behavioral phenotypes. Studying the activation of the SBN in the hummingbird brain can

give us clues about the regulation of social behavior in these naturally aggressive birds and

the use of vocal signals to facilitate social interactions in both males and females.

Hence, to determine the behavioral and neural responses of *O. chimborazo*

hummingbirds to the playback of conspecific HF song, we conducted playback experiments

in the field and assessed neural responses in auditory regions in the forebrain of these

hummingbirds, as well as in SBN nuclei of males and females involved in the regulation of

social behavior. We hypothesized that *O. chimborazo* hummingbirds can hear the HF song

of conspecifics; therefore, we predicted that they would exhibit behavioral responses to the

playback of HF song in the field. We also predicted that the auditory regions in the brains of

hummingbirds exposed to HF song will express higher levels of ZENK protein compared to

control hummingbirds. Together, the behavioral and neural responses will determine

whether this species of hummingbirds can hear frequencies above 10 kHz, allowing them

to use its HF song for communication and social interactions. We also hypothesized that

there are sex-dependent neural responses in the SBN nuclei LS, PVN, POM, BSTm, and TnA

in response to playback of HF song. Thus, we predicted that brain regions associated with

promoting aggressive behavior will be more activated in males whereas those involved in

facilitating sexual behavior will be more active in females in response to the HF song. The

characterization of neural responses in the SBN nuclei will provide insights into the neural

processing of a vocal stimulus with different social valence for females and males, in a

species in which both sexes are highly aggressive.

5.1   Methods

5.1.1   Playback recordings

Stimuli for field playback experiments were assembled using high-quality

recordings of the HF song of *O. chimborazo*, which were recorded at 1 meter from naturally

vocalizing birds using a TASCAM DR-40 recorder (TEAC American Inc., CA, USA) (Duque et

al., 2018). These recordings were collected at Cunugyacu and Chimborazo Lodge for the

subspecies *O. c. chimborazo* and at Rucu Pichincha for the subspecies *O. c. jamesonii.* We

used a single vocalization from each location to generate each of the HF-song playbacks:

two for *O. c. chimborazo* and one for *O. c. jamesonii.* Although the HF song of the two

subspecies differs in structure, the frequency range remains the same (Duque et al., 2018).

Recordings of the selected vocalizations were normalized to 70 dB using *Audacity*

(Audacity Team, 2019). This amplitude level was confirmed using a Sound Pressure Level

(SPL) meter (Brüel & Kjaer, Denmark) at 1 meter in a sound-attenuating chamber. Using

the *seewave* package (Sueur et al., 2008) in *R* (R Core Team, 2013), we created a block of 10

vocalizations, leaving 1-second intervals between vocalizations. We applied a band-pass

filter (10-20 kHz) to delete from the playback any sound outside of this range. Blocks were

repeated at random intervals until the playback stimulus reached five minutes. To create

playbacks of ambient noise, we collected multiple recordings of environmental noise at the

same locations where we recorded HF song (Duque et al., 2018). Then, we selected a

section of the recordings that contained only abiotic noise and was not contaminated with

sounds from other animals. A similar procedure for generating the HF-song playback was

conducted to assemble the playback of ambient noise composed of frequencies below 10

kHz, which is the natural range for noise in this habitat (Duque et al., 2018).

For the controlled ZENK experiment, we followed the procedure described above,

however, vocalizations were assembled in pairs. Blocks of these pairs were repeated at

random intervals until the stimulus had a total duration of 25-minutes. Using *Audacity*

(Audacity Team, 2019), we built a 120-minute stimulus, including silence before and after

the experimental condition. For the control group, which was not exposed to any sound

stimuli, we used *Audacity* (Audacity Team, 2019) to build a 120-minute audio file

containing silence. For the ZENK experiment, we only generated two playbacks of HF song

for the subspecies *O. c. chimborazo* since the experiment was only conducted with this

subspecies at two locations on the slopes of Mt. Chimborazo. Hummingbirds were exposed

to a playback of HF song recorded at a different locale from where the experiment was conducted.

5.1.2 Behavioral experiments

Field experiments were conducted at several locations along the Ecuadorian Andes, namely, at the grasslands in Cotacachi, Pichincha, Antisanilla, Cotopaxi, and Cajas for the subspecies *O. c. jamesonii* and in two locations in the slopes of Mt. Chimborazo for the subspecies *O. c. chimborazo*. After identifying a hummingbird with a defined territory, we placed a rechargeable JBL CLIP 2 speaker (Harman, CT, USA) at one of the individual's preferred perches. We followed a within-subjects design, in which all hummingbirds were exposed to the playback of ambient noise and HF song. We allowed the hummingbird to approach, inspect, and acclimate to the presence of the speaker for at least 30 minutes. After acclimation, we played back the ambient noise to establish a behavioral baseline for 5 minutes. Then, we broadcast the playback of HF song for another 5 minutes. Playback of HF song matched the subspecies living in each field site where we conducted the behavioral experiment; however, the field playback experiments were conducted at different locations from where the original recordings were made.

Two scorers were positioned at least 5 meters away from the speaker and the hummingbird, with clear views of both. Scorers independently scored the behaviors of the hummingbird according to an ethogram (**Table 5**). If a behavior could be attributed to the presence of another animal, it was not recorded by the scorers. Scorers only recorded those behaviors produced by the experimental animal at the onset of each block of sound stimulus for the duration of the playback (5 minutes). Any behavior that was produced during the silent intervals between blocks was not included. After the experiment,

annotations of the two scorers were compared, and only those behaviors that were

consistently reported by the two scorers were selected for analysis.

Table 5. Ethogram

Ethogram		
Aggressive display	A	Males display towards the source of sound, by hovering, opening their wings and tail feathers widely. Sometimes, they will also move the tail feathers from side to side. Displays can sometimes be accompanied by chasing calls.
Fly over	FO	Hummingbird rapidly flies over the speaker at a short distance (1 meter or less) as a sign of aggression. May be accompanied by chasing calls.
Vocalizing	V	Individual vocalizes in response to playback. Annotate type of vocalization: - Chasing calls (CH): long bouts of vocalizations with rapid changes in type of syllables and rich in frequency modulations. Generally produced while flying and chasing another individual. Can be produced during an aggressive display. - Contact calls (CC): single-note vocalizations at lower pitch than HFV. Generally, several renditions separated by short intervals. - Alarm calls (AC): short broadband rasping calls produced to alert of a possible threat. - High-frequency vocalizations (HFV): produced only by males.
Approach	I	Male or female approaches the speaker or the surrounding area but show no signs of aggression. Usually remains in the surrounding area (within a radius of 5 meters from the speaker).
Avoidance	FA	Fly away (FA): Individual flies away from the speaker or the surrounding area after the onset of playback.
Attentiveness	T	The bird stays on its perch but shifts attention: - Head tilt (HT): moving the head from side to side or towards the speaker to improve sound localization in response to playback. This is accompanied by neck extensions to facilitate inspection. - Body posture (BP): adjusting body posture to improve visibility of the area where the sound is produced.

5.1.3 Induction of ZENK expression in the brain

For the controlled ZENK experiment, we collected *O. chimborazo* hummingbirds (n = 12) at two locations around Mt. Chimborazo in Ecuador, at the beginning of the breeding season in 2018 and 2019. They were isolated from each other and hand-fed 20% sucrose solution every 15 minutes until the beginning of the experiment. We built a hardwood sound attenuating chamber (18 x 18 x 18 inches), which had the inner walls covered with acoustic foam. It contained a perch, and two small hand feeders for the hummingbirds to feed during the experiment. Two small lights were provided to facilitate feeding during the experiment. The speaker in a TASCAM DR-40 recorder (TEAC American Inc., CA, USA) was used for playback; a second TASCAM DR-40 recorder was placed inside the chamber to record any vocalizations that the hummingbird produced. Hummingbirds were exposed to 25 minutes of playback stimulus, either silence or HF song, and rested for additional 65 minutes inside the chamber to reach peak ZENK protein levels in the brain (Zangenehpour & Chaudhuri, 2002) (**Figure 25**).
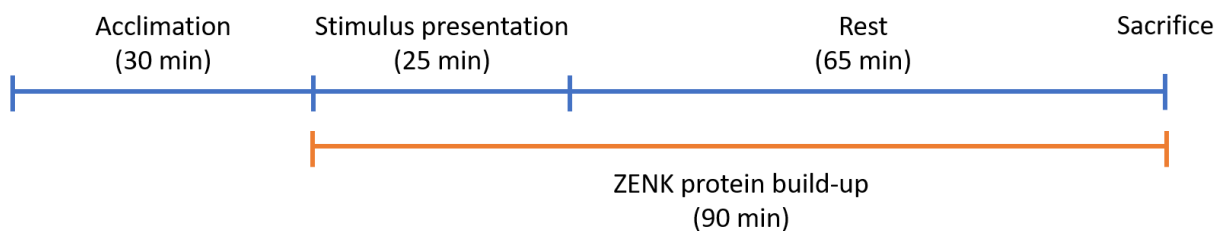
Figure 25. Induction of ZENK expression in the brains of hummingbirds
Timeline showing experimental design for inducing ZENK protein expression in the hummingbird brain in response to a sound stimulus. The control group (n = 5) was exposed to a playback of silence, while the experimental group (n = 7) was exposed to playback of HF song.

Upon completion of the experiment, hummingbirds were anesthetized using 4% Isoflurane and rapidly decapitated. Brains were collected immediately after and preserved

in 4% paraformaldehyde (PFA) solution for 24 hours. Then, the brains were submerged in

10, 20, 30, and 40% sucrose solution with 0.01% sodium azide as a preservative to prevent

fungal growth. The brains were then transported to our laboratory at Georgia State

University where they were embedded in Optimal Cutting Temperature (OCT) medium and

stored at -80 °C until sectioning. Brains were sectioned in a cryostat in 20μg coronal

sections and placed on slides in a sequential order so that the first section of slide A was

adjacent to the first section in slide B. All procedures were conducted in compliance with

the Institutional Animal Care and Use Committee (IACUC) (Protocol #A18049).

5.1.4   Immunohistochemistry

For immunohistochemistry (IHC), we followed standard protocols as described in

(Shahbazi et al., 2011). We used two sets of slides for each brain (A and B). Set A received

the primary antibody and was used to assess our experimental conditions, while set B was

used as a control for background and nonspecific staining during the IHC procedure. We

bathed the sections in multiple washes of Tris Buffer Solution (TBS) followed by a bath in

Triton X (detergent) and Trypsin (porcine enzyme). Then, sections were submerged in

hydrogen peroxide and methanol for 15 minutes. After additional washes in TBS, Normal

Rabbit Serum (5560-0008, SeraCare, MA, USA) was applied on the tissue, which was

incubated at room temperature (RT) for 30 minutes. Then, we applied the primary

antibody to the tissue in set A, while leaving the tissue in set B with additional Normal

serum. We used an EGR-1 polyclonal antibody (AF2818, Novus Biologicals, CO, USA,

dilution 1:500) and let it incubate for 48 hours. Then, we applied a biotinylated rabbit anti-

goat secondary antibody (5570-0009, SeraCare, MA, USA) and let it incubate for 90 minutes

at RT. Peroxidase-labeled Streptavidin (5550-0001, SeraCare, MA, USA) was then applied,

and the tissue incubated for 60 minutes in a dark chamber at RT. Finally, we applied 3,3'-

Diaminobenzidine (DAB) (SK-4100, Vector Labs, CA, USA) and let the sections rest at RT for

15 minutes for staining. We then rinsed the slides and dehydrated the tissue by

submerging it in ethanol at increasing concentrations (40, 70, 90, 100%). Finally, we

submerged the slides in clearing agent before covering them.

To evaluate the specificity of the primary antibody, we conducted protein sequence

alignments of the immunogen sequence of the EGR-1 antibody (Uniprot, Accession #

P18146) ("UniProt," 2019) and the coding sequence (CDS) of the *zenk* gene in the Zebra

Finch (*Taeniopygia guttata*) (GenBank, Accession # EF052676.1) (Malcher et al., 2006)

using protein-protein BLAST (Madden, 2002). We found 93% alignment of amino acid

sequences between the antibody immunogen and the *zenk* CDS. Then, we used the SVMTrip

online software (Yao et al., 2012) to predict putative epitopes in each sequence to which

the EGR-1 polyclonal antibody could bind. SVMTrip provides a total of 10 putative epitopes

for each sequence. Our results showed that at least five putative antigenic epitopes in the

EGR-1 immunogen correspond to putative antigenic epitopes in the coding sequence of the

zenk gene in the Zebra Finch.

5.1.5   Cell counting

To quantify ZENK expression, two scorers blinded to experimental condition and

brain region, counted immune-positive cells in coded pictures. Pictures were modified

using *GIMP* (The GIMP Development Team, 2019) to superpose a 5x5 grid, so that scorers

counted cells only within the 3x3 inner grid on each picture, as shown in **Figure 26A**. This

method guaranteed that only immune-positive cells in the region of interest were counted

while not including cells that could potentially belong to an adjacent region. Scorers

counted multiple alternate sections for each brain region in each animal.
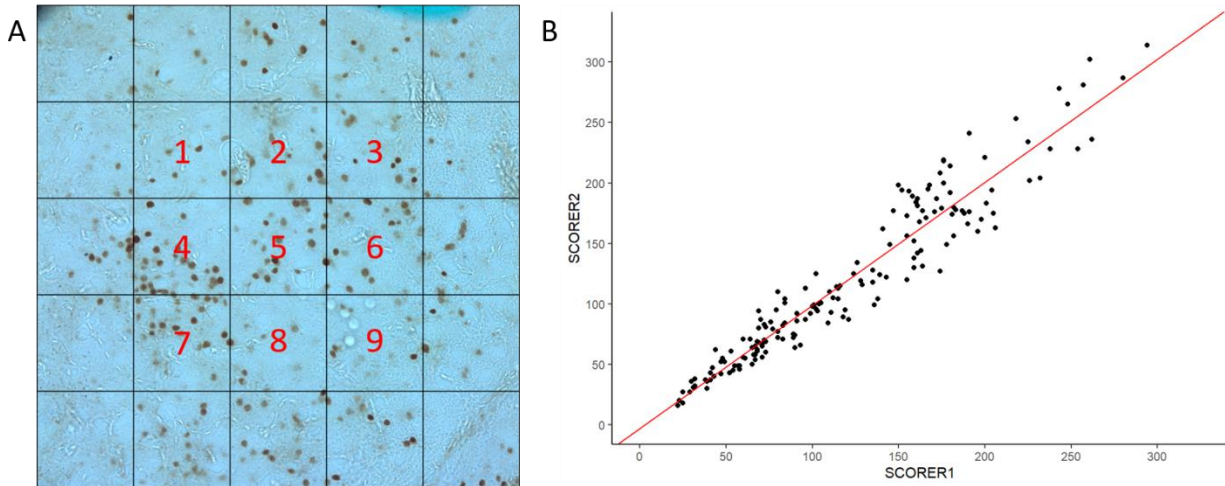
Figure 26. Counts of ZENK-ir cells in the brain of hummingbirds
Two blinded scorers counted ZENK-ir cells in **A)** within the 3x3 inner grid on images of sections of interest. Numbered inner-grid compartments exemplify the counting procedure of a sample section. This procedure guaranteed that any cell from an adjacent area was not counted as part of a region of interest. **B)** Graph showing cell counts for both scorers and the trend line in red (R^2 = 0.9604, p < 0.001).

A coefficient of variation (CV) (standard deviation/mean) < 0.25 between the two

scorers indicated valid counts, otherwise the pictures were recounted by both scorers. In

the final dataset, the coefficient of variation between the scorers was low (mean CV =

0.0906, sd = 0.0611) and the correlation was strong (R^2 = 0.9604, p < 0.001) (**Figure 26B**),

demonstrating that the cell counts were reliable. The average of the cell counts from the

two scorers was then used for our statistical analysis.

5.1.6   Statistical analysis

For the analysis of behavioral responses, we used a McNemar Chi-squared test to

evaluate the effect of HF song in the behavioral responses of hummingbirds in the field,

compared to the behaviors exhibited during a playback of ambient noise. To evaluate the

effect of the presentation of HF song in forebrain auditory regions, a two-way ANOVA was used. Tukey's Honestly Significant Difference (HSD) post-hoc analysis was used to assess pairwise comparisons. All statistical analyses were conducted in *R* (R Core Team, 2013). All plots were generated using the *ggplot2 v.3.3.0* package (Wickham et al., 2019) in R (R Core Team, 2013)*.

5.2   Results

5.2.1   *Behavioral responses to HF song*

First, we evaluated the behavioral responses of *O. chimborazo* to the playback of the conspecific HF song in the field. We identified *O. chimborazo* individuals with defined territories and placed a speaker in one of the hummingbird's preferred perches to simulate an intrusion by another male. We played two different sound stimuli: a) ambient noise, which mostly consisted of wind noise; and b) conspecific HF song. We then recorded behavioral responses to each stimulus. **Table 5** shows an ethogram describing all the behaviors that we assessed in response to playback. We reported only those individuals which were exposed to both experimental conditions (ambient noise and HF song) (n = 13).

The hummingbirds exhibited mainly three behavioral responses to the playback of conspecific HF song: approach to the area surrounding the speaker, head tilts and neck extensions, and changes in body posture (**Figure 27**). These behaviors were produced only by a few birds during the playback of ambient noise. For each behavior, we used a McNemar Chi-squared test to compare responses to HF song versus ambient noise. Regardless of the type of behavior that was scored, more hummingbirds responded to playback of HF song than to that of ambient noise (*approach: X^2 = 9.09, p = 0.003; head tilts and neck extensions: X^2 = 8.1, p = 0.004; body posture: X^2 = 10.083, p = 0.002*). At the onset of

playback of a block of HF song, hummingbirds moved their heads in the direction of the speaker while extending their necks. After a few seconds, they approached the area near the speaker, perching at a nearby perch from which they inspected the speaker for the rest of the experiment (total 5 minutes). After approaching, the hummingbirds continued responding with head tilts and neck extensions to the onset of playback and often shifted their body posture towards the speaker if necessary. Only one hummingbird flew over the speaker in response to the playback. In contrast, during the playback of ambient noise, hummingbirds were foraging undisturbed, maintaining a considerable distance from the perch where the speaker was placed. These behavioral responses show that *O. chimborazo* attends to and responds to the playback of HF song, demonstrating that these hummingbirds can detect this signal in their habitat.
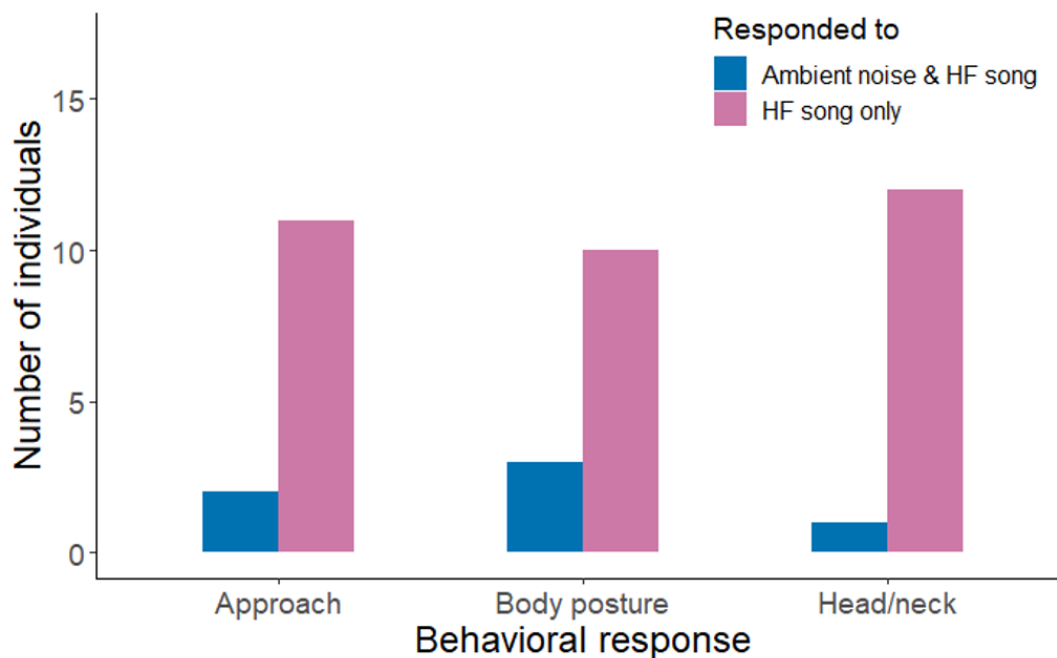
Figure 27. Behavioral responses to conspecific HF song.
Hummingbirds which elicited behaviors during both stimuli (ambient noise and HF song) are shown in blue (approach: 2 hummingbirds; body posture: 3; head/neck: 1), while hummingbirds that only responded to the playback of HF song are shown in purple (approach: 11; body posture: 10; head/neck: 12). Hummingbirds observed (n = 13).

5.2.2   *Neural responses in the auditory forebrain to playback of HF song*

We then assessed auditory responses in the hummingbird brain to frequencies

above 10 kHz. We collected individuals at their roosting site before sunrise (04h30 –

05h00) and isolated them to prevent exposure to external sounds before the experiment.

At the beginning of the experiment, each hummingbird was placed in a sound attenuating

chamber and allowed to acclimate for 30 minutes. Then, it was exposed to either the HF

song (frequencies > 10 kHz) (n = 4) or silence (n = 4) respectively, for 25 minutes (Jarvis et

al., 2000). The hummingbirds were sacrificed 90 minutes after the onset of playback

(**Figure 25**) (M. T. Avey et al., 2008; Bailey & Wade, 2003; Lynch et al., 2012). Using

immunohistochemistry (IHC), we measured the protein expression of the immediate-early

gene *zenk*, a marker for neuronal activity (Feenders et al., 2008; Hoke et al., 2004; Lynch et

al., 2012). We evaluated the secondary auditory regions CMM and NCM, which exhibit

robust ZENK expression in response to salient auditory stimuli, particularly to conspecific

song (M. T. Avey et al., 2005, 2008; Eda-Fujiwara et al., 2016). In addition, in individuals

from both groups, we measured ZENK expression in the hippocampus (HP), which we used

as a control region (Eda-Fujiwara et al., 2012, 2016) (**Figure 28**).
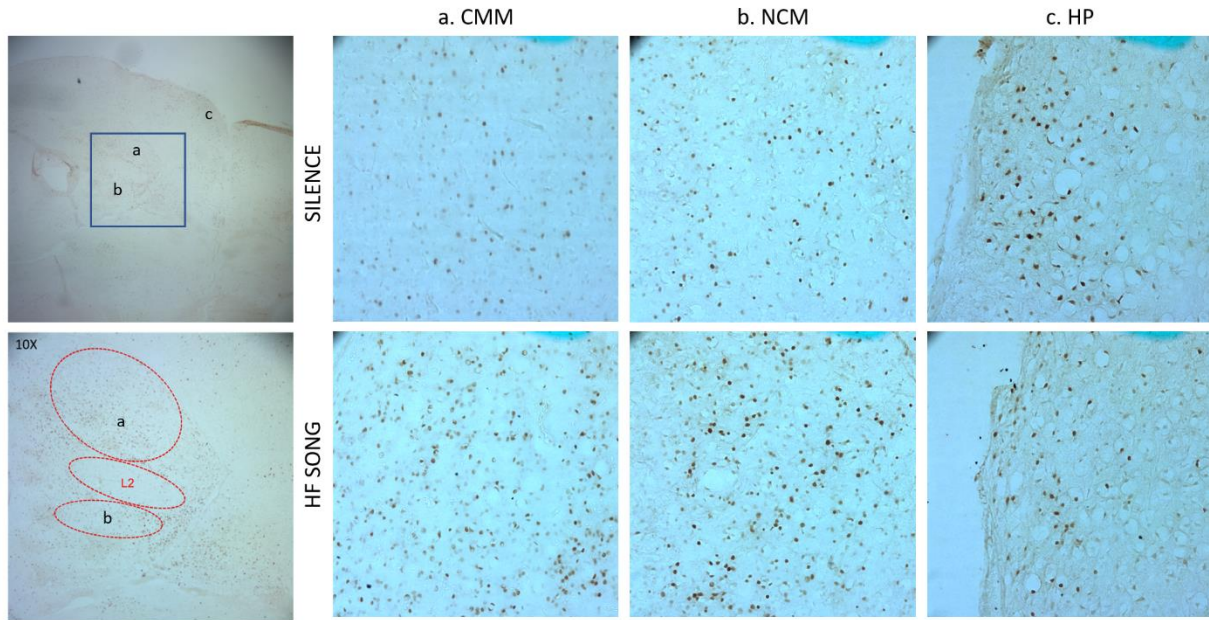
Figure 28. Auditory regions in the hummingbird brain
Top left panel shows representative section of the brain showing the secondary auditory areas CMM **(a)** and NCM **(b)**, and the hippocampus HP **(c)** in a hummingbird exposed to HF song. The blue square delineates the area magnified 10 times in the picture below to show the two secondary auditory areas and, as landmark, L2, the primary auditory region, which expresses little to no ZENK. Additional panels show representative magnified images of each region from brains exposed to silence and to HF song.

A two-way ANOVA showed significant effects of condition (HF song vs. silence), brain area, and the interaction between condition and brain region (*condition:* $F_{(1,162)}$ = 100.652, p < 0.001; *brain area:* $F_{(2,162)}$ = 42.420, p < 0.001; *interaction:* $F_{(2,162)}$ =5.631, p = 0.004). Birds exposed to HF song showed higher ZENK expression in CMM and NCM compared to the controls (*CMM: exp,* $\bar{x}$ = 187.2344 ± 10.2705, *cont,* $\bar{x}$ = 102.7333 ± 7.9956, p < 0.001; *NCM: exp,* $\bar{x}$ =168.4107 ± 7.3816, *cont,* $\bar{x}$ = 104.0689 ± 7.3816, p < 0.001) (**Figure 29**). There were no significant differences in ZENK expression in the hippocampus (HP) between the experimental and control groups (*exp,* $\bar{x}$ = 82.1053 ± 13.4020, *cont,* $\bar{x}$ = 54.7167 ± 3.7703, p = 0.2817). These results show that the auditory regions in the brain of

O. chimborazo responded robustly to the HF song of conspecifics, demonstrating that this

hummingbird species is adapted for high-frequency hearing.
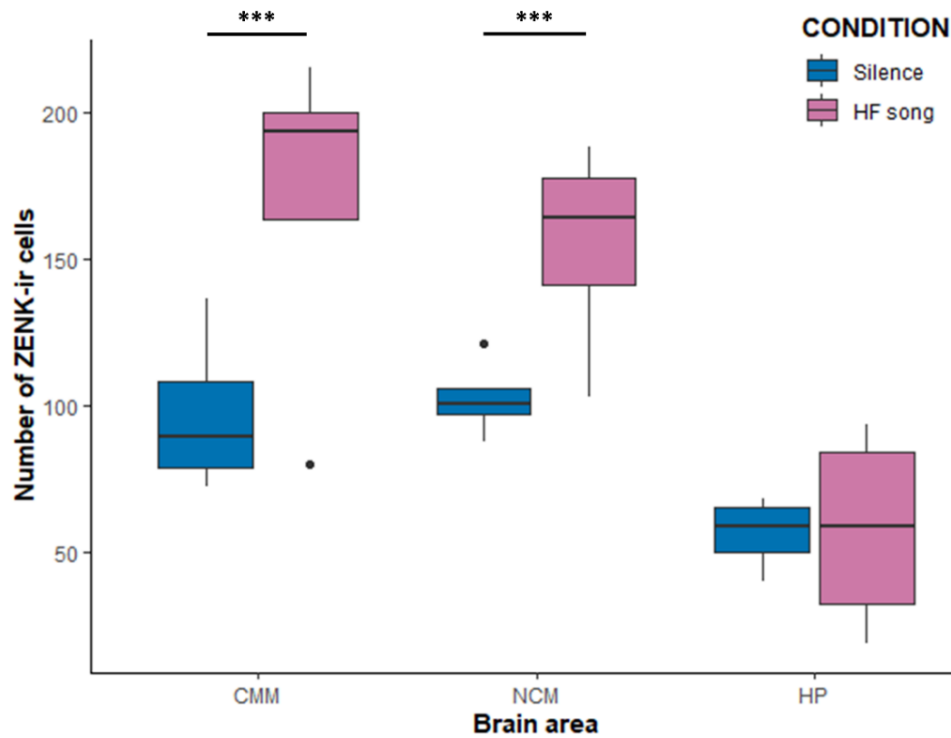
Figure 29. ZENK expression in auditory regions of the hummingbird brain.
ZENK protein expression in the brains of hummingbirds exposed to HF song (n = 4)
(purple) compared to hummingbirds exposed to silence (n = 4) (blue). The boxplot was produced
using median and Interquartile Range (IQR) as measures of centrality. Upper and lower whiskers in
the graph extended to the highest and lowest value, respectively, with a threshold set at 1.5*IQR;
any values beyond this threshold were plotted as outliers. Statistical analysis was done using a two-
way ANOVA, Tukey's HSD Post-hoc test, *** $p < 0.001$.

5.2.3   Neural activity in the SBN of the hummingbird brain in response to

playback of HF song

To evaluate sex-dependent neural responses to the playback of HF song, we

measured ZENK protein expression in key regions of the SBN (PVN, POM, LS, BSTm, and

TnA) in brains of male and female hummingbirds. To determine the overall effect of

acoustic stimulus regardless of sex, we conducted a Two-way ANOVA with experimental

condition (silence or HF song) and brain area as independent variables and the number of

ZENK-ir cells as dependent variable (n = 12, 5 controls and 7 experimental individuals). We

found no significant effect of experimental condition ($F_{(1,45)}$ = 1.846, p = 0.181), but a

significant effect of brain area ($F_{(4,45)}$ = 12.833, p < 0.001); no significant interaction

between the two variables was found ($F_{(4,45)}$ = 0.826, p = 0.516) (**Figure 30**). From the four

outliers shown in the graph, the high values in PVN and TnA in the experimental group

correspond to the same female hummingbird, which exhibited high levels of ZENK

expression for all brain regions evaluated in this part of the study.
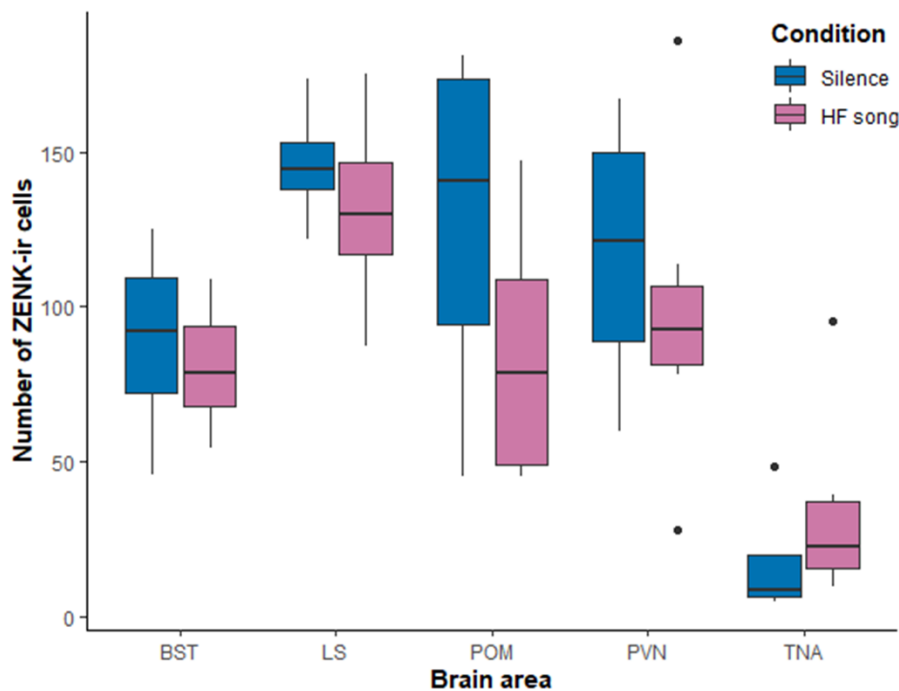
Figure 30. ZENK expression in SBN regions regardless of sex.
Males and females are grouped together in the control (n = 5) and experimental groups (n = 7), respectively. Statistical analysis was conducted using a Two-way independent measures ANOVA with Tukey's HSD post hoc test. No statistically significant difference was found.

We also evaluated patterns of ZENK expression in males and females independently, first to determine whether expression in any region was significantly different in experimental brains from controls. Thus, we conducted Welch's independent t-tests for each brain region between experimental and control males (n = 7, 4 experimental and 3 controls) and females (n = 5, 3 experimental and 2 controls), respectively. We found no statistically significant difference in ZENK expression between experimental and control brains in males or females. Nonetheless, analysis of ZENK expression in the POM of experimental vs. control males approached significance ($t_{3.21.97} = 2.9464$, p = 0.0552) (**Figure 31**).

Since statistical analysis did not show any significant difference between experimental and control brains in males or females, we did not conduct further statistical analysis to test the effect of sex in our current sample. In addition, data for the POM in control brains suggested a potential difference in ZENK expression in this region between control males and females (**Fig. 31**). A Welch's independent t-test showed no statistically significant difference ($t_{1.0429} = -2.9743$, p = 0.1981) in ZENK expression between the sexes despite the apparent trend from the data. In addition, hummingbirds in this experiment did not produce any vocalization in the control or experimental group during the time of the experiment.
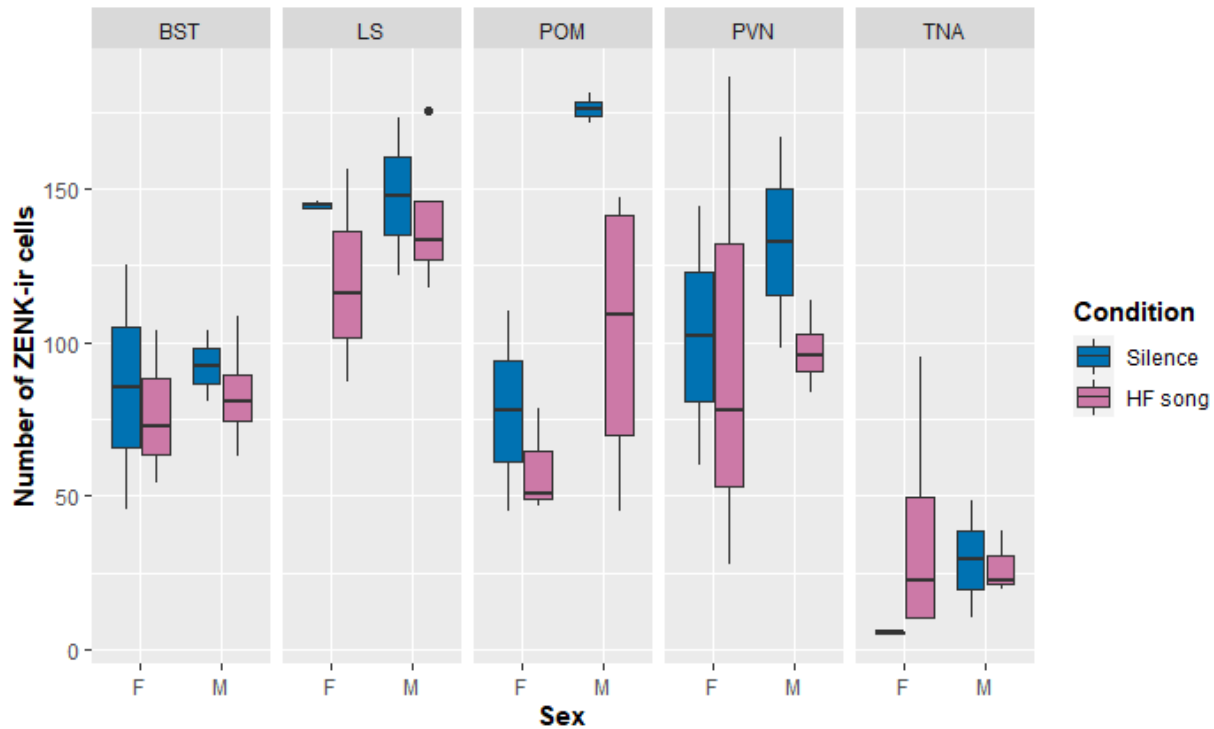
Figure 31. Sex-dependent neural activity in regions of the SBN in response to HF song.
We compared ZENK expression in the SBN of female (F) (n = 5, 2 controls and 3 experimental) and male (M) brains (n = 7, 3 controls and 4 experimental). Welch's independent t-tests were conducted for each sex and brain area. No statistically significant difference was found in any comparison.

5.3   Discussion

5.3.1   HF hearing in hummingbirds

To our knowledge, this is the first evidence of HF hearing in a bird except for some owls (Dyson et al., 1998) which are specialized predators. Unlike owls, which have evolved high-frequency hearing for predation, *O. chimborazo* evolved this feature for conspecific communication. Here, we showed that the Ecuadorian Hillstar (*O. chimborazo*) is adapted to hear its HF song (Endler, 1992). Individuals showed behavioral and neural responses to the HF content of conspecific song (frequencies > 10 kHz). We also report on the use of these vocalizations as part of the courtship displays that males present to females.

5.3.1.1 *Behavioral responses to HF song in the field*

During the behavioral assessment in the field, hummingbirds changed their attention and approached the speaker in response to playback showing that they can hear conspecific HF song. However, *O. chimborazo* hummingbirds did not show strong aggressive responses to the playback of the HF song, which suggests that a sound stimulus alone is not enough to evoke aggressive behavior. This pattern is consistent with our field observations in which *O. chimborazo* males and females do not engage in aggressive encounters with nearby individuals, unless the latter trespass the territory of the former. The use of multiple sensory signals in hummingbird communication has been documented in agonistic and courting contexts (Ficken et al., 2002; Hogan & Stoddard, 2018). Therefore, in this species, the HF song alone may not be enough to elicit a strong aggressive behavioral response in other males. This behavior may help conserve energy, so that the Hillstars engage in aggression with other birds only after they have visually confirmed the intrusion or aggression from another individual (Carpenter, 1976; Ficken et al., 2002).

Here, we also reported that *O. chimborazo* males produce their HF song while courting females in the female's territory. Our observations of the natural history of *O. chimborazo* show that most females tend to roost, forage, breed, and nest near creeks at lower elevations, in contrast to most males, which are usually found at higher elevations (Carpenter, 1976). The running water in these sites creates additional ambient noise, which can reach higher frequencies than those in the wind-dominated noise characteristic of the high-altitude grasslands (Duque et al., 2018). In other species of Andean hummingbirds which also produce HF vocalizations, the profile of the ambient noise suggests that these species evolved vocalizations at high frequencies to avoid signal

masking in their noisy habitat (Duque et al., 2018). This evidence paired with behaviors

produced while vocalizing suggest that *O. chimborazo* males evolved a HF song to avoid

ambient noise in the female's territory during the courtship process. Just like it occurs in

other species, songs can also be used to signal territoriality (Bradbury & Vehrencamp,

2011), which seems to be the case in this species when males vocalize in their own

territories at higher elevations (Duque et al., 2018).

5.3.1.2   *Neural responses to HF song in the forebrain auditory regions*

Our evidence on the neural responses in the secondary auditory regions (CMM and

NCM), which are sensitive to species-specific sounds (M. T. Avey et al., 2008; Hoke et al.,

2004), also show that *O. chimborazo* can hear its HF song. These findings are aligned with

electrophysiological evidence in other species showing that specialized neurons in the

brain auditory nuclei are tuned to spectral and temporal parameters of conspecific songs

(Rose & Capranica, 1984; Wilczynski & Ryan, 2010).

Future studies should focus on identifying sexual differences in the neural activity of

the secondary auditory regions in response to HF song. CMM and NCM are critical for

discrimination between biologically relevant sound stimuli and non-relevant sounds (Amin

et al., 2007; Gentner et al., 2001). Studies looking at the neural responses in the auditory

regions CMM and NCM of male and female brains have shown clear sex-dependent activity

in these regions. In zebra finches (*Taenopigyia guttata*), males have greater expression of

ZENK mRNA in NCM while female neural responses are more robust in CMM (Lampen et

al., 2017). In black-capped chickadees (*Poecile atricapillus*), males have higher ZENK levels

than females in response to conspecific song and calls (M. T. Avey et al., 2008). In addition,

CMM exhibited a complex response dependent on the type of vocalization and sex of the

producer and receiver. Moreover, the forebrain auditory regions are subjected to the

modulatory effects of gonadal steroid hormones and serotonin (Hurley & Hall, 2011;

Maney et al., 2003, 2008; Maney & Pinaud, 2011). Altogether, evidence shows that males

and females perceive conspecific song differently which can result in different behavioral

responses to an acoustic signal. In the Ecuadorian Hillstar, the HF song has a sex-dependent

function compared to other vocalizations in the species' repertoire. Thus, it is important to

understand how auditory processing of this song modulates downstream neural activity,

and consequently, sex-dependent behavioral responses to this social signal.

In addition, studies in other animals have shown that the best frequency sensitivity

for hearing in a species usually matches the dominant frequency in its vocal repertoire

(Green & Swets, 1966; Rose & Capranica, 1984; Wilczynski et al., 1992; Wiley, 2013).

Similar findings have been reported for some anurans that produce ultrasonic sounds, and

whose hearing matches vocal production (Arch & Narins, 2009; Feng et al., 2006). A study

evaluated the hearing curve of the Blue-throated Hummingbird (*Lampornis clemenciae*)

(Pytte et al., 2004), which produces high-frequency sounds as part of its song. The best

frequency sensitivity was between 2–3 kHz in this hummingbird, rapidly declining after

that and not reaching the high frequencies in some components of the song. Nonetheless,

the auditory sensitivity in *L. clemenciae* matched the dominant frequency of the low-

frequency notes in its song, still consistent with previous findings. In contrast to the song of

L. clemenciae, the song of the Ecuadorian Hillstar is produced almost entirely in the shigh-

frequency range, with only a single note reaching as low as 7 kHz, while all other elements

are produced above 10 kHz. We showed neural responses in the forebrain auditory regions

of these hummingbirds which demonstrates that they can hear the HF song, however, the

best frequency for auditory sensitivity in the Ecuadorian Hillstar (*O. chimborazo*) remains

to be determined. Characterizing the hearing curve in this species will allow us to test for

trade-offs in hearing capabilities between low and high frequencies in species producing

HF vocalizations as part of their vocal repertoire (Henry et al., 2011).

Our results suggest that hummingbirds producing HF vocalizations (Duque et al.,

2018; C. R. Olson et al., 2018) have evolved adaptations for the production and perception

of HF sounds, which may not be present in other birds. Recently, two independent research

groups (Monte et al., 2020a; Riede & Olson, 2020) reported on the unusual position of the

avian vocal organ, the syrinx, in hummingbirds. While in most birds, the syrinx is located

inside the thoracic cavity, hummingbirds have a syrinx which is located outside the thorax;

a feature we have also observed in *O. chimborazo*. The position of the syrinx (Riede &

Olson, 2020) paired with other musculature differences (Monte et al., 2020a) may change

the mechanical properties of the vocal organ as well as air pressure while vocalizing,

facilitating the production of HF sounds. In some species of hummingbirds, which have also

encountered environmental pressures to avoid signal masking in acoustically challenging

environments (Duque et al., 2018), these conditions may have come together to promote

the evolution of HF vocalizations as part of the vocal repertoire of these species.

5.3.2   *Sex-dependent neural responses to HF song in the Social Behavior Network (SBN) of the hummingbird brain*

Our assessment of sex-dependent neural activity in regions of the SBN did not yield

any significant results. However, despite the lack of statistical significance, there is an

apparent trend in which ZENK expression in most SBN regions evaluated here is lower in

animals exposed to the playback of HF song than in control brains, exposed to silence (**Fig.**

30). This pattern seems to be reversed in TnA, where ZENK expression is higher in experimental brains. Grouping individuals by sex shows that this pattern is consistent throughout SBN regions in both sexes, except for TnA, where expression seems to be higher in experimental females compared to their controls but no trend in either direction is present between control and experimental males. Furthermore, the data suggested a potential difference in ZENK expression in POM in the brains of males exposed to the HF song compared to controls (**Fig. 31**), which was not supported by the statistical analysis. This case emphasizes the need of increasing our sample size in order to detect meaningful differences between groups.

High levels of activity in BSTm have been associated with regulating social signals in breeding and non-breeding contexts together with activity in LS (Goodson et al., 2005; Heimovics & Riters, 2007). This evidence suggests that BSTm may not be selective to process responses to breeding contexts only, rather to regulate behavior in general social contexts in territorial animals. Photostimulated white-throated sparrow (*Zonotrichia albicollis*) females and those that received estrogen implants exhibited higher levels of ZENK expression in BSTm after exposure to male song compared to controls exposed either to silence or tones (Maney et al., 2008). These findings show that estrogen affects selectivity to the acoustic stimulus to which females respond. However, we did not observe any patterns of ZENK expression in BSTm in experimental females that suggest that this brain region was stimulated by playback of HF courtship song, despite collecting females during the breeding season.

In European starlings (*Sturnus vulgaris*), ZENK expression in the LS is negatively correlated with territoriality and aggression, including production of territorial songs

(Heimovics & Riters, 2007). Low levels of ZENK expression in LS in male and female

hummingbirds, which are naturally territorial, may suggest that playback of HF song could

be perceived as a territorial intrusion, particularly for males. Females that are not in the

appropriate internal state to be receptive to male courtship song probably due to stress or

because HF song is not paired with the appropriate social context, may also perceive this

song as an intrusion.

In contrast, our data on the POM showed interesting trends. There were apparent,

but non-significant, differences in ZENK expression in this region between control males

and females, suggesting that this region may respond differently to non-social stressors

such as handling and captivity. In addition to its role in promoting sexual and courtship

behavior in male birds (Dominguez & Hull, 2005; Goodson, 2005), this region is also

involved in song production during the breeding season (Riters et al., 2000). Our results

showed an interesting trend that almost reached significance ($p = 0.06$) in which

experimental males exhibited lower ZENK levels in POM compared to their controls. This

trend may result from males perceiving playback of Hf song as an intrusion from a

potential competitor. Such perception may be a deterrent from pursuing sexual behaviors

and instead prioritizing aggressive over sexual responses. This interpretation could be

further supported by observed lower levels of ZENK expression in the LS of males exposed

to HF song compared to controls, consistent with studies of ZENK expression in the LS of

territorial or dominant males.

Parvocellular neurons in the PVN produce the neuropeptide arginine vasotocin

(AVT, in birds) or arginine vasopressin (AVP, in mammals) which act as key regulators of

the Hypothalamic-pituitary-adrenal (HPA) axis resulting in the downstream production of

glucocorticoids (Landgraf & Neumann, 2004). Studies of restraint stress in rats have shown

increased AVP mRNA in PVN neurons (Bartanusz et al., 1994). Furthermore, PVN

parvocellular cells increase corticotropin-releasing hormone (CRH) type 1 receptor mRNA

in response to acute stressors (Imaki et al., 2001). This finding shows that in addition to its

regulatory function of the stress response, PVN is highly sensitive to the effects of CRH,

creating a positive feedback loop in response to acute stressors. In our study, the wide

variation in ZENK expression in the PVN in females may be the result of handling stress

rather than a response to the acoustic stimulus. A larger sample size will help us elucidate

the effects of the HF song in the activity of this region in the brains of females despite the

effects of stress.

On the other hand, ZENK expression in TnA appeared to follow an opposite pattern

than those of the other regions of the SBN. Females exposed to HF song seemed to express

higher levels of ZENK in TnA compared to control females, while ZENK levels in males are

similar in this region regardless of experimental condition. The TnA of male and female

birds exhibits robust activation in response to vocalizations compared to noise, even when

the animals are under the effects of anesthesia (Fujii et al., 2016). Likewise, increased

activity in TnA has been observed in birds exhibiting copulatory behaviors (Riters et al.,

2004). Interestingly, females that were photostimulated or implanted with exogenous

estradiol exhibited higher levels of ZENK expression in TnA in response to song compared

to silence and tones, but these results negatively correlated with the number of copulation

solicitation displays produced (Maney et al., 2008). This pattern shows that TnA is involved

in evaluation and processing of acoustic social stimuli, but activation of this area does not

necessarily translate to production of sexual behaviors in females.

Finally, the effects of handling and captivity that were necessary to conduct our experiment will have to be considered in the final interpretation of the results, once the sample size has been increased. Evidence shows that regions in the SBN are not only sensitive to social stressors but also to non-social stressors such as restraint (Cook, 2004; Goodson & Evans, 2004; Imaki et al., 2001). Our own experience with hummingbirds and that of other researchers suggest that hummingbirds do not respond well to captivity; a pattern that can be observed in other species of wild animals undergoing controlled studies (F. Duque, personal observations; Clark C.J., personal communication; Fernandez, R., personal communication).

5.3.3   Conclusion

Altogether, the behavioral responses and the neural activation of critical auditory regions in response to HF song as well as the context in which these vocalizations are produced point to the use of HF vocalizations in conspecific communication in *O. chimborazo*. Our results constitute the first evidence of the use of HF vocal signals and HF hearing in conspecific communication in hummingbirds and any birds. The presence of HF vocalizations in hummingbirds offer a new avenue to study the morphological, peripheral, and central sensory adaptations that facilitate communication and social interactions. These findings also contribute to broadening our understanding of the coevolution of vocal signals and auditory perception to facilitate social interactions in acoustically challenging conditions.

Our study of sex differences in the neural responses of regions in the SBN to a signal with dual function of courtship and territoriality in hummingbirds did not provide statistically significant results. However, it unveiled interesting patterns of neuronal

activity that require further exploration by increasing the sample size in all experimental

groups. Studying how hummingbirds perceive and integrate acoustic social signals will

increase our understanding of the use of vocal communication in mediating complex social

interactions in species in which both sexes exhibit high levels of aggression.

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was involved in the conceptualization, methodology, data collection and analysis in both

manuscripts. I also wrote the initial draft and led the revision of new versions of the

manuscripts until their publication.

6   CONCLUSION

High-frequency vocalizations in hummingbirds are a newly discovered phenomenon that offers opportunities to investigate the evolution of vocalizations and adaptations for their use in communication. The function of HF vocalizations is likely species dependent; however, our observations show that they are used mostly in territorial behavior, and in some species, during courtship like in the Ecuadorian Hillstar. Moreover, the presence of dialects in the courtship HF song of the Hillstar suggest that sexual selection has also played a role in selecting for these signals. Thus, it is likely that female preference for local dialects is acting as a premating barrier between populations of the two subspecies in a contact zone. It also highlights the role of vocal learning in creating signal diversity in hummingbirds, one of the three groups of birds in which this ability has evolved independently.

This research also shows that body size is not an important constraint, as it is in other animals, for vocal production in hummingbirds. We found no correlation between body mass and frequency of vocal production, showing that the production of high-frequency vocalizations is not constrained to the smallest hummingbird species. These results emphasize that high-frequency vocalizations likely result from morphological adaptations in the syrinx rather than from an acoustic allometry alone. It remains to be determined what how the syrinx of these hummingbirds has evolved to enable the production of extraordinary high-frequency sounds in some species of hummingbirds.

Our assessment of ambient noise and habitat structure as ecological factors influencing the evolution of high-frequency vocalizations showed that these signals are likely used for short-range communication. Moreover, it supports the hypothesis that some

hummingbirds have evolved high-frequency vocalizations to avoid signal masking by ambient noise, especially for hummingbirds living in the cloud forest. Our observations in the field suggest that this is likely the case even for the Ecuadorian Hillstar in the high-altitude grasslands. In their habitat, males do not face strong competition from other animals to broadcast their HF signals, however, the noise produced by creeks in females' territories may be responsible for the evolution of a HF courtship song in this species.

Multiple studies in birds had shown that hearing in these animals is restricted to a few frequencies below 8 kHz, raising the question about hummingbird hearing in the species that produce HF vocalizations. Here, we showed behavioral and neural evidence of HF hearing in hummingbirds and the use of HF vocal signals in communication. This finding shows that these birds have adapted to communicate in the high-frequency range, unlike other birds. Finally, we analyzed neural responses to HF song in regions of the brain's SBN in males and females. We did not obtain any significant results, most likely due to the small sample size, but we observed interesting patterns of activity in these regions that deserve further exploration.

The discovery of HF vocalizations in hummingbirds has challenged our current knowledge and understanding of hearing in birds. Furthermore, it has renewed the interest for studying the complexity of vocal communication in these birds, which for a long time was dismissed as extremely simple.

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APPENDICES

Appendix A. Signal transmission in diverse habitats

Here we present distribution plots on experimental data for transmission experiments to measure attenuation, spectral degradation, and temporal degradation in **A)** *A. melanogenys*, **B)** *B. flavescens,* and **C)** *O. chimborazo.* Black line shows the group median, while red dashed line shows the group mean. For attenuation, the x-axis shows the normalized values of amplutide (norm_db). For spectral correlation, x-axis shows values of spectral correlation (SPEC_CORREL), and for temporal degradation, x axis shows the average change in amplitude over time, (dv_mean).
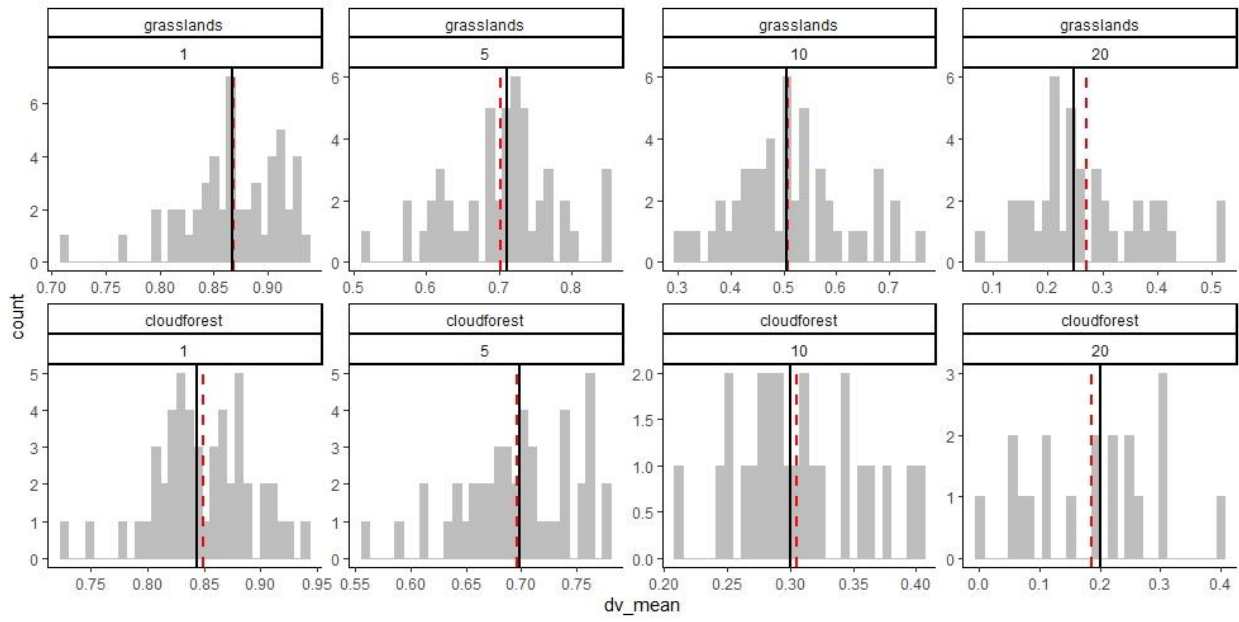
Appendix A.1. Distribution plots for experimental data on attenuation
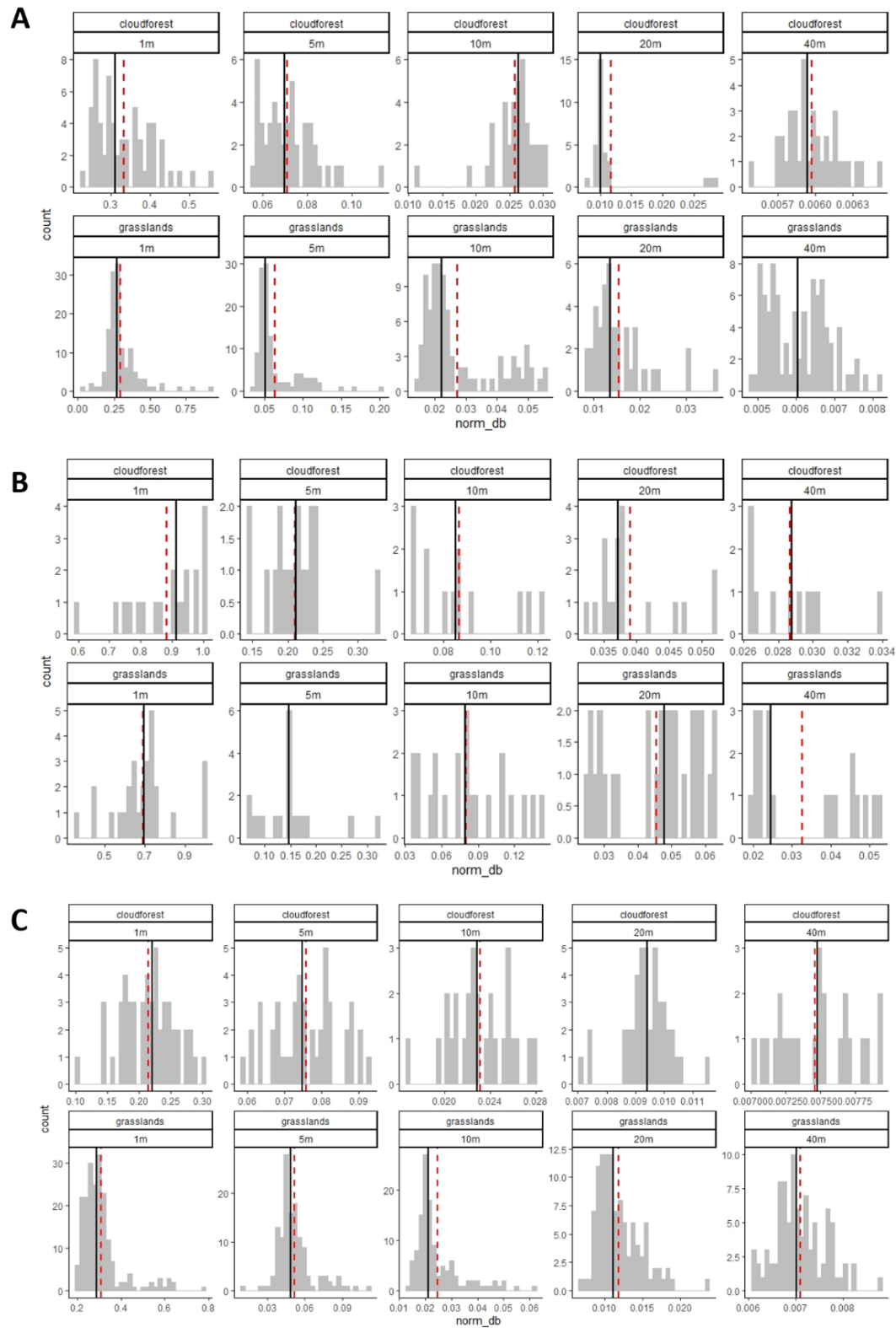
Appendix A.2. Distrubution plots for data on spectral degradation
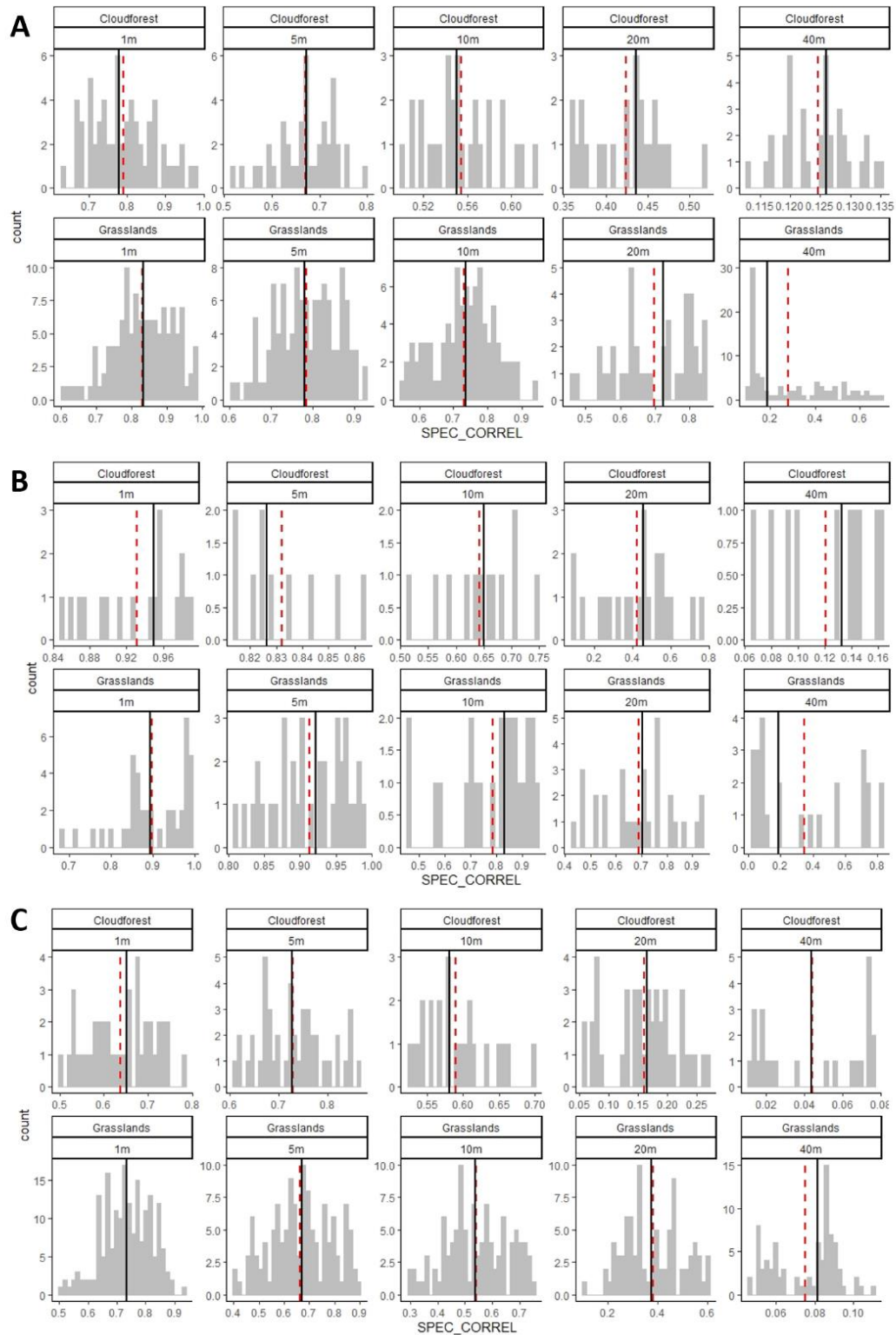
Appendix A.3. Distribution plots for data on temporal degradation

Appendix B. Result of relevant pairwise comparisons for experimental data on signal transmission

Appendix B.1. Sound attenuation of HF vocalizations

   Normalized values of sound amplitude for HF vocalizations of the three species of hummingbirds across distance in the cloud forest and the grasslands. SEM = standard error of the mean.

Species	Distance (meters)	CLOUDFOREST			GRASSLANDS		
		N	Normalized Amplitude (dB)	SEM	N	Normalized Amplitude (dB)	SEM
Adelomyia melanogenys	1 m	63	0.3329	0.009	136	0.2943	0.0097
	5 m	47	0.0713	0.0017	115	0.0635	0.0026
	10 m	38	0.0258	0.0006	92	0.0273	0.0012
	20 m	28	0.01	0.0001	41	0.0154	0.0009
	40 m	37	0.006	0	90	0.0061	0.0001
Boissonneaua flavescens	1 m	20	0.9015	0.0343	28	0.7597	0.0647
	5 m	18	0.2106	0.0098	19	0.1476	0.0143
	10 m	13	0.0868	0.0053	22	0.0804	0.0069
	20 m	22	0.0375	0.0014	30	0.0455	0.0023
	40 m	11	0.0287	0.0007	20	0.0325	0.0028
Oreotrochilus chimborazo	1 m	51	0.2146	0.0059	204	0.3069	0.0067
	5 m	45	0.0759	0.0013	133	0.0518	0.0013
	10 m	27	0.0231	0.0005	123	0.0247	0.0008
	20 m	43	0.0094	0.0001	110	0.0118	0.0003
	40 m	24	0.0075	0.0001	90	0.0071	0.0001

Appendix B.2. Spectral degradation of HF vocalizations over distance

Values of spectral correlation for recordings across distance compared to a reference library. A spectral correlation of 1 shows no change in spectral content with respect to the library, while values closer to zero show increased spectral degradation.

SPECIES	DISTANCE (meters)	CLOUD FOREST			GRASSLANDS		
		N	Spectral correlation	SEM	N	Spectral correlation	SEM
Adelomyia melanogenys	1 m	63	0.7896	0.0104	121	0.8314	0.0077
	5 m	42	0.6713	0.0094	115	0.7855	0.0069
	10 m	28	0.5545	0.0060	89	0.7320	0.0092
	20 m	24	0.4240	0.0086	41	0.6966	0.0170
	40 m	35	0.1247	0.0009	90	0.2817	0.0192
Boissonneaua flavescens	1 m	18	0.9314	0.0110	42	0.8984	0.0121
	5 m	10	0.8323	0.0052	36	0.9133	0.0084
	10 m	12	0.6423	0.0189	25	0.7858	0.0289
	20 m	22	0.4215	0.0394	31	0.6884	0.0257
	40 m	10	0.1209	0.0108	25	0.3455	0.0605
Oreotrochilus chimborazo	1 m	41	0.6385	0.0116	190	0.7333	0.0062
	5 m	45	0.7296	0.0100	126	0.6644	0.0109
	10 m	23	0.5901	0.0096	103	0.5414	0.0114
	20 m	43	0.1610	0.0087	86	0.3825	0.0128
	40 m	24	0.0440	0.0055	84	0.0751	0.0018

Appendix B.3. Temporal degradation in the HF song of O. chimborazo

Values of the ratio of amplitudes (dV) for the syllable to the inter-syllable intervals for the HF song at each distance in the cloud forest and the grasslands. A lower dV value shows increased degradation.

Distance (meters)	CLOUD FOREST			GRASSLANDS		
	n	dV	SEM	n	dV	SEM
1 m	50	0.8488	0.0062	51	0.8676	0.0064
5 m	45	0.6962	0.0076	50	0.7013	0.0102
10 m	25	0.3052	0.0097	50	0.5087	0.0150
20 m	19	0.1850	0.0248	42	0.2708	0.0157