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Nectarivory as a syndrome in birds: insights from feeding morphology,
genetics, and nutritional ecology

By

Cynthia Y. Wang-Claypool

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Rauri C. K. Bowie, Chair

Professor Jimmy A. McGuire

Professor Robert Dudley

Spring 2024

Nectarivory as a syndrome in birds: insights from feeding morphology,
genetics, and nutritional ecology

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By

Cynthia Y. Wang-Claypool

Abstract

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Doctor of Philosophy in Integrative Biology

University of California, Berkeley

Professor Rauri C. K. Bowie, Chair

Nectar-feeding birds have enchanted scientists for centuries due to the remarkable morphological similarities between geographically and phylogenetically distant lineages, extreme life histories, and adaptations to a sugary diet. Though many characters have been attributed to the success of this ecological niche, no singular adaptation is shared across all radiations. Nectarivores range greatly in terms of body size, bill size and shape, tongue morphology, ecology, feeding behavior, and diet composition. Here, I assess nectarivory in birds as a syndrome through the lenses of feeding morphology, genetics, and nutritional ecology. I sought to better understand the evolution of nectarivory by investigating three different aspects of nectar-feeding adaptations: (1) tongues for transporting nectar into the throat; (2) the evolution of genes underlying bill morphology; and (3) the ecology of ethanol in the diets of birds. In chapters 1 and 2, I explore the skeletal morphology of the feeding apparatus and the genetic underpinnings of bill size and shape in Sunbirds (Passeriformes: Nectariniidae). In Chapter 3, I measure the prevalence of ethanol, a by-product of the fermentation of the sugars in nectar, with a focus on birds that feed on nectar and fruit. Specifically, I compare the prevalence of dietary ethanol within several species of hummingbirds and between hummingbirds and other bird species that serve as exemplars of other diet categories.

In Chapter 1, I describe the tongue apparatus in sunbirds and explore the patterns of size and shape variation in the morphology of the skeletal elements using several phylogenetic comparative methods. When compared to their non-nectarivorous relatives, sunbirds have evolved a highly modified tongue apparatus to feed on nectar, yet sunbird species vary in the degree to which they specialize on nectar. To better understand the evolution of sunbird tongues, I first described the sunbird tongue apparatus and the function of key components by reconstructing the skeletal elements and associated soft tissues *in situ* of an Olive Sunbird (*Cyanomitra olivacea*). The functional significance of sunbird tongue morphology in nectar-feeding was hypothesized by comparing it to previous studies of avian tongue anatomy and function. Then I investigate patterns in morphological diversity across sunbirds and the relationship between size and shape, phylogeny, and feeding ecology by reconstructing the

tongue skeleton from 41 CT scans representing sunbirds (Nectariniidae), flowerpeckers (Dicaeidae), and their relatives (Irenidae, Chloropseidae, Modulatricidae, Promeropidae). Two-dimensional and three-dimensional morphometrics were used to quantify size of the individual skeletal elements and the shape of the basihyal-urohyal. Two-dimensional data were supplemented with previously published bill measurements and body masses in the AVONET dataset. The morphology of the Olive Sunbird tongue apparatus supports previous hypotheses that sunbirds feed on nectar by generating a negative-pressure space (hermetic suction) with the tongue body and upper palate. I also describe a structure on the paraglossalia unique to sunbirds that likely provides structural support to the tongue body. The morphometric results suggest a complex interplay between bill size, tongue size and shape, and diet (as a proxy for feeding behavior) that is partially driven by phylogenetic history. Diet predicts shape depending on how body mass is included as a co-variable. Lastly, I compare the tongue apparatus between other nectarivorous radiations - hummingbirds and Australian honeyeaters - and discuss the differences in form and function of nectar-feeding adaptations across birds.

In Chapter 2, I explore whether known candidate genes for bill morphology are implicated in the evolution of sunbird bills. Selection is expected to have occurred at the ancestral branch of sunbirds after the split from flowerpeckers (Dicaeidae) which coincides with an increase in bill length. I surveyed genes previously implicated in studies of bill size and shape. I generated sequence data of 11 candidate genes and phylogenetically informative sites for 139 taxa using a target capture method, including sunbirds (Nectariniidae), flowerpeckers, and several outgroups. Using a multilocus phylogeny constructed from the same individuals, exonic regions of these genes were tested for positive selection at the site level and at the branch level. I found evidence of episodic positive selection at a total of 28 sites across the protein *ALX1*, *BMP4*, *LPP*, *RNASEH2B*, and *SHH*. Fourteen sites were detected in *LPP* alone, six of which occurred in one exon, and seven were detected in *RNASEH2B*. No statistically significant, elevated rates of evolution was detected for the ancestral branch of sunbirds, nor for any other clades with elongated beaks. Although positive selection was not detected where I hypothesized, the results do not preclude these genes potentially being implicated in phenotypic changes in other ways (e.g., changes in gene expression).

Chapter 3 focuses on the nutritional ecology of feeding on nectar. Ethanol is a naturally occurring molecule produced via fermentation of sugar-rich foods by yeast. Birds that regularly consume fruit and nectar may accordingly be ingesting ethanol on a chronic basis, although this possibility has not been documented in the wild. However, ethanol catabolism is followed by production of secondary metabolites such as ethyl glucuronide (EtG), which can be measured to provide a retrospective view of dietary exposure. We hypothesized that avian species with diets rich in fermentable sugars consume non-trivial amounts of ethanol, and therefore accumulate detectable levels of EtG. Assays for the presence of EtG in avian feathers yielded positive results for 10 of 17 avian species; EtG was also present at substantial levels in the livers of two of five study species. We found that EtG was present in nectarivorous hummingbirds, but also in species in other trophic niches (3 granivores, 1 omnivore, 3 invertivores, and 1 vertivore). Dietary exposure to ethanol may thus be much more widespread than has previously been recognized, and diverse features of avian nutritional ecology (e.g., secondary consumption via ingestion of prey items) may contribute to its accumulation in tissues.

Dedication

*To Stefan Michael Claypool
for being there every step of the way*

Always

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I am deeply grateful to the family members, friends, and mentors around the world who have supported me on my journey. I feel fortunate to have made so many connections with you all!

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General Introduction

The striking similarities of nectar-feeding adaptations across many groups of birds around the world have inspired questions for centuries. It has been estimated that nectarivory has evolved upwards of 22 times across the avian phylogeny (Pauw 2019). While many lineages of birds feed opportunistically from flowers, it is the specialists that provide opportunity to ask questions about the myriad ways in which nectarivory has evolved, and what tradeoffs were made in other aspects of their natural history to facilitate nectarivory.

Despite the similarities between the many radiations of nectarivorous birds, no single adaptation is exclusive to this ecological niche and the extent of convergence is variable depending on the trait of interest. Tubular tongues unite many nectarivores (e.g., hummingbirds, sunbirds, Australian honeyeaters, and flowerpiercers), but not all, and their tongues vary greatly in structure upon closer examination. Even the presence of tongue bristles, which is often attributed to a diet of nectar, can be found on the tongues of non-nectarivorous birds (see figures in Gardner 1925). Some proposed adaptations for nectarivory also vary greatly within a lineage, as is the case with tongue morphology in sunbirds. Several traits have been elucidated through studies of feeding biomechanics of hummingbirds (Rico-Guevara 2017), Australian honeyeaters (Hewes et al. 2023) and sunbirds (Cuban et al., in prep). Although recent studies on nectar-feeding birds have redefined our understanding of how birds feed on nectar, many aspects of the evolution of nectarivory remain incomplete with many questions unanswered.

For my dissertation, I assessed from several perspectives how nectarivory fits the definition of a syndrome: when distantly related species evolve a collection of correlated traits, often in response to an evolutionary driver (Sinnott-Armstrong et al. 2022). This serves as a framework around which a deeper understanding of traits relevant to nectar-feeding, their underlying mechanisms, and the factors driving their evolution in a given radiation can be inferred. Data on these traits are subsequently analyzed with regard to variation within the focal clade and to related taxa, then compared to what is currently known for said traits.

Background on Sunbirds (Passeriformes: Nectariniidae), Flowerpeckers (Passeriformes: Dicaeidae), and Hummingbirds (Caprimulgiformes: Trochilidae)

Sunbirds (Nectariniidae) are a large radiation of small passerine birds distributed throughout much of Africa extending to the Middle East, India, South-east Asia, and northern Australia (Cheke and Mann 2001; Winkler et al. 2020a). A majority of the over 145 species in the family consume nectar, small insects, and spiders in varying proportions, with some species also feeding on pollen, fruit, and berries (Cheke and Mann 2001; Winkler et al. 2020a). A non-exhaustive list of dietary outliers include: *Euchloridia fraseri* which is thought to be entirely insectivorous (Cheke and Mann 2020a, 2001); *Nectarinia famosa* is noted to have consumed small lizards (Cheke and Mann 2020b, 2001); small snails and nematodes have been found in the stomach contents of *Cinnyris notatus* (Cheke and Mann 2001; Kirwan et al. 2021) and *Anthodiaeta collaris* (Cheke and Mann 2020c); tree sap in *Cinnyris amethystina* (Cheke and Mann 2020d) and *Cinnyris coccinigastrus* (Cheke and Mann 2020e); bits of flowers in *Cinnyris chloropygius* (Cheke and Mann 2020f); and seeds, perhaps incidentally, in *Arachnothera magna* (Amar-Singh et al. 2023); *Cinnyris notatus* (Kirwan et al. 2021); *Cyanomitra verticalis* (Cheke and

Mann 2020g); *Euchloridia seimundi* (Cheke and Mann 2020h); *Kurochkinogramma hypogrammicum* (Cheke and Mann 2020i); and *Leptocoma brasiliana*, (Hoyo et al. 2020). A few species are also known to be nectar-robbers (Dhanya et al. 2013; Fujita 2000; Jayanthi et al. 2015). Bill size and shape in sunbirds varies in length and curvature, from short and straight with a slight curve at the tip, to long and decurved (Cheke and Mann 2001). Their tongues, specifically the keratinous portion, are tubular and vary in terms of total length, degree of bifurcation and fringe-like structure of the tongue tip, and shape and structure of the tongue body (Cheke and Mann 2001). Variations in tongue structures are likely correlated with differences in the biomechanics of feeding between sunbirds and hummingbirds. Sunbirds are hypothesized to employ hermetic suction to extract nectar (D. Cuban unpubl. data; Cuban et al. 2022) whereas hummingbirds use elastic filling (Rico-Guevara et al. 2015) and fluid trapping (Rico-Guevara and Rubega 2011).

Flowerpeckers (Dicaeidae), the sister-group to sunbirds, are found in India, Southeast Asia, and Australia (Winkler et al. 2020b). They primarily consume fruit and nectar with their short, thick, and slightly curved bills (Winkler et al. 2020b). Other food items include seeds and arthropods, though many species prefer mistletoe berries (Winkler et al. 2020b). In contrast to sunbirds, their tongues range from broad, flat, and non-tubular, to long, narrow and tubular (Cheke and Mann 2001).

Hummingbirds (Trochilidae) are a highly speciose family of caprimulgid birds found throughout the Americas, including the Caribbean, but primarily in Central and South America (Winkler et al. 2020c). Bill size and shape of the over 360 species is highly variable, varying in length from one head length to more than the length of the body as well as in curvature from slightly recurved to straight to strongly decurved (Winkler et al. 2020c). In contrast to sunbirds, hummingbirds subsist only on nectar and small insects and other invertebrates (Winkler et al. 2020c) and variation in their tubular tongues is primarily in the length of certain sections (Rico-Guevara 2014). Hummingbird and sunbird tongues differ in terms of how the tubes are formed. Moving from the proximal tongue to the distal tip, in sunbirds the lateral margins curl dorsomedially to form a pair of tubes much like the Greek letter omega (ω), whereas in hummingbirds the sides of the tongue invaginate to form laterally inverted C-shapes (ω ; Paton and Collins 1989). The skeletal elements also vary, with the length of some bones being held constant regardless of bill length while the length of others are not (Zusi 2013). Recent studies have changed our understanding of how they feed. For over a century it was believed that nectar flowed through their tubular tongues via capillary action (Gardner 1925; Kim et al. 2012; F. A. Lucas 1895; Scharnke 1932). However, the work by Rico-Guevara and colleagues determined that nectar is transported through a series of mechanisms involving both the tongue and bill working in unison (Rico-Guevara et al. 2015, 2023; Rico-Guevara and Rubega 2011, 2017).

The independent natural histories yet superficially convergent adaptations for nectarivory of sunbirds and hummingbirds lend themselves well to different types of questions about the evolution of nectar-feeding in birds. Sunbirds, with their diverse yet primarily nectarivorous diet and variable tongue shape, and their relatives including the flowerpeckers are a good system in which to test the relationship between diet, morphology, and phylogeny. Additionally, sunbirds are also more suitable for investigations into the genetic basis of bill morphology as much of the work has been done in oscine passerine birds, of which sunbirds are a member. On the other hand, because the diet of hummingbirds is more restricted, they are better suited to questions regarding the nutritional ecology of nectar.

Of particular interest in my dissertation are patterns in how nectar-feeding birds interact with their environment compared to non-nectarivorous birds based on their internal feeding anatomy, the genetic mechanisms coding for their long and thin bills, and their nutritional ecology. My dissertation is composed of three chapters: (1) Adaptations to nectarivory and patterns of morphological diversity in the hyoid apparatus of sunbirds; (2) Evaluating candidate genes for bill morphology in sunbirds and relatives; and (3) The proof is in the plumage: dietary ethanol exposure in birds revealed by evaluating ethyl glucuronide levels in feathers. The first two chapters focused on sunbirds (Passeriformes: Nectariniidae) of the eastern hemisphere. The third chapter focused on hummingbirds (Caprimulgiformes: Trochilidae) of the western hemisphere. I addressed the following knowledge gaps: (1) the relationship between the anatomy of the protrusible tongue apparatus, phylogeny, ecology, and feeding mechanism in sunbirds and relatives; (2) the evolutionary patterns in known candidate genes for bill morphology in sunbirds and relatives; and (3) the prevalence of ethanol in the nutritional ecology of nectarivorous birds relative to non-nectarivorous birds in North America.

Chapter 1

Adaptations to nectarivory and patterns of morphological diversity in the hyoid apparatus of sunbirds

1.1 Introduction

The avian tongue plays a central role in the feeding behaviors of many groups of birds. The size and arrangement of the tongue apparatus (the tongue and its associated musculature and skeletal elements) influence its range of motion. In turn, these factors affect a bird's ability to drink and to grasp, manipulate, and swallow food. Woodpeckers (Piciformes: Picidae) have a barbed tongue for spearing food items from within the holes they drill into wood (Bock 1999), frugivorous broadbills (Passeriformes: Calyptomenidae: *Calyptomena*) use their tongues to mash soft fruits against their palate and to move large food items into the throat (Zubkova 2019a), duck species (Anseriformes: Anatidae) can be grouped on the basis of their distinct feeding modes made possible by morphological differences in certain tongue structures (Z. Li and Clarke 2016), and hummingbirds (Caprimulgiformes: Trochilidae) employ a concerted series of mechanisms involving the bill and tongue to transport nectar into the throat (Cuban et al. 2022; Rico-Guevara 2017; Rico-Guevara et al. 2015, 2023; Rico-Guevara and Rubega 2011, 2017). Thus, in many aspects the tongue arguably is equally as important as the bill in feeding, yet historically much of the focus has been on the bill.

Lucas (1895) noted early on that “the tongues of birds have not exactly been overlooked, but somewhat neglected, by ornithologists...” This sentiment still rings true today over a century later; although morphological diversity in avian tongues has been investigated from various perspectives, the functional consequences of much of that diversity remains unknown. Past surveys of avian tongues emphasized its potential taxonomic value in comparative anatomical studies, particularly the relationships between form, function, and phylogeny. Most studies have focused on the entoglossum as a taxonomic character (Beecher 1953; Gadow 1899; Gardner 1925; F. A. Lucas 1895, 1896; Pratt 1992; Rand 1967), though at least two studies examined tongue bones (Bates 1930; George 1961). In addition to the entoglossum, much of the tongue apparatus is composed of small and delicate muscles that have been challenging to study (Engels 1938) as can be seen in Figures 1.1 and 1.2.

Few studies have explored the factors shaping tongue morphology across a given taxonomic group, and these often varied in terms of their focus. By far the broadest treatments of passerines were by George (1958, 1961, 1962) and Webster (1988, 1997, 2003, 2007; Webster and Webster 1999) of primarily North American oscines. George (1958, 1961, 1962) examined the basihyal across 500 species of passerine birds, noting its potential as a taxonomic character and how shape variation in key bones is likely a function of muscle attachments and muscle size. Through close dissection of the anatomy of the tongue apparatus, inferences could be made about a species' ability to move its tongue along the three cardinal planes – sagittal, transverse, and frontal. George (1958) noted that basihyal depth (i.e. dorsoventral height) and shape of the ceratobranchial likely covaries with bill height and feeding behavior. He observed that thick-billed, seed-eating birds had greater basihyal depth compared to species with thinner bills. Additionally,

the ceratobranchial also ranged in cross-sectional shape from oval to dorsally flattened, the latter of which he attributed to the muscles necessary for lateral tongue movements (George 1961). He observed that both features, high basihyal depth and a dorsally flattened ceratobranchial, were more frequently found in families such as Passerellidae and Cardinalidae. These families exhibit seed-husking behavior in which the tongue is instrumental in manipulating a seed along the cutting edge of the bill to remove its tough exterior. This lateral movement would necessitate an enlarged m. stylohyoideus and m. ceratoglossus posterior, and therefore a higher basihyal depth and flatter ceratobranchial, respectively, for muscle attachments. On the other hand, Webster for the most part found basihyal shape was too consistent among species within Thraupidae (Webster 1988), Emberizinae (Webster and Webster 1999), and Icteridae (Webster 2003) to be used as a generic or specific character given the state of avian taxonomy at the time. Urohyal shape (depressed or not depressed) was informative of generic separation within Estrildidae (Webster 2007). Richards and Bock (1973) found little variation between the tongue musculature of the species surveyed within Hawaiian Honeycreepers (Fringillidae: Drepanidinae), possibly due to shared phylogenetic history, but hypothesized that the method of food acquisition is more likely to be correlated with the evolution of morphological adaptations than qualities of the food items themselves. Notably, the Hawaiian Honeycreeper radiation included several nectarivorous species whose tongue morphologies are like that of sunbirds. Thus, additional studies of tongue variation are needed to broaden our understanding of the evolutionary processes that have contributed to the convergence of ecological niches across birds.

Study of the avian tongue apparatus involves not just assessing its basic anatomical structure, but also its biomechanics, sensory capabilities, and its axes of variation. The feeding biomechanics of hummingbirds is the best understood of nectar-feeding birds (Kim et al. 2012; Rico-Guevara et al. 2015; Rico-Guevara and Rubega 2011). Technological advancements in high speed videography coupled with quantitative models of nectar consumption have provided a deeper understanding of the functional morphology of hummingbird tongue adaptations, as well as those of Australian Honeyeaters (Passeriformes: Meliphagidae) (Hewes et al. 2023; Rico-Guevara 2017; Rico-Guevara et al. 2023). Although these studies are narrow in taxonomic scope, they nevertheless form a solid foundation upon which comparisons can be made between unrelated clades of nectarivores. Recent reviews of nectar-feeding adaptations (Hewes et al. 2022) and feeding biomechanics (Cuban et al. 2022) have highlighted additional clades of nectarivorous birds in need of experimental investigation such as True Parrots (Psittaciformes: Psittacoidea), the passerine families of Sunbirds (Nectariniidae), Sugarbirds (Promeropidae), Flowerpeckers (Dicaeidae), Sunbird-asities (Philippitidae), Hawaiian Honeycreepers (Fringillidae: Drepanidinae), Flowerpiercers (Thraupidae: *Diglossa*), and White-eyes (Zosteropidae).

Nectarivores represent a remarkable shift in feeding ecology compared to birds with other diets, owing to their often highly specialized morphologies. Much of our understanding of nectar-feeding mechanisms is based on hummingbirds. This speciose family likely evolved from short-billed, insectivorous ancestors, as this is the primary phenotype and diet of their caprimulgiform relatives (Prum et al. 2015). However, their diet is restricted to nectar and arthropods and their tongues appear to be morphologically similar across the family (Weymouth et al. 1964; Zusi 2013). Study of the relationship between morphology and ecology may be more tractable in a system that includes intermediate representatives as morphological adaptations

towards optima on a fitness landscape may become more apparent as diets become more specialized. Thus, because of their dietary spectrum and phylogenetic relationships to non-nectarivorous taxa, sunbirds are an ideal system in which to study the evolution of nectar-feeding adaptations. The family of sunbirds (Nectariniidae) is among the largest groups of nectar-feeding birds with approximately 136-145 species (Bowie and Fjeldså 2020). Although considered nectar-specialists, sunbirds will also consume arthropods, fruits, and seeds, with at least one taxon that is considered entirely insectivorous (*Euchloridia fraseri*; Cheke and Mann 2001). Thus, sunbird tongue morphology likely varies more broadly than is the case for hummingbirds and is subject to evolutionary pressures for different feeding behaviors and mechanisms.

The phylogenetic relationships of sunbirds with respect to non-nectarivorous relatives also affords the opportunity to study adaptations to nectarivory. Sunbirds are sister to the Flowerpeckers (Dicaeidae), followed by the outgroups Sugarbirds (Promeropidae), Spot-throat and allies (Modulatricidae), Fairy Bluebirds (Irenidae), and Leafbirds (Chloropseidae) in the superfamily Passeroidea within the order Passeriformes (Bowie and Fjeldså 2020). Although the exact relationship between these families is not fully resolved, Promeropidae and Modulatricidae are basal relative to Nectariniidae and Dicaeidae, and Irenidae and Chloropseidae are on the next branch in the phylogeny (Figure 1.3.A-B). These families, except for Modulatricidae, include nectar in their diet, but only Nectariniidae and Promeropidae are typically considered nectarivores. Other trophic niches represented in this assemblage include frugivory, insectivory, and omnivory following the trophic niche definition in the AVONET dataset (Tobias et al. 2022). In comparison, hummingbirds (Caprimulgiformes: Trochilidae) specialize on nectar and their closest relatives, swifts, are aerial invertivores. Morphological and phylogenetic comparative studies of hummingbirds are therefore more limited as no intermediate forms exist among extant taxa.

The tongues of sunbirds and their relatives appear primarily as descriptions of the entoglossum in the literature (Figure 1.3.A-B), with occasional forays into its fluid-holding capacity. Early reviews of bird tongues remarked on similarities between Nectariniidae and other nectarivorous families, in particular the bristle-like tips of the entoglossum (F. A. Lucas 1895, 1896; Rand 1967; Scharnke 1932). Bates (1930) was among the first to have depicted the tongue skeleton of a sunbird, followed shortly thereafter by Moller (1931) who described the tongue musculature, select bones, and the entoglossum, and noted the elongated epibranchials in sunbirds. Schlamowitz et al. (1976) and Paton and Collins (1989) quantified variation in fluid-holding capacity across several species of sunbirds; the latter also compared rates across sunbirds, hummingbirds, and Australian honeyeaters. Beecher (1953) briefly described sunbird and flowerpecker tongues in addition to the nectar-adapted tongue and bill tip of *Chloropsis cochinchinensis*. Several studies focused on describing the external characteristics of the entoglossum: *Kurochkinogramma hypogrammica* (Gill 1971); Dicaeidae (Morioka 1992); *Dicaeum dayakorum* (Saucier et al. 2019), and *Dicaeum nigrilore* (Rand 1961). Downs (2004) compared measurements and gross morphology of seven species of sunbirds and Gurney's Sugarbird (*Promerops gurneyi*) using scanning electron microscopy (SEM). Most recently, El-Mansi et al. (2023) described the tongue and palate of *Cinnyris habessinicus* using a combination of gross anatomical, histological, and SEM analyses. Overall, these studies highlighted characteristics commonly found in nectarivores and identified the multifaceted ways that these birds may have evolved to feed on nectar.

The goal of the first part of this study is to describe and identify key components underlying the nectar-feeding mechanism in sunbirds. To do so, I conducted a digital dissection of a sunbird specimen by isolating intrinsic tongue muscles and tongue bones. I infer the functional significance of sunbird tongue morphology by comparing it to previous studies of avian tongue anatomy. I then use this understanding of sunbird tongue anatomy and build on Richard and Bock's (1973) hypothesis regarding passerine nectar-feeding mechanisms.

The goal of the second part of this study is to: (1) identify regions of variance in the tongue skeletal morphology and bill size and characterize diversity in basihyal shape across Nectariniidae and their relatives, and (2) investigate patterns of basihyal shape variation in relation to phylogeny and ecology at the interfamilial level and within sunbirds. To do so, I reconstructed tongue skeletal elements using micro-Computed Tomographic (microCT) scans and made use of linear measurements and geometric morphometric methods to study size and shape respectively. Finally, I tested the relationship between size, shape, phylogeny, and ecology using phylogenetic Generalized Least Squares (PGLS) methods.

I predict that differences in basihyal shape will primarily reflect differences in food type (and by extension, feeding behavior and mechanism) using trophic niche as a proxy. The basihyal of species that primarily move their tongues laterally, such as when manipulating fruit within the bill, will be taller and more laterally compressed to accommodate the enlarged stylohyoideus and ceratoglossus posterior muscles. The basihyal of species that primarily protrude and retract their tongues or move the tongue vertically will be more dorsoventrally flattened, due to the increased size of the hypoglossus muscle, which is used in moving the tongue vertically. Based on our understanding of nectar-feeding in hummingbirds, I also hypothesize that nectarivores will have markedly longer hyoid horns than non-nectarivores due to the complex mechanics necessary for rapid tongue protraction and retraction when feeding on nectar.

1.2 Materials and Methods

1.2.1 A primer on the passerine tongue apparatus

The tongue apparatus is intricately linked to a bird's dietary preferences and foraging strategies. In general, the shape of a bone and how it articulates with neighboring structures reflect its function and the stresses it experiences during movement. Therefore, a familiarity with overall morphology is essential in understanding the biomechanics of avian feeding. I follow the terminology established by avian anatomists including the works of Engels (1938), Richards and Bock (1973), Manegold (2008), and Zubkova (2019b). The passerine tongue can generally be broken down into three layers: the cornified exterior, the underlying skeletal elements, and the soft tissues including muscles, ligaments, and connective tissues. I use the term "tongue" as opposed to "hyoid" following Richards and Bock (1973) because I focus on specific mechanisms of the tongue and not its association with the jaw and skull. Emphasis is placed on the tongue bones. General variation is noted and discussed in greater detail in the Results and Discussion section.

The "tongue", also referred to as the entoglossum, is the structure that occupies the oral cavity (Figure 1.2). The entoglossum is most analogous to the tongue of mammals. Unlike for mammals, however, the avian tongue is supported by bones (Figure 1.2.C). The entoglossum, sometimes referred to as the tongue body, is triangular in shape, and sits within the dorsal surface of the mandible. The keratinous tip ranges in the depth of bifurcation and the extent of

its fimbriated structure (Figure 1.3.A-B). In sunbirds, the rostral two-thirds of the entoglossum is tubular, forming two parallel tubes (Figure 1.2.C). The tubular structure unfurls caudally and the margins transition from medially- to laterally oriented. These lateral margins continue caudally for about 1 mm past the point of the processus paraglossalis rostromedialis (Ppr; Figure 1.1). The caudal third of the sunbird tongue is trough shaped.

The tongue skeleton consists of three bilaterally paired elements and one fused bone, the typical condition found in passerines (Figure 1.1.A). The entoglossum is supported by paired paraglossalia bones (singular: paraglossalium; two paraglossalia: paraglossal; Hill et al. 2015) and the lingual root is supported by the fused basihyal and urohyal bones. The paraglossalia articulates with the rostral head of the basihyal on an articular facet (facies articularis paraglossobasihyalis; Homberger and Brush 1986). In passerines, the shape of this joint ranges from that of a complex saddle joint (Wang-Claypool pers. obs.) to a ball-and-socket joint (George 1962).

The basihyal is fused with the urohyal and is sometimes referred to as basihyal-urohyal or just the basihyal for simplicity (Figure 1.1). It articulates with the paired ceratobranchial on either side. Distal to the basihyal, the ceratobranchial articulates with the epibranchial. The paired ceratobranchial and epibranchial together form the “hyoid horns”. In species with long hyoid horns, the bones encircle the skull along the ventral and caudal sides, with the longest hyoid horns continuing along the dorsal surface of the skull (Figure 1.1.A). Most of the structures are ossified and visible in microCT scans except for the rostral portion of the paraglossal, caudal portion of the urohyal, and distal epibranchial tips. These structures are not as ossified and therefore not discussed here.

Movement of the tongue apparatus in all three axes (sagittal, coronal/frontal, and transverse) requires the complex coordination of three sets of muscles as outlined by Richards and Bock (1973). The overall protraction and retraction of the tongue is mediated by one set of muscles, with the m. brachiomandibularis responsible for protraction, the m. stylohyoideus and m. tracheohyoideus responsible for retraction of the whole tongue, and the m. thyrohyoideus protracting the keratinized part of the tongue. Except for the m. thyrohyoideus (see below), these structures are categorized as extrinsic muscles, meaning the muscles extend from the skull or other parts of the body to the tongue apparatus. The third set of muscles, categorized as intrinsic tongue muscles because they extend from one part of the tongue relative to another, are the m. ceratoglossus anterior, m. hypoglossus, m. ceratoglossus posterior, m. thyrohyoideus, and m. ceratohyoideus (m. ceratoglossus anterior = m. hypoglossus anterior, m. hypoglossus = m. hypoglossus obliquus in Richards and Bock 1973). I focus primarily on the first four intrinsic muscles as they are responsible for movement of the paraglossal-basihyal joint (i.e., tongue joint) and the basihyal-ceratobranchial joint. Based on our current understanding of nectar-feeding mechanisms in sunbirds (D. Cuban unpubl. data; Cuban et al. 2022), these four muscles together are inferred to play a significant role in transporting nectar from the tongue tip to the oral cavity.

1.2.2 Digital dissection of an Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421)

Anatomical studies prior to the advent of micro-Computed Tomographic (microCT) scanning methods often required destructive dissection techniques. MicroCT methods have brought about a new frontier by not only increasing the accessibility and availability of specimens, but also generating morphological data compatible with numerous evolutionary, functional, and biomechanical studies. When done in conjunction with contrast-enhanced protocols (Gignac et

al. 2016; Gignac and Kley 2014), these scans enable the visualization of both hard and soft tissues in situ (Figure 1.1).

Inferences of muscle attachments and orientations were based on a fluid-preserved, whole-body specimen of Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421) in the collection of the Museum of Vertebrate Zoology. MVZ RCKB1421 was stained in 3% Lugol's Iodine Solution for seven days to visualize soft tissues as part of a collaboration with David Cuban (University of Washington). The specimen was then CT scanned using a Nikon Metrology XT H 225 ST scanning system at 100 kV and 450 microamps with a voxel size of 22.4 μm at the University of Washington. The CT image stacks were imported into 3D Slicer (Fedorov et al. 2012) using the ImageStacks module within the SlicerMorph extension (Rolfe et al. 2021). The segmentation was performed based on attenuation differences between bone and soft tissues in the iodine-stained specimen. The skeletal elements of the tongue apparatus were segmented using a combination of the threshold tool and manual segmentation, and soft tissues were manually segmented. Segmentations were then converted to three-dimensional (3D) surface mesh models using 3D Slicer (Fedorov et al. 2012).

Identification of muscles of interest and inferences of their function were informed by past descriptions of passerine feeding anatomy and follow the terminology used therein (Engels 1938; George 1958, 1961; Richards and Bock 1973; Zubkova 2019b, 2019a). Apart from the addition of a new character introduced here (Ppr; Table 1.1), the terminology of characters on the skeletal elements follow that of Homberger (1986) and Manegold (2008). Because cartilaginous structures (e.g., non-ossified sections of bone such as the rostral paraglossalia, caudal urohyal, and distal epibranchial, as well as ligaments, tendons, and aponeuroses) are known to be resistant to iodine staining (Z. Li et al. 2016) and therefore difficult to visualize, their placements and functions were inferred as best as possible. Thin muscles could not be segmented with confidence due to insufficient image resolution and were therefore omitted from the dissection (e.g., m. ceratohyoideus, m. serpihyoideus, and so on). Note that the homology of these muscles described here should be considered uncertain until multiple specimens can be examined.

1.2.3 Sunbird tongue morphology data

Three-dimensional (3D) Computed Tomographic (CT) scans of fluid-preserved birds from across 44 individuals, representing 42 species, 18 genera, and six families were downloaded from MorphoSource (Appendix Table 1). The sunbird ingroup comprised specimens of 31 species representing 12 of the 17 genera recognized by Bowie and Fjeldså (2020). Representatives from Dicaeidae, Irenidae, Chloropseidae, Modulatricidae, and Promeropidae were chosen for outgroup comparisons because recent DNA analyses, as well as the phylogeny presented in Chapter 2, place them as close relatives of the Nectariniidae (Bowie and Fjeldså 2020; Oliveros et al. 2019).

Scans were performed using the following CT scanners: GE Phoenix Nanotom S, GE Phoenix v tome x L 240, GE Phoenix v tome x s, and Nikon Metrology XT H 225 ST. The resulting voxel sizes ranged from 5.99 μm to 112 μm depending on whether the focus of the scan was of the head or the whole body. Trade-offs exist between scan object size and image resolution; the smaller the region of interest in the scan, the smaller the voxel size, and the higher the resolution of the resulting images and digital reconstruction. To achieve taxonomic breadth in my dataset,

I used female, male, and sex unknown individuals. Specimens were determined to be subadults or adults using the degree of skull ossification as a proxy for age (Baird 1964). Although 15 individuals were identified as possible subadults with incomplete skull ossification, they were nevertheless included in the dataset to maximize the number of species in downstream analyses. Given the limited sampling, it is not possible to assess the effect of age. Segmentation and visualization of bones followed the same protocol as the digital dissection above. A list of voucher specimens included in this study and their respective institutions can be found in Appendix Table 1. Figure 1.5 depicts representatives from each genus.

Both traditional and landmark-based geometric morphometric (GM) methods were used to analyze size and shape. Traditional morphometrics uses standard univariate measurements such as length and mass from the AVONET dataset (Tobias et al. 2022). GM methods quantify shape by considering the coordinates of points relative to one another when location, scale, and rotational effects have been removed (Zelditch et al. 2012). Landmarks reflect structures of known homology that can be consistently placed on 2D images or 3D surfaces and with high confidence (Zelditch et al. 2012). Shape can also be captured using semi-landmarks which reflect a user-specified number of points evenly spaced along curves between landmarks (Zelditch et al. 2012).

A set of landmarks, semi-landmarks, and measurements were collected from the tongue apparatus using the MarkUps module inside of SlicerMorph (Rolfe et al. 2021). Landmarks were used to generate the following distances: paraglossal width, paraglossal length, anterior paraglossal length, basihyal length, basihyal-urohyal length, ceratobranchial length, and epibranchial length (Figure 1.4; Table 1.2). Univariate measures were based in part on previous work by Li and Clarke (2016). New semi-landmarks were developed for this study: basihyal articular surface outline (ASO) and basihyal-urohyal midsagittal surface outline (MSO; Figure 1.4; Table 1.2). ASO captures shape variation in the basihyal head, whereas MSO captures basihyal-urohyal height throughout its length.

Because many specimens had incomplete data for body mass and certain measurements were not possible to acquire from the scans, additional continuous morphological traits were sourced from the AVONET dataset (Tobias et al. 2022). These were beak width, beak depth, beak length at nares, beak length at culmen, and body mass (Tobias et al. 2022). Additionally, trophic niche for each species was assigned following the AVONET dataset (Tobias et al. 2022). In brief, species were assigned to the trophic niche and trophic level from which they obtained at least 60% of their resources. Trophic niche and trophic level were classified as “omnivore” if multiple categories were utilized in relatively equal proportions. Bill curvature was not included in morphometric analyses due to lack of available data.

1.2.4 Phylogenetic comparative analyses

All statistical analyses were performed in R version 4.3.1 (R Core Team 2023). All phylogenetic analyses were conducted using the phylogeny generated in Chapter 2 (Figure 2.1) with taxonomic revision following Bowie and Fjeldså (2020). The tree was pruned to the species used in the analysis (Appendix Table 1). If a species in the morphological dataset was not in the phylogeny, a congener was used instead. Taxa were filtered out either because there were multiple representatives of one species or no suitable substitute was available to represent the taxon in the phylogeny (e.g., *Kakamega poliothorax* and *Arcanator orostruthus*). The tree was

then converted to an ultrametric tree using the ‘chronos’ function in the *ape* package (Paradis and Schliep 2019).

Raw continuous trait data collected from the scans and from the AVONET dataset (Tobias et al. 2022) were log-transformed prior to all analyses. The ‘phyl.resid’ function in *phytools* was used to remove the effects of size and to take into account phylogenetic non-independence among species by regressing each trait against body mass (Revell 2012). The residuals were treated as the size-corrected data for analysis of the continuous trait data. Morphological variation in the continuous trait data was visualized by plotting the positions of each species in morphospace. I used the ‘phyl.pca’ function in *phytools* (Revell 2012) to perform a phylogenetically-informed Principal Component Analysis (pPCA) with a covariance matrix that incorporated the phylogenetically size-corrected traits and body mass (Huie et al. 2021). Phylogenetic PCA morphospace, where the phylogeny is projected onto morphospace (“phyломorphospace”; Figure 1.6) was projected using the ‘phylomorphospace’ function in the *phytools* package (Revell 2012). Paraglossal anterior length was excluded from interfamilial analyses because the processus paraglossalis rostrolateralis (Ppr; new term defined in this study) is only found in the Nectariniidae. The process paraglossalis rostrolateralis was depicted in a drawing in Bates (1930) but otherwise never again mentioned in the literature.

The raw landmark coordinates of the basihyal articular surface outline (ASO) and basihyal-urohyal midsagittal surface outline (MSO) were aligned (i.e., Procrustes transformed) as independent shapes and as combined shapes using the ‘gpagen’ function in the R package *geomorph* (Adams et al. 2022; Baken et al. 2021). The ‘gm.prcomp’ function in *geomorph* (Adams et al. 2022; Baken et al. 2021) was then used on the aligned semi-landmarks to perform phylogenetically-informed principal component analysis. Phylomorphospace of basihyal shape (Figure 1.7) was projected using the ‘phylomorphospace’ function in the *phytools* package (Revell 2012).

I investigated the relationship between trophic niche and basihyal outline shapes using phylogenetic Generalized Least Squares (PGLS). Analyses were run on ASO and MSO separately and ASO and MSO combined. This was first implemented using the ‘procD.pgls’ function in *geomorph* (Adams et al. 2022; Baken et al. 2021). I used the Procrustes coordinates as the response variable and trophic niche as the predictor variable with two covariables representing size – log body mass and log centroid size. Size (allometry) is known to be a contributing factor to shape in birds (Bright et al. 2016). Each size covariable was run independently and in unison. The first was log body mass. The second was log centroid size (the square root of the sum of squared distances of the landmark to their centroid, obtained from general Procrustes analysis) as a proxy for basihyal size, with the centroid being representative of the size of an object independent of shape (Pandelis et al. 2023).

A second PGLS implementation was performed using the ‘glS’ function in the *nlme* package (Pinheiro et al. 2023) and ‘ANOVA’ in the *car* package (Fox and Weisberg 2019) using simplified shape data to provide an additional layer of robustness to the results (see Pandelis et al. 2023). The ‘glS’ function used PC scores for principal components 1 and 2 of basihyal shape respectively as the shape variables. I accounted for phylogenetic structure by specifying an evolutionary covariance matrix under Brownian motion using the function ‘corBrownian’ from the *ape* package (Paradis and Schliep 2019). Size was also accounted for by using both log body mass and log centroid size as proxies.

1.2.5 Broad comparisons of representatives from three major nectarivorous radiations

The tongue skeletons of three major radiations of nectar-feeding birds were compared in order to identify potential morphological correlates to feeding behavior and feeding mechanism. The tongue skeletons of an Anna's Hummingbird (Caprimulgiformes: Trochilidae: *Calypte anna*; UWBW uncataloged) and Eastern Spinebill (Passeriformes: Meliphagidae: *Acanthorhynchus tenuirostris*; nhmuk:1964.17.104) were segmented and visualized using the same protocol as the digital dissection above.

1.3 Results

1.3.1 Digital dissection: entoglossum shape, tongue musculature, and inferred functional roles in nectar-feeding birds

The entoglossum of *Haagneria olivacea* (MVZ RCKB1421) is long and narrow (Figure 1.1.CD). The microCT scan can be roughly divided into two sections – the tongue body (Figure 1.1 sections 1-3) and the tubular tongue (Figure 1.1 sections 4-5 and beyond). The tongue body has a trough-like shape whereas the tubular tongue is shaped more like a straw. The tongue body is an isosceles triangle in dorsal view (Figure 1.1.C). The caudal margin is U-shaped and is slightly narrower. It forms the papillary crest and terminates in a pair of fleshy alae linguae on the left and right sides. These alae linguae are supported internally by the processus paraglossalis caudalis (Ppc; Figure 1.1.C). The lateral margins are thick caudally when in parallel with the alae linguae (Figure 1.1 sections 1-3). As the margins become thinner rostrally, the margins simultaneously orient dorsally (Figure 1.3 section 3) before curling medially (between sections 3-4) to form two tubes (section 5 and beyond). Interestingly, the processus paraglossalis rostralateralis underlies this transitional area (Ppr; Figure 1.3.C). The distal tip is not visible in the scan but is assumed to be slightly bifurcated (Figure 1.3; see also Cheke and Mann 2001).

The four main intrinsic muscles, points of attachment, and inferred functional roles in movement of the tongue joint (i.e., the articulation of the paraglossal and the basihyal) are described here:

1. **Ceratoglossus anterior (cga):** The m. ceratoglossus anterior is a tiny muscle nestled between the paired paraglossalia within the ventral side of the tongue body (cga; Figure 1.1 and Figure 1.8).
 - Attachment: In MVZ RCKB1421, anteriorly it is uncertain where the ap. ceratoglossus insertionis (acg; Figure 1.1 and Figure 1.8) attach rostrally on the paraglossal. Posteriorly it is connected to the rostral tip of the basihyal.
 - Action: In sunbirds, the function of the m. ceratoglossus anterior is not immediately apparent. It may be partially involved in slightly shortening the tongue body (Figure 1.1.C-D, Figure 1.8.B, C, and E). Through its hypothesized connection with the m. ceratoglossus posterior, contraction of the m. ceratoglossus anterior may bend the tongue body downwards (see cgp below).
 - Context: In Old World suboscines, the acg “ends on the ventral sides of the cartilaginous ends of the paraglossalia and “dissolves” in the connective tissue surrounding their rostralateral edge” (Zubkova 2019b); contraction of the m. ceratoglossus anterior primarily serves to bend the entoglossum in an arch, thereby lowering the tongue tip downwards (Zubkova 2019a). Zubkova (2019b)

notes that the m. ceratoglossus anterior is practically absent or not described in most songbirds. It is absent in honeycreepers (Richards and Bock 1973).

2. **Ceratoglossus posterior (cgp):** This muscle envelopes the dorsal half of the length of the ceratobranchial (Figure 1.1.CD). It extends rostrally under the stylohyoideus, lateral to basihyal-ceratobranchial joint, where it then connects the rostroventral margin of the basihyal head via a tendon (ap. ceratoglossis, acg; Figures 1.1.D, 1.8.B). The body of the ceratoglossus posterior itself is enveloped in a similar manner by the stylohyoideus.
 - Attachment: It originates from a depression on the dorsal surface of the distal ceratobranchial. The exact nature of its point of insertion is unclear, though faint evidence of soft tissue ventral to the m. hypoglossus and thickened rostroventral margin of the basihyal head suggests that it connects to the rostral tip of the basihyal head.
 - Action: Bilateral contraction retracts the paraglossal-basihyal unit. Unilateral contraction with the m. hypoglossus may cause the tongue to move to the ipsilateral side.
 - Context: In Old World suboscines, the m. ceratoglossus posterior originates on the ceratobranchial and attaches via a tendon (aponeurosis ceratoglossis; ap. ceratoglossis) to the ventromedial protrusions of the paraglossalia directly in front of the tongue joint (Zubkova 2019b). Richards and Bock (1973) observed in Hawaiian Honeycreepers that: “this muscle need shorten only slightly to accomplish its action – hence its short fibers. But it may have to depress the tongue against resistance and therefore must develop considerable force - hence the large physiological cross-section.” El-Mansi et al. (2023) refer to this muscle as the m. genioglossus lateralis.
3. **Hypoglossus (hg):** This is a short yet wide muscle extending from the underside of one processus paraglossalis caudalis (Ppc; also referred to as paraglossalium horn) of one side, under the basihyal, to the underside of the Ppc. The width of muscle extends from the ventral surface of the basihyal head to the middle of the basihyal neck.
 - Attachment: It attaches to the medial-facing concavity of the Ppc. In MVZ RCKB1421 it also appears to partially attach to the ventral side of the basihyal (see Context below).
 - Action: Bilateral contraction of the m. hypoglossus brings the paraglossal horns downwards towards the basihyal and raises the distal tip of the tongue upwards towards the upper palate (Figure 1.8.D).
 - Context: Although the m. hypoglossus appears to be one broad, transverse muscle passing beneath the basibranchial between the caudolateral processes of the paraglossalia, histological sections of *Cinnyris habessinicus* show fibers attaching to the basihyal deep within the hypoglossus (hr hypoglossus rostralis in Figures 10A and 10D from El-Mansi et al. 2023). This condition is similar to structures observed by George (1961) in New World nine-primaried oscines and ploceid assemblages (Figure 7c in George, 1961). The m. hypoglossus also appears to be attached to the underside of the basihyal head (Figure 1.1.D), a condition which has not been noted in previous descriptions of passerine tongue musculature. Additionally, the paraglossal tips are noted to be connected by a symphysis at their

rostral end (Manegold 2008). Bilateral contraction of the m. hypoglossus may bring the paraglossal horns closer medially and cause flexion in the flattened midsection of the paraglossal (Figure 1.8.E).

4. **Thyreohyoideus (th):** a small muscle that extends from the cricoid cartilage (anterior head of the trachea) and runs parallel along the length of the basihyal (Figure 1.1.BD)
- Attachment: It originates from the ventral surface of the cricoid cartilage and attaches along a concavity on the lateral surface of the basihyal. Its rostral-most point of attachment is nestled deep between the hypoglossus on the lateral side and the basihyal on the medial side.
 - Action: Bilateral contraction of the m. thyreohyoideus brings the basihyal and the cricoid cartilage closer together, thus retracting the tongue (Figure 1.8.E). Unilateral contraction with the m. thyreohyoideus could cause the tongue to move to the ipsilateral side.
 - Context: Richards and Bock (1973) reported the m. thyreohyoideus as originating from the cricoid cartilage by two separate heads. Due to the resolution of the scan, the condition in MVZ RCKB 1421 was not able to be confirmed. Richards and Bock (1973) also report little to no variation in the m. thyreohyoideus between the species studied.

For clarity, the inferred functional roles in movement of the tongue, particularly of the tongue joint, are divided into the three axes:

- Transverse axis (rostral-caudal movement): Movement of the tongue apparatus is primarily driven by extrinsic tongue muscles (e.g., m. brachiomandibularis, m. stylohyoideus, and m. tracheohyoideus; see Introduction).
- Sagittal axis (lateral movement): Some contralateral movement of the paraglossal may be possible through contraction of one side of the m. hypoglossus and the m. ceratoglossus posterior.
- Frontal axis (dorsal-ventral movement): Movement on the frontal axis occurs at two points:
 - 1) articulation between the paired paraglossalia and the basihyal head (i.e., the tongue joint) is likely tied to the vertical movement of the tongue as a whole, involving the m. hypoglossus (Figure 1.8.D) and likely the m. ceratoglossus posterior (Figure 1.8.C). Angles of the articulating facets vary from primarily lateral-facing to rostradorsal-facing. Variation in the morphology of this joint likely contributes to or limits the range of motion depending on the species.
 - 2) Articulation between the basihyal and the paired ceratobranchials. The basihyal assists in the pulling down of the entoglossum seal alongside the hypoglossus and possibly the ceratoglossus anterior and posterior, creating a negative pressure space (Figure 1.8.D). This likely also involves the thyreohyoideus muscle, which helps to retract the tongue.

1.3.2 Osteology of the tongue in sunbirds and relatives

CT scans were of a sufficient resolution such that tongue skeletons could be reconstructed, segmented, and compared (Figures 1.3.A-B, 1.9). Below is a broad description of each skeletal element in the tongue across sunbirds and their relatives.

1.3.3 Paraglossalium

The paraglossalium is highly variable in shape across the examined passerines. In dorsal view, it generally has the shape of a right triangle and the rostralateral margin is straight. An exception can be seen in sunbirds, which have a projection of variable size protruding from the lateral margin approximately halfway along the length of the tongue (processus paraglossalis rostralateralis, Ppr, a new term). Non-sunbirds lack this process. In *Modulatricidae* (Figure 1.5.17A-C) and *Eurylamidae* (Zubkova 2019b), the paraglossalium narrows into a pointed, cylindrical shape and meets along the midline. In some passerines, the paraglossalia is more wedge-shaped with the medial margins thicker than the lateral margins (Figure 1.5.14A-C). The caudal margin is incised, and separates a pointed caudolateral process, herein referred to as the paraglossal horn, from a caudomedial process which articulates with the rostradorsal face of the basihyal head. A ventral process, processus paraglossalis ventralis (Ppv; Manegold, 2008) can be found in some passerine species in the region rostral to the paraglossal-basihyal joint (*Chloropseidae*; Figure 1.5.16A-C). The paraglossal horns are excavated on its medial surface where the hypoglossus muscle attaches. There is also substantial variation in the thickness of the paraglossalium outside of sunbirds, particularly in species with shorter bills (*Dicaeidae*; Figures 1.5.13A-C, 1.5.14A-C). *Modulatrix* is the only species observed where the paraglossalia are cylindrical throughout.

The sunbird paraglossalium (Figure 1.1) represents a strong departure from the typical passerine condition. The paraglossal is composed of paired paraglossalia which are variable in shape and size. The typical morphology of the paired paraglossalia in *Nectariniidae* is as follows: it is generally flattened, of complex shape, and can be roughly divided into three sections:

(1) The rostral third of each paraglossalium is simple and rod-like, with the distal ends lying parallel to each other. The paired paraglossalia may meet medially here but do not appear to articulate based on the resolution of the scans available for use in this study.

(2) The bone in the middle third of each paraglossalium is dorsoventrally flattened and wide. From the lateral margin emerges a rostralateral projection (Ppr; Figure 1.1.C). This structure follows the buccal edge, terminating approximately where the lateral edges of the cornified tongue begin curling medially (Figure 1.2.C). This projection was depicted in a figure as early as 1930 (Bates 1930), but has not been discussed or otherwise mentioned in the literature since. It appears to be unique to sunbirds. The distance between the paraglossal tip and the rostralateral projection varies in length (anterior paraglossal length, APGL; Table 1.3). The average APGL of *Nectariniidae* is 2.54 mm. *Aethopyga* has the shortest APGL on average (1.85 mm, $n = 3$ species), with *A. linaborae* having the shortest APGL at 1.64 mm, and the shortest paraglossalium among the sunbirds (5.54 mm, $n = 3$ species). *Arachnothera longirostra* has the longest APGL at 3.76 mm, though *Ar. magna* has the longest paraglossalium (12.79 mm). The prominence also varies, with some species appearing more like a widening of the paraglossalium with no obvious projection (Figure 1.5.11A) and others possessing a distinct point (Figure 1.5.6A). The paired paraglossalia are angled rostromedially towards each other, forming a wide V-shape

with a subtle bowl-like channel (foramen interparaglossale; Manegold 2008) along the middle third of the paraglossal until it articulates with the head of the basihyal. This V-shape varies from lens-shaped to a shallow bowl shape and can be seen in the entoglossum (Figure 1.2.BD; see also Figure 1A in Downs 2004).

(3) The posterior third is composed of a thickened, widened region that then narrows to a caudolateral process (Ppc) or “horn” on each paraglossalia. On the ventral side of the widened region is a plane of articulation (articular facet) with the head of the basihyal. The caudolateral processes diverge laterally before returning medially. Each process is slightly curved and flattened, with the flattened region facing ventromedially, creating an area for the attachment of the hypoglossus muscle (Hg). Variation is present in the paraglossal horns, particularly in the orientation of the concave surface and robustness of the shape. Of particular note is the presence of a structure similar to the Ppv in *Chloropsis* (Figure 1.5.16C) in *Anthreptes* (Ppv?; Figure 1.5.11C-D) and *Arachnothera* (Ppv?; Figure 1.5.12C-D). The designation is questionable because the shape and location relative to the basihyal-paraglossal articulation are so different that they may not be homologous structures. The Ppv of *Anthreptes* and *Arachnothera* has a hook-like morphology as opposed to the straight projection in *Chloropsis*. It projects ventrally then curves medially under the basihyal head.

1.3.4 Basihyal and Urohyal

The shape and proportions of the basihyal-urohyal as a three-dimensional structure varies from being dorsoventrally flattened in *Aethopyga* (Figure 1.5.10), laterally compressed in *Dicaeum* (Figure 1.5.13), to being cylindrical throughout in Modulatricidae (Figure 1.5.17). Interestingly the taxa with flattened basihyals possess a slight sagittal ridge along the dorsal midline (Figures 1.5.9A, 1.5.10A).

The structure of the basihyal head, which articulates with the paraglossalia on its dorsolateral surface, is variable both within sunbirds and between sunbirds and non-sunbirds. It is primarily oriented ventrally and at a slight angle rostrally. In dorsal view, the outline of the basihyal head can appear anywhere from pointed (Figure 1.5.17B) to round (Figure 1.3.5B) to spade-shaped (Figure 1.5.12B). The length of the basihyal head also varies, as does its width. In profile, the rostral outline ranges from a low slope (Figure 1.5.12C) to strongly curved (Figures 1.5.6C, 1.5.17C). This variation in morphology likely affects the articulation of this joint. The range of motion of the basihyal and the paired paraglossalia likely ranges from a saddle joint (Figure 1.5.17A-C) to a hinge-like joint (Figure 1.5.16A-C). Notably in sunbirds, the rostroventral margin has a thickened ridge or flange, likely reinforcing the bone where the aponeurosis ceratoglossis inserts. In Dicaeidae, the contralateral surfaces of the basihyal head are divided by a tall, narrow dorsal crest of the basihyal, which extends from approximately one-half (Figure 1.5.14C) to one-third (Figure 1.5.13C) the length of the basihyal-urohyal.

The region caudal to the basihyal head and anterior to the articulation with the ceratobranchials, referred to here as the “neck”, varies in its shape in cross-section. The three broad categories previously established by George (1958, 1961) were generally corroborated by the species in this study – (1) laterally compressed, (2) round or oval, or (3) intermediate. The degree of compression varies depending on the species, but also where it occurs along the length of the neck. Lateral compression was most evident in the height of the basihyal. The non-sunbirds fall into the categories (1) laterally compressed or (2) round. Basihyal necks in sunbirds have a

complex intermediate shape. A slight lateral crest is visible on dorsoventrally compressed basihyal necks (Figures 1.5.9A-C, 1.5.10A-C), likely due to insertion of the paired thyrohyoideus and stylohyoideus muscles. The exception in sunbirds is *Euchloridia fraseri* (Figure 1.5.2A-C) whose overall basihyal-urohyal shape is gracile and reminiscent of that of Modulatricidae (Figures 1.3.B, 1.5.17A-C). The region of the basihyal anterior to the ceratobranchial joint is also highly variable in width as well as shape. The ventral surface of the basihyal between the basihyal-ceratobranchial joints is slightly concave in some species (e.g., *Haagneria olivacea*), though it is difficult to determine its extent in some scans due to low resolution.

The urohyal primarily varies in height and width. In some species it has a cylindrical shape (Figure 1.5.4A-C) and in others a dorsoventrally flattened shape (Figure 1.5.15A-C). In some species of sunbirds, the thickness of the fused basihyal-urohyal abruptly thins caudal to the basihyal-ceratobranchial joint. This results in a lateral profile reminiscent of a quill nib (1.7A-B). For most species analyzed in this study, the urohyal is of consistent width throughout its length. The exception is *Kakamega poliothorax* in which the caudal third of the dorsoventrally depressed urohyal broadens slightly, the shape of which is reminiscent of a spoon (Figure 1.3.B).

1.3.5 Ceratobranchial and Epibranchial

The ceratobranchial is a gracile, slightly curved bone that is variable in length, although not to the same extent as the epibranchial. There is some variation of the shape of the cross-section, ranging from oval to crescent-shaped due to a convex hollowing of its dorsomedial surface. The latter shape possibly relates to the strength of muscle contractions, particularly the m. ceratoglossus posterior and the m. brachiomandibularis (George 1961). This is referred to by George (George 1961) as “excavated ceratobranchial” when examining cardueline finches and Hawaiian honeycreepers.

In sunbirds, the epibranchial is a narrow bone and highly variable in length. When retracted, long epibranchials will extend dorsally along the occiput on either side of the cerebellar prominence (see also Zusi 2013), with the longest epibranchials continuing rostrally and its distal tips even with the orbits. The distal tips are either rounded (Figure 1.5.17A), slightly flattened (Figure 1.5.11A), or both flattened and widened (Figure 1.5.8A). In some *Dicaeum*, the distal tips are flattened and ever-so-slightly bifurcated (Figure 1.5.13A). For other sunbirds and non-sunbirds, the epibranchial tapers to a narrow, cylindrical tip. Linear measurements can be found in Table 1.3. In sunbirds, the epibranchial is longer than the ceratobranchial, ranging from 11.44 - 33.83 mm for the epibranchial and 4.94 - 10.03 mm for the ceratobranchial. On average, the epibranchial was 2.94 times longer than the ceratobranchial, and ranged from 1.79 and 1.98 times on the lower end, to 3.73 and 3.83 times in length on the higher end (*Lamprothreptes longuemarei*, *Euchloridia fraseri*, *Cyanomitra alinae*, and *Arachnothera longirostra*, respectively). In non-sunbirds, the epibranchial is shorter than the ceratobranchial, ranging from 7.76 - 13.48 mm for the epibranchial and 5.28 - 12.98 mm for the ceratobranchial, though in *Promerops cafer* the epibranchial is only 0.5 mm shorter than the ceratobranchial.

1.3.6 Phylomorphospace of tongue skeleton and bill measurements

Lengths of the tongue skeletal elements taken from CT scans and bill measurements from the AVONET dataset (Tobias et al. 2022) were input into a phylogenetically-informed Principal Component Analysis. When considering both tongue and bill measurements, the first two

principal components in all families account for 72.02% of the variance (Figure 1.6.A). PC1 accounts for 58.4% of variation for all traits (excluding the anterior paraglossal length - see Methods). PC1 loads for decreasing beak length (both beak length at nares and culmen length) and epibranchial length. PC2 accounts for 13.62% of variation and is associated with increasing beak depth, basihyal length, and ceratobranchial length. Species in the same phylogenetic clade cluster closely together in morphospace, except for the family Promeropidae (Figure 1.6.). Nectariniidae and Promeropidae cluster towards the left on PC1, indicating generally longer bills and epibranchials (Figure 1.6.A). This is perhaps driven by convergence in trophic niche as evidenced by the clustering of nectarivores (Figure 1.6.A2). However, this convergence is less evident when only the tongue is considered (Figure 1.6.B). Though *Promerops* is closer to nectariniids along the PC1 axis, the distance between nectariniids and non-nectariniids becomes more distinct along the PC2 axis, indicating generally longer basihyal-urohyal lengths in the latter group. When only tongue lengths were considered, the first two principal components account for 79.21% of the variance (Figure 1.6.B). PC1 accounts for 60.79% of variation and loads for decreasing epibranchial length and paraglossal length. PC2 accounts for 18.42% of variation and is associated with decreasing basihyal-urohyal length.

When viewed through the lens of trophic niche, frugivores, invertivores, and nectarivores form clusters in morphospace, though omnivores overlap with each of the other three. Both nectarivores and invertivores tend to cluster towards the left on PC1 and frugivores towards the right (Figures 1.6.A2, 1.6.B2). Overlap between multiple niches is most obvious in sunbirds, the most well represented family. Sunbirds form a tight core with an outer periphery of points in both Figure 1.6.A and 1.6.B. This suggests that although the morphology is generally similar for members of this clade, several species remain distinct despite correcting for size. Notably several of these peripheral sunbirds are categorized as insectivores by Tobias et al. (2022).

1.3.7 Phylomorphospace of basihyal shape

The first two principal components of the ASO shape in all families account for 87.66% of the variance (Figure 1.7.A). PC1 accounts for 78.39% of variation and loads for basihyal head width and the angle of the rostral margin in dorsal view (Figure 1.7.A1). PC1 also loads for convexity of the articular surface in frontal view (from saddle shaped to flat; Figure 1.7.A2). PC2 accounts for 9.27% of variation and is associated with the basihyal head length. The first two principal components of the MSO shape in all families account for 57.68% of the variance (Figure 1.7.B). PC1 accounts for 40.52% of variation and loads primarily for basihyal height, especially the region caudal to the basihyal head. PC2 accounts for 17.16% of variation and is associated with overall basihyal height, particularly of the basihyal head itself. The first two principal components of the merged ASO and MSO landmarks in all families account for 60.43% of the variance (Figure 1.7.C). PC1 accounts for 48.47% of variation and loads for overall basihyal height and basihyal head length. PC2 accounts for 11.96% of variation and is associated with height of the basihyal neck and basihyal head.

Species in the same clade generally clustered together when ASO was considered (Figure 1.7.A,C). Clades tend to have slightly greater overlap when only MSO was analyzed. Within the trophic niche plots, there is significant overlap between the four categories. Most notable are the placements of Promeropidae (in pink) and *Euchloridia fraseri* in Figure 1.7.B1 (the blue point partially covered by red representing Chloropseidae).

1.3.8 PGLS with basihyal ASO and MSO data

When the relationships between two different measures of shape (multivariate Procrustes coordinates versus univariate principal components), trophic niche, and two size co-variables, and log(mass) versus log(centroid size) were analyzed, multivariate and univariate measures were significantly predicted by different variables (Table 1.4). Basihyal articular surface outline (ASO) was significantly predicted by log(mass) for both multivariate and univariate shape analyses (procD.pgls $p = 0.044$, gls $p = 0.03157$). Univariate shape analyses using principal component 1 (PC1) scores found trophic niche and log(centroid size) to be significant (or near significant) predictors of basihyal-urohyal midsagittal outline shape (MSO; Table 1.4).

1.3.9 Broad comparisons of the tongue skeleton between three radiations of nectarivores (Caprimulgiformes: Trochilidae, Passeriformes: Nectariniidae, Passeriformes: Meliphagidae)

Several differences are apparent in the overall shape of the paraglossal, the articulation of the tongue joint, depth of the basihyal-urohyal, ceratobranchial shape in cross section, and proportions of the basihyal and the urohyal. Variations in these structures are likely related to variation in points of muscle attachment and muscle size, which are likely governed by the mechanics underlying different feeding behaviors.

In general, the tongue bones of Anna's Hummingbird are greatly simplified compared to Olive Sunbird and Eastern Spinebill. Given our current understanding of nectar-feeding mechanisms in hummingbirds (Cuban et al. 2022), the tongue joint of hummingbirds likely experiences decreased physical demands compared to sunbirds and honeyeaters. Capture of insect prey primarily involves flexion of the bill to widen the gape (Yanega and Rubega 2004). The paired paraglossalia sit on either side of the basihyal in *Calypte* (Figure 1.9.B-C), dorsolaterally in *Haagneria* (Figure 1.9.E-F), and dorsally in *Acanthorhynchus* (Figure 1.9.H-J). The paraglossal horns are angled differently across all three groups. The concave surface is oriented more medially in *Calypte* and *Haagneria*, whereas it is oriented ventrally in *Acanthorhynchus*. The paraglossal of *Acanthorhynchus* is more reminiscent of *Chloropsis* (Figure 1.9.A-C), though curiously the narrowing at the rostral third appears more akin to *Haagneria*.

The basihyal-urohyal is strikingly different between *Calypte* and the passerines *Haagneria* and *Acanthorhynchus*. The basihyal head in *Calypte* is laterally compressed whereas the passerines are more dorsoventrally flattened. The basihyal head of the honeyeater genus *Acanthorhynchus* is most reminiscent of the sunbird genus *Arachnothera* with its shovel-like outline when viewed from above (Figure 1.9A-D). However, *Acanthorhynchus* is far more dorsoventrally flattened along the entire length of the basihyal-urohyal. Interestingly, the urohyal is highly reduced in *Calypte* (see also Figure 12 in Zusi, 2013). Previous work suggests that, unlike in oscines, the urohyal does not support the larynx in hummingbirds (Zusi 2013) and thus may experience less mechanical stress. In *Acanthorhynchus* the urohyal is much thinner, narrower, and proportionally longer than in *Haagneria*.

Lastly, the articulation between the basihyal and the ceratobranchials is also strikingly different between hummingbirds and passerines. In hummingbirds, the ceratobranchials articulate with the dorsal surface of the basihyal as opposed to the ventrolateral orientation of the sunbird and spinebill. The ceratobranchial is considerably flattened in the honeyeater compared to that in the hummingbird and the sunbird.

1.4 Discussion

1.4.1 Digital dissection informs tongue form and function relationships

MicroCT scans of iodine-stained specimens provide valuable insight into soft tissue structures and inform biomechanical studies. Anatomical structures can be studied in situ in relation to neighboring soft and hard tissues, while also maintaining the integrity of the specimen. Visualization of the overall tongue musculature and the corresponding muscle insertions and origins shed light on the potential mechanical stresses experienced by the tongue skeleton. Variation in osteology, such as crests and grooves on the basihyal (see below), can then be used to infer the mechanics underlying feeding behaviors associated with different diets.

Another notable structure is the unique shape of the lateral margin of the entoglossum tongue body, coinciding with the processus paraglossalis rostralateralis (Ppr; Figure 1.2 between slices 2 and 3). While moving distally along the tongue, the cross-sectional outline of the entoglossum transitions from an open V-shaped trough with lateral “flaps” (Figure 1.2 sections 1-2) to a closed U-shaped tube (Figure 1.2 sections 3-4). To my knowledge, the Ppr is wholly unique to sunbirds and likely serves to structurally reinforce the gradual curling of the keratinous tongue. Its existence and placement lend further credence to the proposed mechanics underlying nectar-feeding in sunbirds, namely the “seal” of the tongue body against the palate and the generation of a pressure differential as the tongue is depressed (see below; D. Cuban unpubl. data). The theory is further supported by the estimated internal tongue volume of the examined specimen aligning with the measured rate of feeding in live sunbird feeding trials (D. Cuban unpubl. data).

At the core of the proposed nectar-feeding mechanism in sunbirds is the creation of a pressure differential (i.e., vacuum) to transport nectar from the tongue tip to the throat. One hypothesis is that the negative pressure is being generated in the buccal cavity, much like how a person sucks through a straw (Liversidge 1967). However, in birds the nares open directly into the buccal cavity, and there is no mechanism, either a flap or a muscular valve, with which birds can seal the internal nares (Richards and Bock 1973). A more likely mechanism proposed by Richards and Bock (1973) in their monograph on Hawaiian Honeycreepers (Passeriformes: Fringillidae: Drepanidini) forms the basis of the most supported hypothesis (D. Cuban unpubl. data). In brief, nectar is drawn up from the tongue tip through a bellows-like movement at the base of the tongue (El-Mansi et al. 2023; Richards and Bock 1973). A vacuum can form when the tongue body is pressed flat against the upper palate against which a seal is formed using the lateral and caudal edges of the trough-shaped caudal entoglossum (Figure 1.2.C-D). This seal is composed of the thin, lateral margins of the tongue body and the fleshy alae linguae along the caudal margin (Figure 1.2.C-D). The cavity would then increase in volume by angling the caudal processes of the paraglossalia (processus paraglossalis caudalis, Ppc; Figure 1.8.C) upwards, thereby creating negative pressure and drawing nectar through the tubular tongue. When the tongue apparatus has been sufficiently retracted into the mouth, angling of the Ppc ventrally would break the seal along the alae linguae (Figure 1.8.D), thus releasing a small amount of nectar towards the back of the throat. Additional amounts of nectar would be transferred into the bill via cohesive pulling of liquid along the structure of the tongue as the tongue is protruded and retracted (Cuban et al. 2022).

1.4.2 Relationships between ecology and linear measurements

When body mass was accounted for in analyses of tongue skeleton and bill measurements (Figure 1.6.A), the main finding that principal components 1 and 2 load for bill measurements is not surprising. In a comprehensive analysis of phenotypic diversity for extant birds, Pigot et al. (2020) found that beak size and shape significantly predicts trophic niche in nectarivores. The co-loading of PC1 with bill length and epibranchial length corroborates our understanding of the functional role of the tongue in birds whose diet is predominantly composed of nectar in comparison to occasional consumers of nectar.

When considering only the tongue module (Figure 1.6.B), the inverse relationship between PC 1 (epibranchial and paraglossal length) and PC2 (basihyal-urohyal length) suggests that adaptation for nectarivory in this clade may have initially occurred in the relative proportions of skeletal elements rather than the length of any single element. Both the epibranchial length and the basihyal-urohyal length appear to be more labile within sunbirds than in, for example, hummingbirds. Zusi (2013) observed that across nine species of hummingbirds ($n = 1$ per species), the epibranchial shows the most variation among species, whereas the relative lengths of the basihyal and the ceratobranchial are approximately constant regardless of bill length. Hyoid horn length has been proposed to be correlated with the ability of the tongue to be protracted further (Richards and Bock 1973). The extent of tongue protrusion from the bill, and consequently a bird's ability to drink from deep nectaries, is dependent on the length of the epibranchials and brachiomandibularis muscles (Richards and Bock 1973).

Though not within the purview of this study, it is plausible that the tongue skeleton is highly modular, with each element of the tongue skeleton under different selective pressures. Here I propose that the adaptiveness of proportionally longer paraglossalia observed in sunbirds is related to its role in supporting the tongue body. Long paraglossalia may increase rigidity along a longer proportion of the entoglossum length, thus extending the region that can withstand increased negative pressure when the tongue is depressed. Conversely, Richards and Bock (1973) speculated that shorter paraglossalia in Hawaiian honeycreepers would provide less structural support, thus increasing flexibility in the tongue and thereby facilitating feeding on nectar deep within floral nectaries. Both the above hypotheses remain to be tested. Ancestral state reconstruction of the shape of each component of the hyoid would shed more light on this aspect of tongue evolution. High resolution scans of stained specimens would also aid in determining the size of various muscles and infer their contribution to tongue function.

1.4.3 Relationships between ecology and shape measurements

An interesting relationship exists between basihyal shape, size (body mass and centroid size), and ecology. The first method of analysis with *procD.pgls* using Procrustes coordinates as the input for shape found body mass to be the only significant predictor of shape. This result is unsurprising given that the outgroup families Promeropidae, Modulatricidae, and Irenidae are approximately three to eight times larger than the average sunbird (Table 1.3) and have relatively simple basihyal head shapes (Figures 1.3.B, 1.7.A1). The second method of analysis using principal components found trophic niche and both size measures to be significant predictors (Figures 1.7.B1-B2, 1.7.C1-C2). Minimal overlap between the families occurs along the axis of PC1, indicating their distinctness in basihyal-urohyal MSO morphospace (Figures 1.7.B1, 1.7.C1).

Similarly, frugivores do not overlap at all with the other trophic niches when MSO and the merged ASO+MSO shapes are considered (Figures 1.7.B2, 1.7.C2).

These results tentatively support my hypothesis that basihyal shape reflects ecology as a proxy for feeding behavior. The varying significance of size as a covariable was to be expected given the size distribution of the surveyed families (Table 1.3). Sunbirds are generally quite small compared to the non-nectarivorous outgroups. This perhaps hints at the relationship between ecology, body mass, and metabolism, in which a nectarivorous diet limits the maximum body mass (Brown et al. 1978).

1.4.4 Basihyal shape: patterns in variation and form and function relationships

Contrary to studies using traditional dissection methods that observed little to no variation in the basihyal at lower phylogenetic levels (e.g., genus, species; George 1958, 1961; Webster 1988, 1997, 2003, 2007; Webster and Webster 1999), several genera within sunbirds had distinct basihyal-urohyal shapes when reconstructed from high resolution microCT scans. For instance, the genera *Leptocoma* (Figure 1.5.9A) and *Aethopyga* (Figure 1.5.10A) possess a distinct combination of features, including strong dorsoventral compression, a lateral outgrowth of the basihyal neck, and a dome-shaped basihyal head. The basihyal ASO of *Arachnothera* (Figure 1.5.12A-D) and *Anthreptes* (Figure 1.5.11A-D) are reminiscent of a shovel when viewed from above.

Analysis of basihyal ASO and basihyal-urohyal MSO shape, separately and combined, provides some insight into the complex relationship between phylogeny, morphology, allometry, and ecology. Families form tight clusters when evaluating ASO shape (Figure 1.7.A). However, overlap between families increases when considering only MSO shape (Figure 1.7.B) and merged ASO and MSO shape (Figure 1.7.C). Log(centroid size) was a significant predictor of shape. This is perhaps unsurprising considering allometry is known to partially explain morphological disparity in avian skulls (Bright et al. 2016). Previous studies have suggested that the evolutionary change in the tongue apparatus is correlated with shifts in diet and feeding behavior (Manegold 2008; Richards and Bock 1973). Ecology, here characterized by trophic niche, predicts shape (Table 1.1). *Euchloridia fraseri* (Nectariniidae) and *Promerops cafer* (Promeropidae) represent instances where basihyal-urohyal morphology suggests an evolutionary trajectory away from nectarivory for the former and towards nectarivory for the latter. *Euchloridia fraseri* is thought to be entirely insectivorous (Cheke and Mann 2001), a hypothesis corroborated by the unusual location of *E. fraseri* in both linear measurements (Figure 1.6) and basihyal shape analyses (Figure 1.7.B-C) where it is intermediate between the core sunbird cluster and non-sunbirds, perhaps representing a return to insectivory. The circumstances for *Promerops* are reversed – it is the sole nectarivorous non-sunbird in this study, yet its shape is more sunbird-like in one or both PC1 and PC2 (Figures 1.5.18B-C, 1.6A). This suggests the existence of a region in morphospace where certain shapes are advantageous for nectarivorous diet; however, the significance of trophic niche is likely partially driven by the four frugivorous taxa outside of sunbirds. Another clue to the functional anatomy of sunbirds can be seen in *Leptocoma* and *Aethopyga*. The broad basihyal of these Asian genera are quite similar to that of the *Philepitta* asities (Passeriformes: Philepittidae), which, in addition to nectar, consume soft-shelled fruits by strategically crushing them against the palate using their tongue (Zubkova 2019a, 2019b). Although little is known about the diets of most *Leptocoma* and *Aethopyga* species, grapes are noted as one of the foods

of *L. zeylonica* (Cheke and Mann 2001). Broader taxonomic sampling is needed to account for all the diversity present in sunbirds. Additionally, higher resolution scans are needed to better infer muscle attachments (e.g., Figure 1.5.2A-C versus Figure 1.5.14A-C, Figure 1.3.B *Arachnothera magna* versus *A. longirostra*).

1.4.5 Nectarivory as a syndrome

Recent work on nectar-feeding adaptations in hummingbirds have spurred similar research in other nectarivorous radiations that increasingly highlights the diversity of mechanisms implicated in different stages of the drinking process (Cuban et al. 2022; Hewes et al. 2022, 2023). Broad comparison between representatives from three different radiations of nectar-feeding birds have yielded several insights into morphological adaptations to nectarivory.

All three representative species are ecological analogues in their respective distributions (Collins and Paton 1989). They possess elongated hyoid horns composed of the ceratobranchials and epibranchials, a feature noted by Paton and Collins (1989). These structures provide surface area for the brachiomandibularis and stylohyoideus muscles involved in protraction and retraction of the whole tongue apparatus, a fundamental movement in nectar-feeding (Richards and Bock 1973). However, upon closer examination as George (1961) astutely noted, the ceratobranchial in passerines are variable in their cross-sectional shape, ranging from cylindrical to crescent-shaped. Inside the “hollow” sits the origin of the ceratoglossus posterior muscle, which likely inserts onto the basihyal head in sunbirds (Figure 1.1) and the paraglossals in other birds (Zubkova 2019b). One can imagine that size of the ceratoglossus is likely to be correlated with movement at the tongue joint (George 1961), though exactly how is yet to be determined.

The foremost difference can be found in the paraglossalia. In Anna’s Hummingbird, the paired paraglossalia are proportionally the smallest elements in the tongue skeleton, whereas in Olive Sunbird and Eastern Spinebill they are among the largest elements, second only to the hyoid horns. This suggests that the paraglossalia of the latter two radiations are subject to comparatively stronger mechanical stresses than hummingbirds. Structural differences make sense in light of how liquid is initially collected by the tongue. Hummingbird tongues fill via expansive filling where the grooves of the keratin spontaneously expands upon contact with liquid and requires no muscle action (Cuban et al. 2022; Rico-Guevara et al. 2015; Rico-Guevara and Rubega 2017). Sunbird tongues draw up nectar at the distal tongue tip when negative pressure is generated by depression of the tongue body whilst the lateral margins of the tongue form a seal against the upper palate (this study; D. Cuban unpubl. data). The relationship between form and function in honeyeaters is less clear. A study of five honeyeater species found interspecific variation in tongue filling mechanics, including expansive filling and capillary filling (Hewes et al. 2023). Unfortunately, our sampling did not include the species used by Hewes et al. (2023). Nevertheless, the morphology of *Acanthorhynchus tenuirostris* (Figure 1.9.G-J) suggests honeyeaters may employ a different mechanism than sunbirds. Our understanding of the relationship between form and function will continue to grow as sampling size increases for morphological and biomechanical studies.

1.5 Conclusions

Based on the available information on the tongue morphology and natural history of sunbirds, the results of this study of the tongue skeleton give further credence to part of the

hypothesis of Richards and Bock (1973): evidence of evolutionary adaptations for different diets is expected to be found in the passerine tongue skeleton. Trophic niche does predict basihyal shape to some degree, depending on the region of interest. All nectarivores are characterized by long epibranchials, but variation in the tongue skeleton within and between nectarivorous radiations implies a complex relationship between phylogeny, ecology, and morphology. This study highlights the value of studying avian tongue morphology, not just in nectar-feeding clades but in other groups where the tongue plays a major role in food handling. The results support previous findings that dietary shifts and therefore modifications in feeding behavior are a primary driver of morphological evolution. While historically much attention has been paid to bill size and shape, the results of this study indicate that there is just as much, if not more, variation in the tongue yet to be fully explored.

It must be noted that this study is by no means comprehensive of all modes of nectar-feeding. At least two sunbird species has been observed to rob nectar (Dhanya et al. 2013; Fujita 2000; Jayanthi et al. 2015), and it remains to be seen whether specialized nectar-robbing taxa such as flowerpiercers in the genus *Diglossa* (Passeriformes: Thraupidae) evolved unique morphologies and mechanisms for drinking nectar. MicroCT Scan resolution (i.e., voxel size) is also a key factor in being able to reconstruct the basihyal; based on the scans used in this study, voxel size should be as low as is feasible and no higher than 40 μm to visualize the basihyal-paraglossal joint and the basihyal-ceratobranchial joints. Broader sampling of both the bill and the tongue apparatus will lead to a more holistic understanding of the evolutionary dynamics underlying diet-mediated radiations in birds. This study will inform future work of functional morphologists and provide a foundation for studying the biomechanics of feeding, particularly how morphological variation shapes performance. Subsequent work tying variation in musculature of the tongue apparatus, entoglossum shape, bill shape, and feeding performance will enable a deeper understanding of the evolution of nectar-feeding in birds.

1.6 Tables and Figure

Table 1.1: Glossary of tongue elements and letter designations. Terminology was adapted from Manegold (2008), Richards and Bock (1973), Zubkova (2019a).

Element	Name	Abbreviation
Muscle	M. ceratoglossus anterior (hypoglossus anterior in Richards and Bock, 1973)	cga
Aponeurosis	Ap. ceratoglossis insertionis	acg
Muscle	M. ceratoglossus posterior	cgp
Aponeurosis	Ap. ceratoglossis	acg
Muscle	M. ceratohyoideus	ch
Muscle	M. hypoglossus, also hypoglossus posterior (George, 1962), hypoglossus obliquus (Richards and Bock, 1973), hypoglossus rostralis	hg
Muscle	M. brachiomandibularis	bm
Muscle	M. genioglossus	gg
Muscle	M. stylohyoideus	sth
Muscle	M. serpihyoideus	sh
Muscle	M. mylohyoideus	mh
Muscle	M. thyreohyoideus	th
Muscle	M. tracheohyoideus	trh
Skeleton	Paraglossal	Pg
Process	Processus paraglossalis rostralateralis (new term). A projection on the lateral edge of the paraglossalium, typically at the midlength of the paraglossal, pointed rostrally and usually on a plane elevated above the rostral tip of the paraglossalia. Depicted but not noted in Bates (1930).	Ppr
Process	Processus paraglossalis caudalis	Ppc
Process	Processus paraglossalis ventralis (Manegold, 2008)	Ppv
Articular facet	Facies articularis paraglossobasihyalis (Homberger and Brush, 1986; Manegold, 2008)	
Skeleton	Basihyal	Bas
Skeleton	Urohyal	Ur
Skeleton	Ceratobranchial	Cb
Skeleton	Epibranchial	Ep
Other	Entoglossum	ent
Other	Cricoid cartilage	cri

Table 1.2: Description of characters used for linear and geometric morphometric analyses.

Continuous traits were based on previously published methods. Several continuous traits and semi-landmark curves are used for the first time in this study.

Trait or Landmark	Name	Abbreviation	Definition	Reference if applicable
Continuous trait	Beak length at culmen	BLAC	Length of the bill from bill tip to the base of the bill at junction with skull	Tobias et al. (2022)
Continuous trait	Anterior paraglossalia length	APGL	Linear distance from the rostral tip of the paraglossal to the <i>process paraglossalis rostromedialis</i> (ppr)	New metric
Continuous trait	Paraglossalia length	PGL	Length of paraglossal, from rostral tip to end of caudal process	Li and Clarke (2016)
Continuous trait	Paraglossal width	PGW	Width at widest part, typically anterior to the paraglossal horns	Li and Clarke (2016)
Semi-landmarked curve	Articular surface outline of the basihyal	ASO	One landmark at the rostral-most point of the basihyal, 10 semi-landmarks constrained to the model around the margins of the facets that articulate with the paired paraglossalia.	New metric
Continuous trait	Basihyal-urohyal length	BUL	A composite bone, the joint between the basihyal and the urohyal is indiscernible. Length of the combined basihyal and urohyal, from rostral point of the basihyal to caudal end of the urohyal.	Li and Clarke (2016)
Continuous trait	Basihyal length	BHL	Length of the basihyal, from anterior point of the basihyal to the midpoint between the articulation with the left and right ceratobranchial	Li and Clarke (2016)
Continuous trait	Basihyal head width	BHW	Width of the basihyal head at widest point	Partly based on Webster (2003)
Semi-landmarked curve	Midsagittal outline of the basihyal-urohyal	MSO	Two landmarks representing the rostral-most point of the basihyal head and the caudal-most point of the urohyal. 58 semi-landmarks between the two landmarks, on both the dorsal and the ventral surfaces (29 each)	New metric
Continuous trait	Ceratobranchial length	CBL	Here length of the curve along the ventral surface, starting at the basihyal-ceratobranchial joint and ending at the ceratobranchial-epibranchial joint	Li and Clarke (2016)
Continuous trait	Epibranchial length	EPL	Here length of the curve along the ventral surface and continues to the top of the skull, starting at the ceratobranchial-epibranchial joint and ending at the distal tip of the epibranchial	Li and Clarke (2016)

Table 1.3: Linear measures of tongue skeletons taken from 3D reconstructed skulls. For taxa represented by more than one individual, an average for the genus or family is also provided. Incomplete measures are either noted as not applicable (N/A) or not visible (N/V). * = from the AVONET dataset (Tobias et al. 2022). ** = considered a subspecies of *Dicaeum agile*. See abbreviations in Table 1.2.

Family	Genus	Species	Mass (g)*	BLAC (mm)*	APGL (mm)	PGL (mm)	PGW (mm)	BHW (mm)	BHL (mm)	BUL (mm)	CBL (mm)	EPL (mm)
Promeropidae	<i>Promerops</i>	<i>Promerops cafer</i>	34.70	36.8	N/A	12.62	2.01	0.78	5.57	9.12	13.48	12.98
Modulatricidae	<i>Modulatrix</i>	<i>Modulatrix stictigula</i>	30.50	17.5	N/A	6.87	2.65	0.82	4.65	9.23	10.94	7.23
Chloropseidae	<i>Chloropsis</i>	<i>Chloropsis hardwickii</i>	32.30	24.9	N/A	12.39	2.23	0.72	5.90	10.73	11.55	8.11
Irenidae		Family average	72.60	27.5	N/A	9.38	3.88	1.24	6.67	12.33	12.98	10.49
	<i>Irena</i>	<i>Irena cyanogaster</i>	80.30	28.7	N/A	9.02	3.87	1.27	7.18	12.67	13.03	11.20
		<i>Irena puella</i>	64.90	26.3	N/A	9.74	3.90	1.21	6.16	11.99	12.93	9.78
Dicaeidae		Family average	9.16	11.3	N/A	4.63	2.34	0.78	3.67	6.34	8.61	5.81
	<i>Dicaeum</i>	Genus average	9.10	11.5	N/A	4.54	2.37	0.81	3.66	6.26	8.61	5.76
		<i>Dicaeum aeruginosum</i> **	9.00	10.5	N/A	4.19	2.36	0.78	3.41	6.14	8.54	5.28
		<i>Dicaeum anthonyi</i>	11.60	11.4	N/A	4.55	2.59	0.75	3.96	6.42	9.38	5.72
		<i>Dicaeum australe</i>	8.70	11.9	N/A	4.51	2.54	0.95	3.97	6.53	8.76	6.19
		<i>Dicaeum trigonostigma</i>	7.10	12.1	N/A	4.91	2.00	0.74	3.31	5.92	7.76	5.86
	<i>Pachyglossa</i>	<i>Pachyglossa olivaceus</i>	9.40	10.6	N/A	4.97	2.20	0.70	3.70	6.70	8.63	5.98
Nectariniidae		Family average	9.91	22.7	2.54	7.19	2.12	1.16	2.50	4.36	6.35	18.66
	<i>Aethopyga</i>	Genus average	6.87	22.2	1.85	5.54	1.83	0.89	2.08	3.68	5.55	15.85
		<i>Aethopyga flagrans</i>	6.70	19.2	1.93	5.92	1.90	0.91	2.19	3.96	5.75	16.26
		<i>Aethopyga linaraborae</i>	7.20	22.5	1.64	5.96	1.92	0.94	2.20	3.72	5.95	19.86
		<i>Aethopyga siparaja</i>	6.70	19.0	1.98	4.73	1.67	0.83	1.85	3.35	4.94	11.44
	<i>Anthobaphes</i>	<i>Anthobaphes violacea</i>	9.40	23.1	N/V	N/V	2.04	1.08	2.33	4.26	6.21	20.22
	<i>Anthodiaeta</i>	Genus average	8.43	19.9	2.52	6.71	1.97	1.15	2.33	3.97	5.93	16.89
		<i>Anthodiaeta collaris</i>	7.00	15.3	2.08	5.75	1.96	1.17	2.10	3.63	5.49	13.74
		<i>Anthodiaeta hartlaubi</i>	10.50	22.7	2.96	7.52	1.89	1.17	2.67	4.67	6.58	18.19
		<i>Anthodiaeta jugularis</i>	9.00	20.7	2.67	6.72	2.07	1.19	2.28	3.91	5.95	16.72
		<i>Anthodiaeta sovimanga</i>	7.20	20.7	2.36	6.87	1.95	1.06	2.27	3.68	5.69	18.90
	<i>Anthreptes</i>	<i>Anthreptes malacensis</i>	11.30	20.3	3.48	7.52	2.89	1.21	3.27	5.89	7.59	16.51
	<i>Arachnothera</i>	Genus average	21.60	44.3	3.40	10.47	2.67	1.40	3.55	5.89	8.47	28.66
		<i>Arachnothera longirostra</i>	12.50	41.8	3.76	8.15	2.40	1.12	3.05	4.98	6.90	26.48
		<i>Arachnothera magna</i>	30.70	46.8	3.04	12.79	2.93	1.68	4.05	6.81	10.03	30.83
	<i>Cinnyris</i>	Genus average	9.86	21.9	2.52	7.43	1.97	1.18	2.54	4.38	6.28	19.39
		<i>Cinnyris adelberti</i>	9.80	19.8	2.06	7.00	1.94	1.33	2.30	4.10	5.82	17.34
		<i>Cinnyris amethystina</i>	13.70	24.8	3.49	9.66	2.10	1.18	3.05	5.25	7.34	22.83
		<i>Cinnyris chloropygius</i>	6.20	19.8	2.33	6.37	1.72	1.08	2.21	3.62	5.54	16.57
		<i>Cinnyris cupreus</i>	8.90	21.1	2.20	6.79	1.89	1.11	2.42	4.35	6.21	19.53
		<i>Cinnyris erythrocerus</i>	8.80	19.4	2.31	6.19	1.89	1.11	2.38	4.15	6.07	17.15
		<i>Cinnyris notatus</i>	14.60	31.1	3.17	9.89	2.63	1.46	3.25	5.16	7.86	28.30
		<i>Cinnyris pulchellus</i>	7.00	17.6	2.11	6.10	1.65	1.02	2.19	4.00	5.15	14.00
	<i>Cyanomitra</i>	Genus average	11.17	24.3	2.87	8.07	2.36	1.22	2.68	4.28	6.61	22.36
		<i>Cyanomitra alinae</i>	12.60	29.1	3.23	9.31	2.61	1.46	2.94	4.64	7.30	27.25
		<i>Cyanomitra ursulae</i>	6.50	19.2	2.02	6.32	2.14	1.02	2.28	3.82	5.93	17.41
		<i>Cyanomitra verticalis</i>	14.40	24.7	3.36	8.58	2.32	1.17	2.83	4.38	6.61	22.42
	<i>Euchloridia</i>	<i>Euchloridia fraseri</i>	11.30	19.4	2.54	6.20	2.22	1.18	2.38	5.09	6.14	12.16
	<i>Haagneria</i>	<i>Haagneria olivacea</i>	9.70	23.4	2.70	7.31	1.96	1.15	2.28	3.90	6.57	21.68
	<i>Lamprothreptes</i>	<i>Lamprothreptes longuemarei</i>	11.40	18.5	2.54	6.47	2.58	1.28	2.95	5.46	7.08	12.71
	<i>Leptocoma</i>	<i>Leptocoma sperata</i>	6.40	15.3	2.60	6.27	2.29	0.77	2.27	4.08	5.74	15.17
	<i>Notiocinnyris</i>	Genus average	7.93	21.6	2.25	6.84	2.02	1.19	2.27	4.07	6.06	17.45
		<i>Notiocinnyris chalybeus</i>	7.70	21.8	N/V	N/V	1.76	1.16	2.22	3.85	5.48	16.69
		<i>Notiocinnyris manoensis</i>	9.50	21.9	2.55	7.94	2.24	1.38	2.64	4.74	6.63	19.21
		<i>Notiocinnyris mediocris</i>	8.20	19.3	2.20	7.01	2.11	1.04	2.06	4.17	5.88	17.65
		<i>Notiocinnyris regius</i>	6.60	18.8	2.01	6.03	2.00	1.18	1.97	3.60	5.65	16.49
		<i>Notiocinnyris reichenowi</i>	6.80	21.5	2.06	6.43	2.06	1.21	2.36	3.82	5.97	14.81
		<i>Notiocinnyris stuhlmanni</i>	8.80	26.5	2.41	6.78	1.97	1.17	2.36	4.27	6.77	19.83

Table 1.4 Summary of statistics from PGLS analyses. Multivariate basihyal shape data was analyzed using ‘procD.pgls’ and the first two principal components were analyzed using ‘gls’. Analyses were run alternatively with either log(mass), log(centroid size), or both. Significant results ($P < 0.05$) are highlighted in bold and near-significant results ($0.5 < P < 0.1$) are underlined. ASO = articular surface outline of the basihyal head, MSO = midsagittal surface outline of the basihyal-urohyal.

Module	Trophic Niche (procD.pgls)	Log(mass) (procD.pgls)	Log(cs) (procD.pgls)	Trophic Niche (PC1 - gls)	Log(mass) (PC1 - gls)	Log(cs) (PC1 - gls)	Trophic Niche (PC2 - gls)	Log(mass) (PC2 - gls)	Log(cs) (PC2 - gls)
ASO	Z=-0.8434, P=0.803	Z=1.7148, P=0.044		P=0.92769	P=0.03157		P=0.7572	P=0.5316	
ASO	Z=-0.39569, P=0.661		Z=0.17495, P=0.437	P=0.83902		P=0.16637	P=0.6690		P=0.7734
ASO	Z=-0.75097, P=0.763	<u>Z=1.63261, P=0.057</u>	Z=0.04488, P=0.481	P=0.9275	P=0.1129	P=0.9067	P=0.7688	P=0.5745	P=0.8991
MSO	Z=-0.08253, P=0.525	Z=-0.69309, P=0.748		P=0.02221	P=0.81175		P=0.1816	P=0.3326	
MSO	Z=0.05447, P=0.472		Z=0.83105, P=0.228	<u>P=0.06841</u>		<u>P=0.0643</u>	P=0.2305		P=0.6913
MSO	Z=0.01482, P=0.496	Z=0.24862, P=0.409	Z=0.95871, P=0.170	P=0.035	P=0.10156	P=0.01278	P=0.2009	P=0.3736	P=0.8522
ASO + MSO	Z=-0.07608, P=0.525	Z=-0.47225, P=0.678		P=0.02225	P=0.8815		P=0.3333	P=0.1574	
ASO + MSO	Z=P=0.06717, P=0.474		Z=1.01873, P=0.167	<u>P=0.070788</u>		P=0.001344	P=0.2318		P=0.7834
ASO + MSO	Z=0.02922, P=0.487	Z=0.25572, P=0.407	Z=1.0954, P=0.133	P=0.02673	P=0.03213	P < 0.0001	P=0.3088	P=0.1293	P=0.5209

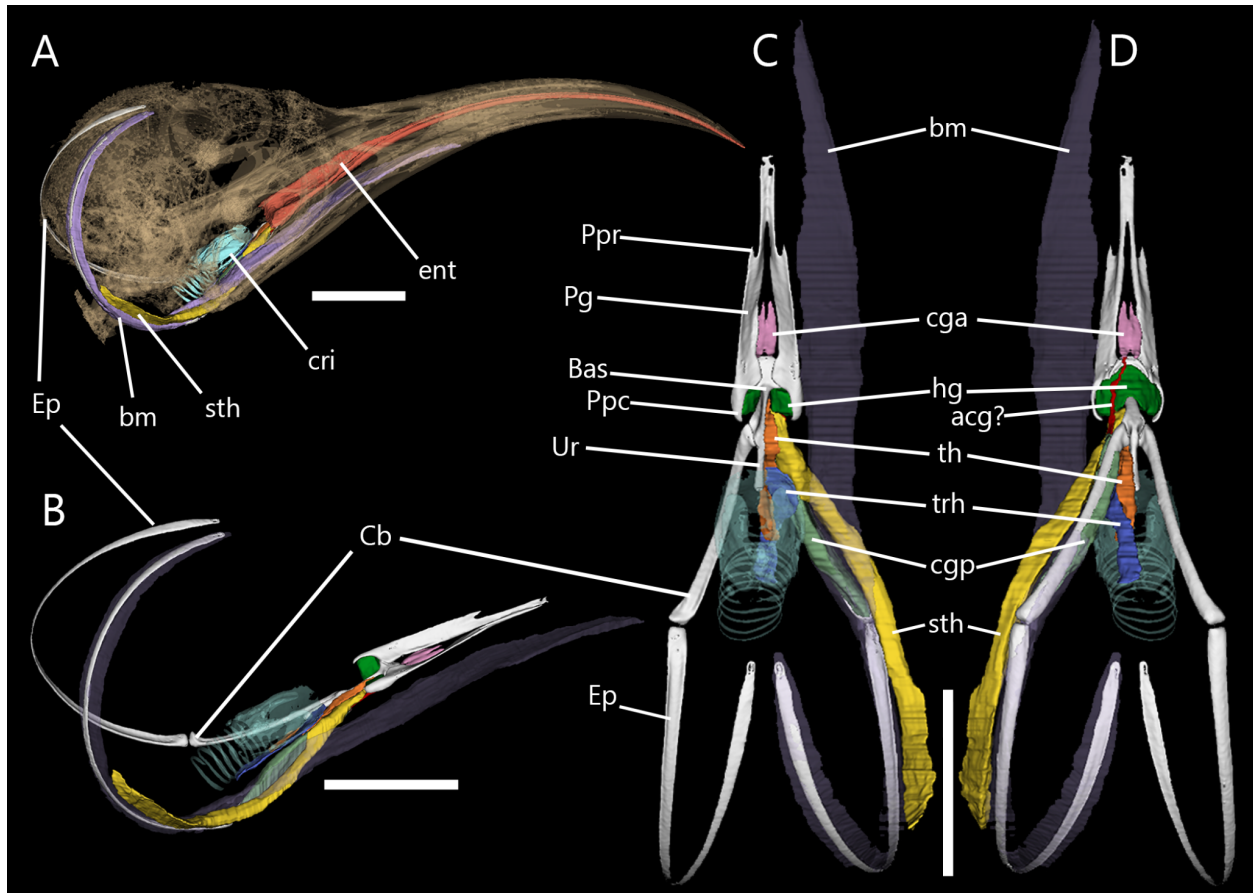


Figure 1.1: Skull, tongue bones, entoglossum, and select hyoid muscles of the Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421). (A) Lateral view of the skull, tongue bones, entoglossum, and select muscles in situ. The cricoid cartilage (cri) and m. brachiomandibularis (bm) are transparent in views (B-D). (B) Dorsal oblique view of the tongue apparatus without the skull, (C) dorsal view, (D) ventral view. acg? – possible ap. ceratoglossis; Bas – basihyal; bm – m. brachiomandibularis; Cb – ceratobranchial; cri – cricoid cartilage; cga – m. ceratoglossus anterior; cgp – m. ceratoglossus posterior; ent – entoglossum; Ep – epibranchial; hg – m. hypoglossus; Pg – paraglossalium; Ppc – processus paraglossalis caudalis; Ppr – processus paraglossalis rostralateralis; sth – m. stylochoyoideus; th – m. thyreochoyoideus; trh – m. tracheochoyoideus; Ur – urohyal. Scale bar = 5 mm.

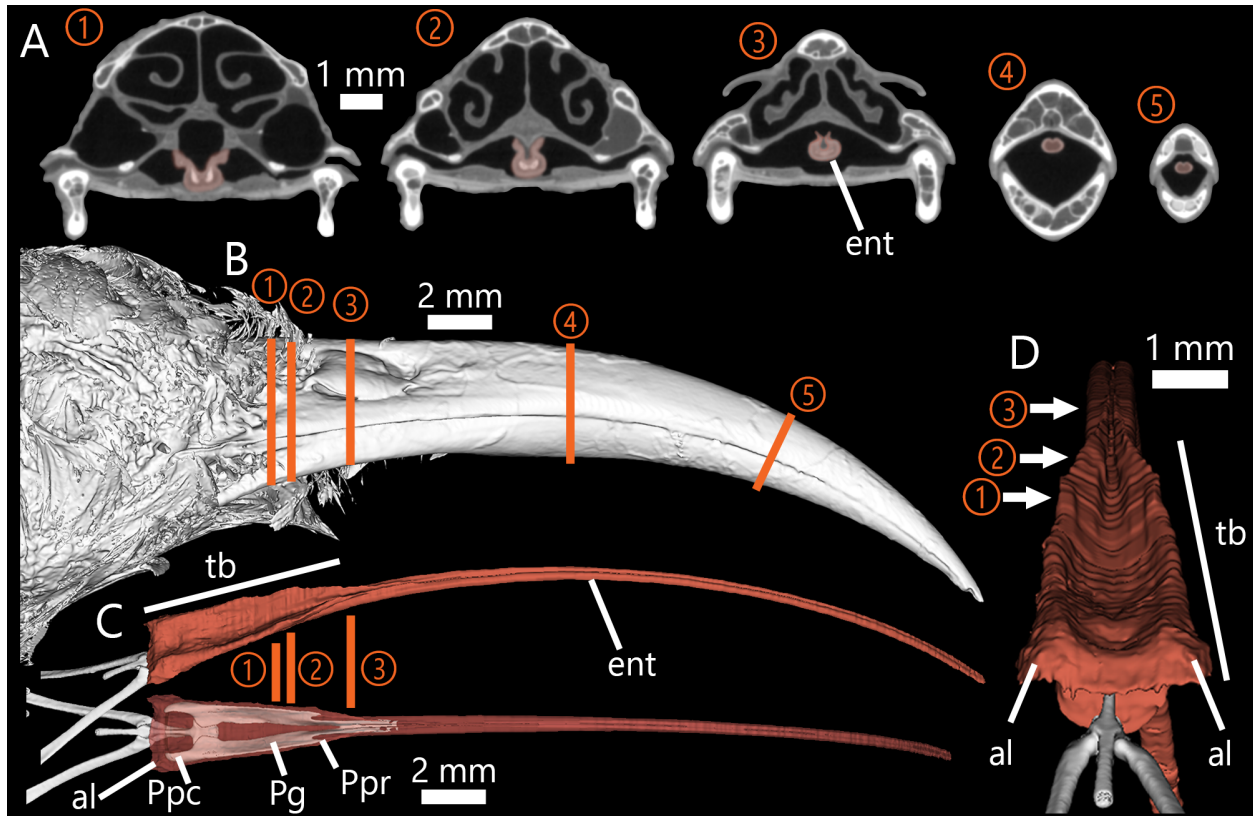


Figure 1.2: Surface reconstruction and cross section of the bill and entoglossum from a microCT scan of an Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421). Voxel size is 22.4 μm . (A) Frontal cross section of both the entoglossum (shaded in red) and bill, (B) lateral view of the head, (C) the entoglossum is shown in (C top) oblique dorsolateral view as fully opaque, (C bottom) in dorsal view as slightly translucent to show the positioning of the paraglossalia and processes, and (D) oblique caudal dorsal view. The numbers correspond to the same transverse slice across all views. al – alae linguae; ent – entoglossum; Pg – paraglossalium; Ppc – processus paraglossalis caudalis; Ppr – processus paraglossalis rostralateralis; tb – tongue body.

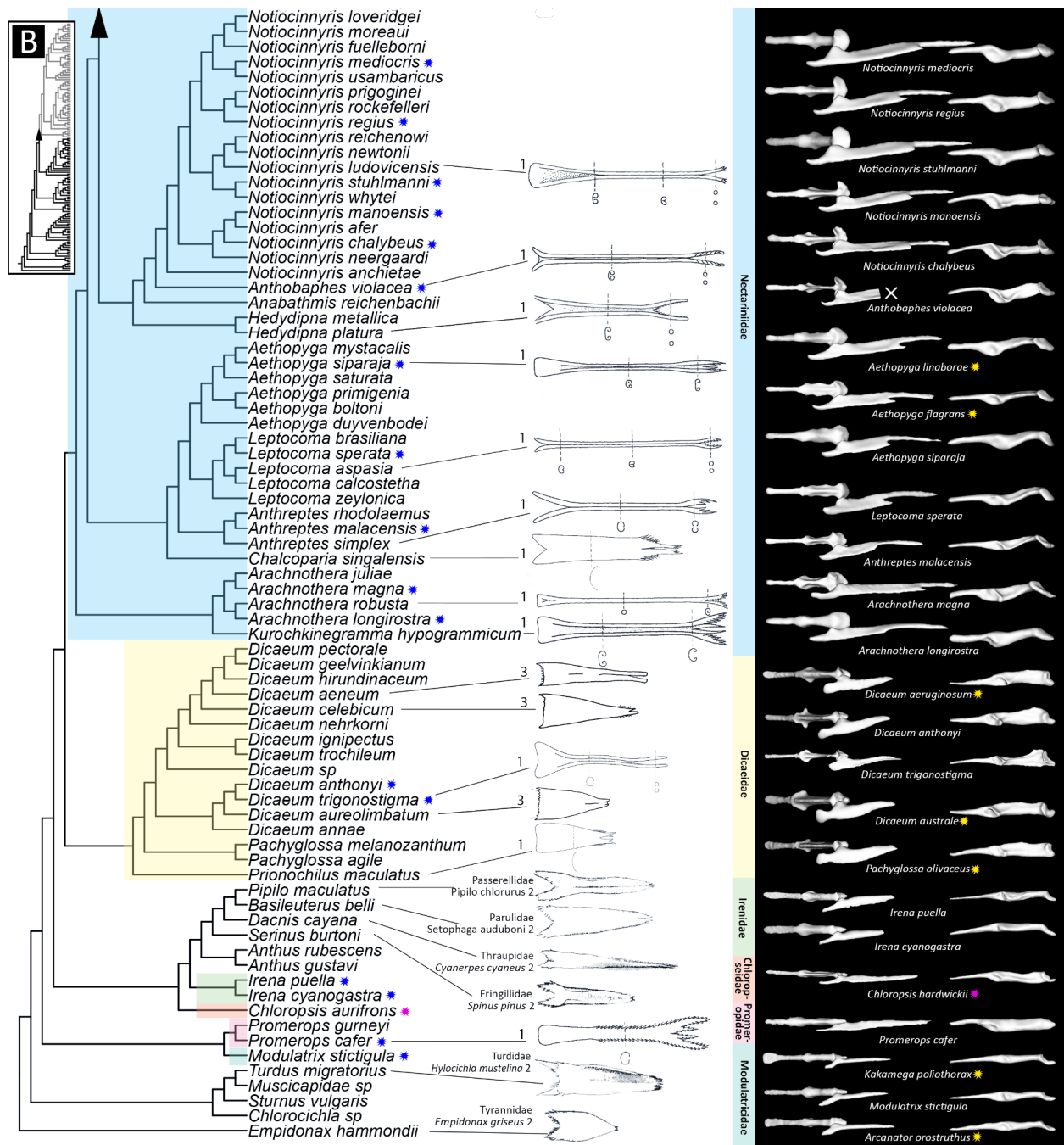


Figure 1.3.A-B: Entoglossum drawing, basihyal-urohyal with right paraglossalia, and basihyal-urohyal morphology across the ultrametric phylogeny of Nectariniidae and outgroups. Entoglossum drawings (not to scale), several of which show the shape in cross section, are adapted from (1) Cheke and Mann (2001), (2) Gardner (1925), (3) Morioka (1992), (4) Scharnke (1932), and (5) Schlamowitz (1976). The entoglossum drawings and the basihyal-urohyal with right paraglossalia are in dorsal view (not to scale) and the basihyal-urohyal size is held roughly constant. The plane of the paraglossalia is approximately parallel to the transverse plane. The basihyal (not to scale) are in the right lateral view and the length is held roughly constant. Rostral half of *Anthobaphes violacea* was not included in the scan.

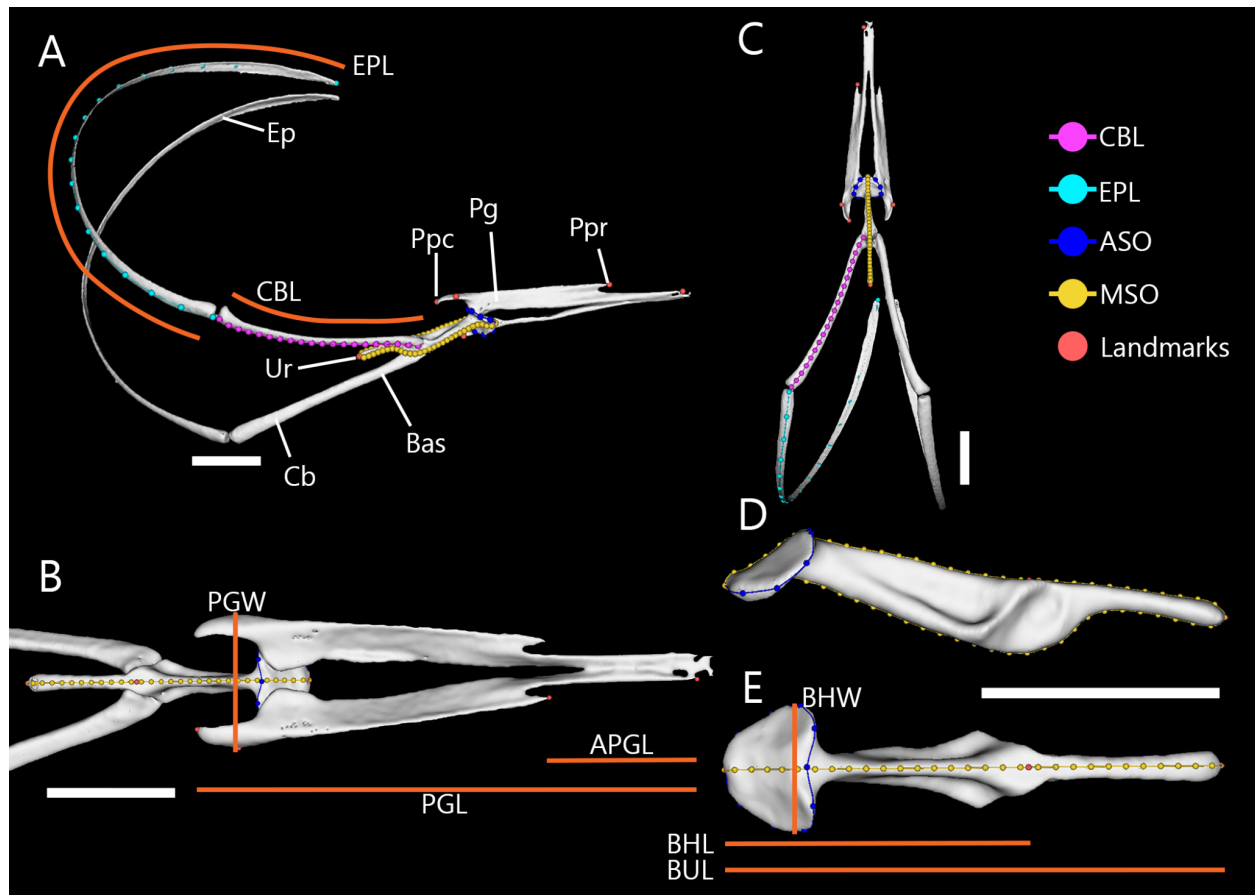


Figure 1.4: Hyoid measurements and semi-landmark curves used in morphometric analyses. The tongue skeleton depicted is from the Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421), viewed at several angles to illustrate the placement of landmarks, semi-landmarks, and curves: (A) an oblique ventrolateral angle, (B) dorsal view of the paraglossalia and the basihyal, (C) ventral view of the tongue skeleton, (D) left lateral view of the basihyal, and (E) dorsal view of the basihyal. Curves used for geometric morphometrics are the basihyal anterior surface outline (ASO; blue) and the basihyal-urohyal midsagittal outline (MSO; yellow). Curves used for measuring ceratobranchial length (CBL; pink) and epibranchial length (EPL). APGL – anterior paraglossalia length; BHL – basihyal length; BUL – basihyal-urohyal length; CBL – ceratobranchial length; EPL – epibranchial length; PGL – paraglossalia length; PGW – paraglossal width. Scale bar = 2 mm.

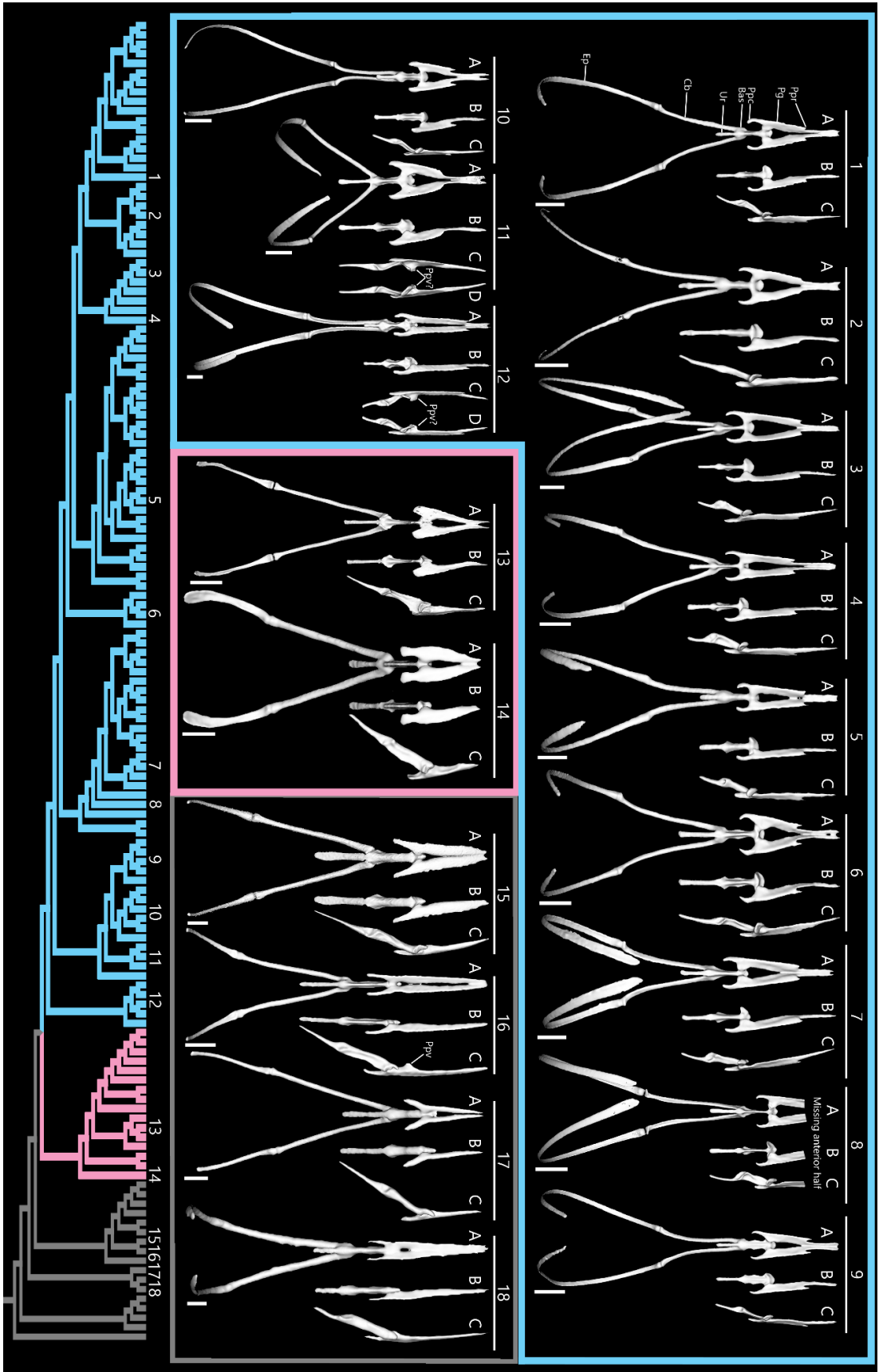


Figure 1.5: Representative tongue skeletons across an ultrametric phylogeny of Nectariniidae and outgroups. One representative from each genus is depicted, where the number above the skeleton corresponds to its placement on the phylogeny. Blue represents the sunbird (Nectariniidae) clade, pink the flowerpecker (Dicaeidae) clade, and grey the outgroups. (1A-C) Collared Sunbird (*Anthodiaeta collaris*), (2A-C) Fraseri's Sunbird (*Euchloridia fraseri*), (3A-C) Blue-headed Sunbird (*Cyanomitra alinae*), (4A-C) Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421), (5A-C) Buff-throated Sunbird (*Cinnyris adelberti*), (6A-C) Western Violet-backed Sunbird (*Lamprothreptes longuemarei*), (7A-C) Miombo Double-collared Sunbird (*Notiocinnyris manoensis*), (8A-C) Orange-breasted Sunbird (*Anthobaphes violacea*), (9A-C) Purple-throated Sunbird (*Leptocoma sperata*; FMNH:Bird:345062), (10A-C) Flaming Sunbird (*Aethopyga flagrans*), (11A-D) Brown-throated (*Anthreptes malacensis*), (12A-D) Streaked Spiderhunter (*Arachnothera magna*), (13A-C) Orange-bellied Flowerpecker (*Dicaeum trigonostigma*), (14A-C) Olive-backed Flowerpecker (*Pachyglossa olivaceus*), (15A-C) Asian Fairy-bluebird (*Irena puella*), (16A-C) Orange-bellied Leafbird (*Chloropsis hardwickii*), (17A-C) Spot-throat (*Modulatrix stictigula*), and (18A-C) Cape Sugarbird (*Promerops cafer*). A = Dorsal view, B = dorsal view with left paraglossalium removed, C = left lateral view with left paraglossalium removed, D = right lateral view with left paraglossalium removed. Abbreviations: Bas – basihyal; Cb – ceratobranchial; Ep – epibranchial; Pg – paraglossalium; Ppc – processus paraglossalis caudalis; Ppr – processus paraglossalis rostralateralis; Ppv? – possibly the processus paraglossalis ventralis; Ur – urohyal. Scale bar = 2 mm.

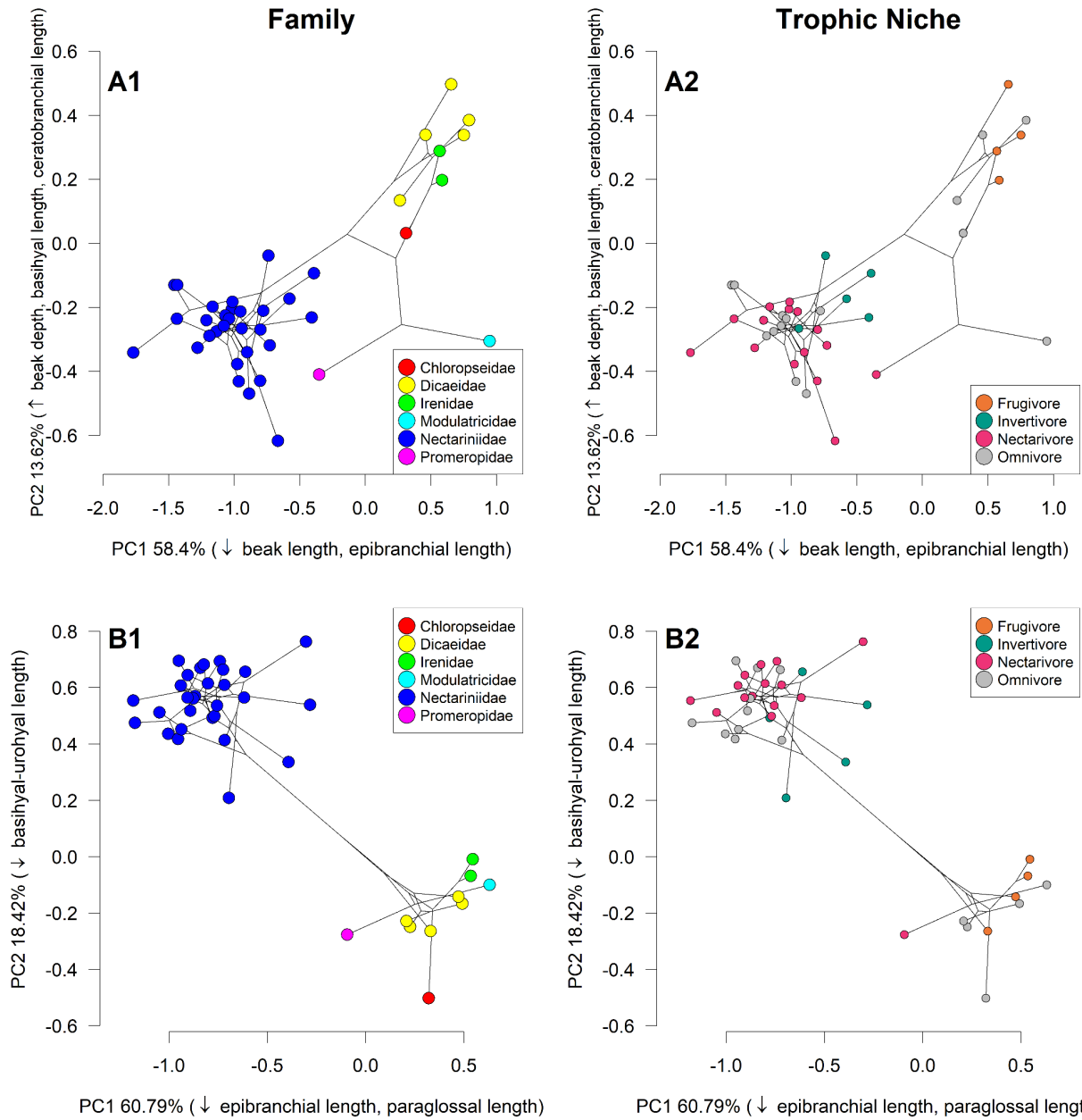


Figure 1.6: Phylomorphospace of PC1 and PC2 from a phylogenetic PCA. Measurements were corrected for size. (A) The tongue and the bill and (B) only the tongue with respect to (1) family and (2) trophic niche.

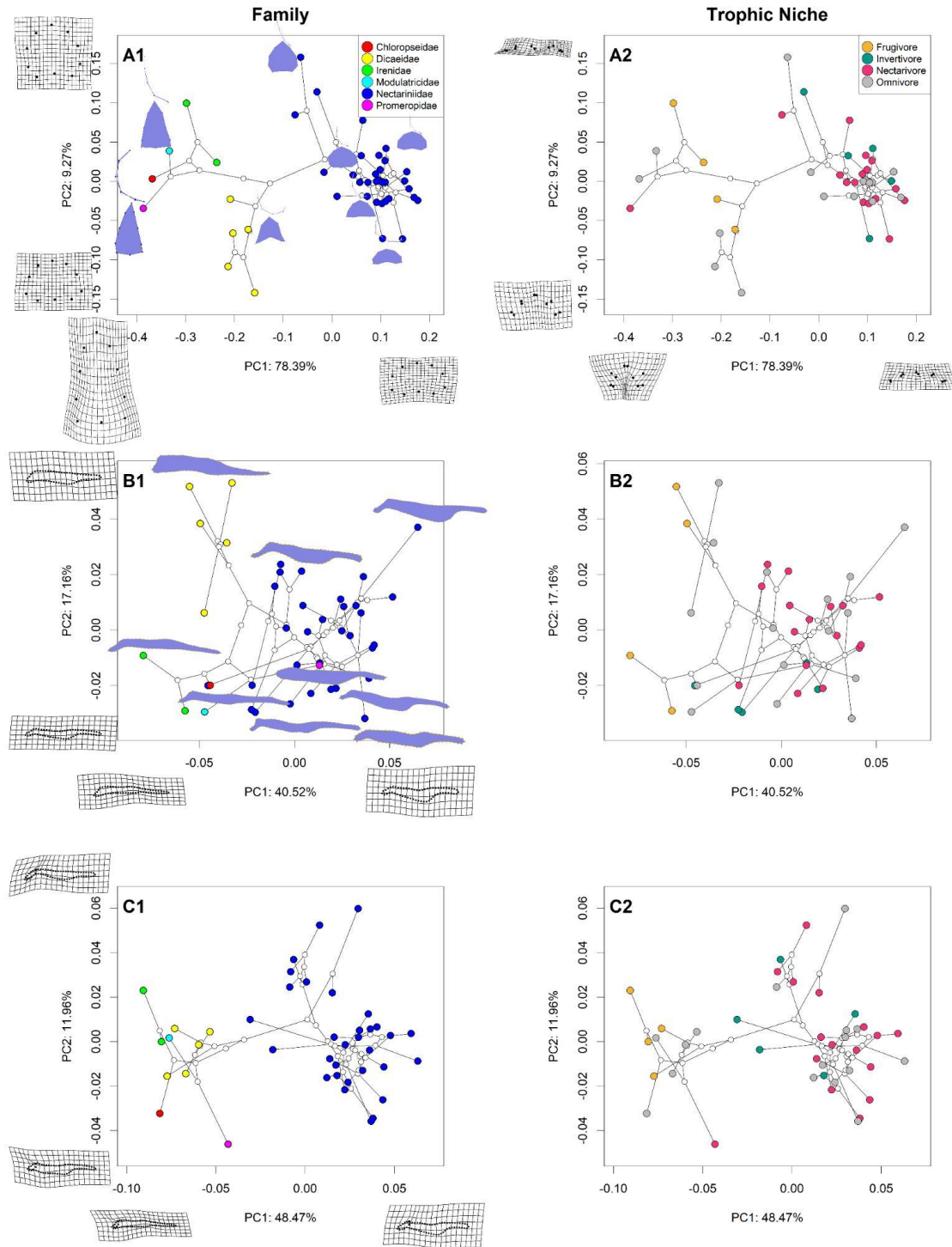


Figure 1.7: Principal components of shape (PC1, PC2) of three sets of landmarks. (A) Articular surface, (B) midsagittal surface, and (C) merged articular and midsagittal surface landmarks with respect to (1) family and (2) trophic niche. Shape deformations along PC1 and PC2 are illustrated with deformations of (A1) dorsal basihyal articular surface shape, (A2) rostral basihyal articular shape, (B1) lateral basihyal midsagittal shape, and (C1) lateral merged articular and midsagittal surface shape. Note the location of Promeropidae (pink) with respect to Nectariniidae (blue).

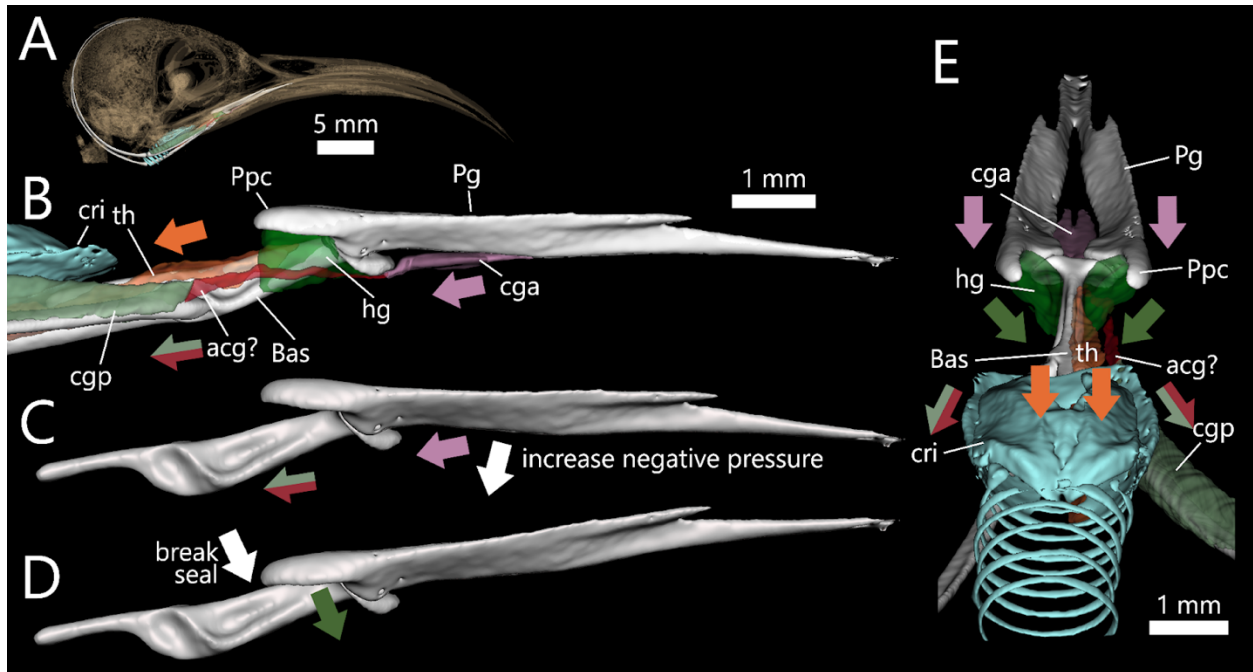


Figure 1.8: The tongue bones and intrinsic hyoid muscles of the Olive Sunbird (*Haagneria olivacea*; MVZ RCKB1421). Colored arrows correspond to muscles of the same color and represent the direction of movement. White arrows represent the overall effect of the muscle contractions. (A) Lateral view of the skull, tongue bones, and select muscles in situ. The cricoid cartilage (cri) is opaque in views A, B and E. (B) Right lateral view of the tongue apparatus without the skull, (C) movement of the paraglossalia relative to the basihyal-urohyal when the hypoglossus muscle contracts and lifts the rostral tip of the paraglossalia, (D) movement of the paraglossalia relative to the basihyal-urohyal when the ceratoglossus anterior and the ceratoglossus posterior muscles contract and lowers the rostral tip of the paraglossalia. acg – ap. ceratoglossus; Bas – basihyal; cri – cricoid cartilage; cga – m. ceratoglossus anterior; cgp – m. ceratoglossus posterior; hg – m. hypoglossus; Pg – paraglossalium; Ppc – processus paraglossalis caudalis.

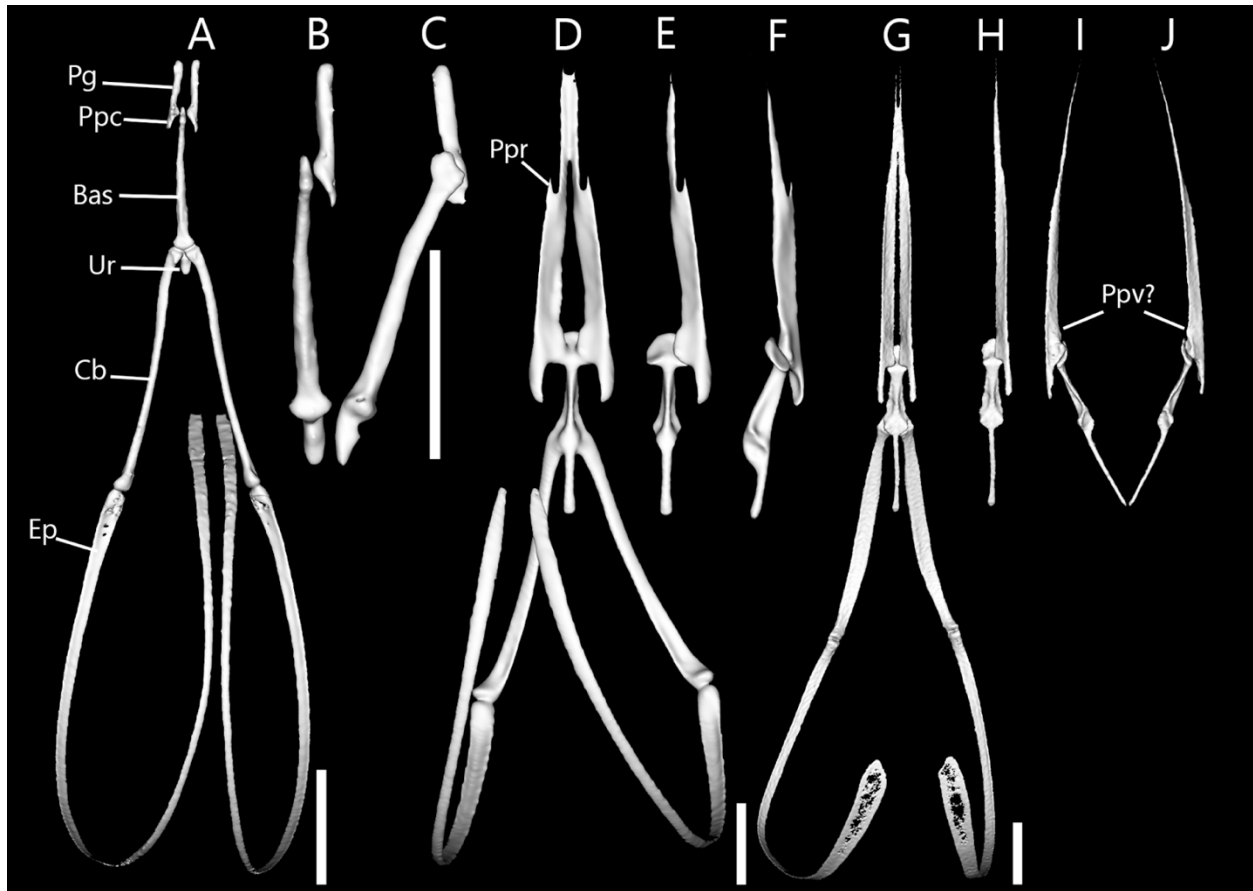


Figure 1.9: Tongue skeleton of Anna's Hummingbird, Olive Sunbird, and Eastern Spinebill. (A-C) Anna's Hummingbird (Caprimulgiformes: Trochilidae: *Calypte anna*), (D-F) Olive Sunbird (Passeriformes: Nectariniidae: *Haagneria olivacea*), and (G-J) Eastern Spinebill (Passeriformes: Meliphagidae: *Acanthorhynchus tenuirostris*). (A, D, G) Dorsal view, (B, E, H) dorsal view with left paraglossalium removed, (C, F, J) left lateral view with left paraglossalium removed, (I) right lateral view with left paraglossalium removed. Abbreviations: Bas – basihyal; Cb – ceratobranchial; Ep – epibranchial; Pg – paraglossal; Ppc – processus paraglossalis caudalis; Ppr – processus paraglossalis rostralateralis; Ppv? – possible processus paraglossalis ventralis; Ur – urohyal. Scale bar = 2 mm.

Chapter 2

Evaluating candidate genes for bill morphology in sunbirds and relatives

2.1 Introduction

Avian bill size and shape have been central to our understanding of evolutionary biology since Charles Darwin wrote about the divergence of the Galápagos Finches (Darwin 1859). Despite being a porous, light-weight structure, the bill has adapted to sustain multiple physical forces while performing several discrete functions. For example, the bill must exhibit fracture resistance to mechanical stress during feeding (Soons et al. 2010), while also serving a role in thermoregulation (Benham and Bowie 2021; Tattersall et al. 2017). Recent meta-analyses across birds have found correlations between bill size and shape and dietary niche, such as birds that feed on floral nectar having long, thin bills (Felice et al. 2019; Pigot et al. 2016). Given the adaptive nature of bill morphology and the extent of its variation across both large and small spatial scales, the genetic basis of bill size and shape has been an active area of research.

Studies of genes and associated molecular pathways that may underlie variation in beak morphology have identified several mechanisms by which small changes in genotype can affect phenotype. Many of these genes have been identified in Galápagos Finches, an adaptive radiation of passerine birds in which species have evolved different bill sizes and shapes as a way to partition food resources and reduce competition (Grant and Grant 2008). Robust-billed species are capable of breaking through harder seeds that thin-billed species cannot, whereas thin-billed species can consume delicate seeds more efficiently (Bowman 1961). Developmental studies have revealed how signaling pathways can affect bill length independent of bill width and depth (Abzhanov et al. 2004, 2006; Lamichhaney et al. 2015). Variation in genes such as *ALX1* (Lamichhaney et al. 2015) and *HMGA2* (Almén et al. 2016; Chaves et al. 2016; Lamichhaney et al. 2016) have been shown through genomic studies to explain a large amount of variance in the Galápagos Finch radiation (particularly the genera *Geospiza*, *Camarhynchus*, *Platyspiza*, and *Certhidea*). Recent studies on bill shape in the Galapagos Finches have identified additional loci of large-effect, but with unknown roles in craniofacial development (e.g., *LPP* and *RNASEH2B*, Enbody et al. 2023). Studies in other passerines have identified additional genes of varying effect sizes (Armstrong et al. 2018; Bosse et al. 2017; Lundregan et al. 2018; vonHoldt et al. 2018). When considered collectively, no one gene has been consistently implicated across all studies, which suggests that beak morphology is a polygenic trait and underpinned by a complex genetic architecture.

Nectarivory in birds is estimated to have 22 independent origins across the avian phylogeny (Pauw 2019). Nectarivores tend to have long and slender bills, though phylogenetic history and feeding behavior can lead to much variation (Chapter 1; Hewes et al. 2022). Although hummingbirds (Caprimulgiformes: Trochilidae) are perhaps the most well-known nectarivorous birds, most studies investigating the genetic basis of variation in bill morphology have been performed on songbirds (Passeriformes). Thus, investigations into the genetic basis of bill morphology of a nectarivorous species might be best served by studying nectarivorous songbirds because of our greater knowledge of the genetics of bill morphology in passerines. Sunbirds (Passeriformes: Nectariniidae) are a large radiation (>140 species) distributed across the Eastern

Hemisphere. They are in many ways the ecological analogues of hummingbirds (Cheke and Mann 2001). The diet of a majority of species is primarily nectar, supplemented by arthropods, fruits, and/or berries to varying degrees (Cheke and Mann 2001; Winkler et al. 2020a). Variation in bill size is primarily along bill length, though bill width and depth are also variable to a lesser extent (Figure 2.1A-C). The degree of downward curvature of the bill also varies, ranging from straight to moderately curved, though curvature is not assessed here. Since many sunbird species have elongated bills relative to related clades of passeriform birds, strong selection on bill length is hypothesized to have occurred early in their evolution. The sister group to sunbirds, the flowerpeckers (Passeriformes: Dicaeidae), are also known to occasionally eat nectar but are primarily frugivorous (Winkler et al. 2020b). Therefore, elevated rates of evolution in genes associated with bill elongation are expected to occur at the base of the sunbird radiation after the split between sunbirds and flowerpeckers.

I explored the genetic basis of bill morphology in sunbirds by analyzing known candidate genes for bill size and shape within a phylogenetic framework. I studied how selection may have acted on these candidate genes in the sunbird radiation using a recently developed multilocus phylogeny that includes sunbirds (Nectariniidae), flowerpeckers (Dicaeidae), and several outgroups (Bowie et al., in prep.). I tested for signatures of selection at the site level within the protein-coding regions of each gene and tested for elevated rates of evolution at specific branches in the phylogeny. I hypothesized that sunbirds utilize similar genetic mechanisms to underlie an increase in beak length as identified in previous studies of passerine birds, particularly at functionally important binding sites within a protein-coding region, which would result in signals of selection acting on these genes. I also predicted that sunbirds have an elevated rate of evolution in bill genes at the base of the radiation relative to their sister clade, the flowerpeckers, which have strong, short, and thick bills (Cheke and Mann 2001).

2.2 Materials and Methods

2.2.1 Data collection

Sequence data was acquired using a target capture method. Sequence-capture probes were designed to capture exonic regions of 11 genes implicated in studies of the genetics of bill morphology (Table 2.1). We assembled a target file of genetic sequences that could be used to design sequence-capture probes that would enrich the targeted DNA for sequencing. We began by extracting genetic sequences for loci of interest from the reference genome from the Zebra Finch (*Taeniopygia guttata*, GenBank assembly GCA_003957565.4) (Warren et al. 2010), which is a model avian species. However, in some cases we used genetic sequences from the Collared Flycatcher (*Ficedula albicollis*, GenBank assembly GCA_000247815.2) (Ellegren et al. 2012), which also has a high quality reference genome. The targeted loci that we extracted for this study were the phylogenetically useful RELEC loci (Karin et al. 2019) and candidate genes for beak size/shape (Table 2.1). We then used the program Alibaseq (Knyshov et al. 2021) to extract sequences matching the Zebra Finch (or Collared Flycatcher) loci from genomic data for the Black Sunbird (*Leptocoma aspasia*, GenBank assembly GCA_013399835.1) and the Red-banded Flowerpecker (*Dicaeum eximium*, GenBank assembly GCA_013396995.1) (Feng et al. 2020). We did not use the annotations associated with the sunbird or flowerpecker genomes to extract our loci of interest, because these genomes were not as high quality as the Zebra Finch and Collared Flycatcher genomes and were much more fragmented. In Alibaseq, we extracted the targeted region plus

an extra 50 bp flanking each side of the targeted sequence. We then compared the size of the loci extracted from the sunbird and flowerpecker to the original Zebra Finch sequence. For mapping, we preferentially used the sunbird sequence over the flowerpecker sequence for probe design when sequences from the two genomes were equivalent in length. However, if the flowerpecker sequence was greater than 30 bp longer than the sunbird sequence, we used the flowerpecker sequence in our target file. If either the sunbird or flowerpecker sequence was >200 bp smaller than the Zebra Finch sequence, we retained both the Zebra Finch and sunbird/flowerpecker sequences in our target file. We used the Zebra Finch sequence alone in our mapping file if the sunbird/flowerpecker sequence was smaller than 150 bp (i.e., less than the probe size) in total length. The resulting DNA sequences for the candidate genes, RELEC loci, and other loci for different projects were sent to Roche to design a custom Kapa HyperExplore sequence-capture kit.

DNA was extracted from 479 individuals, including both modern samples (those with tissues obtained at time of preparation) and historical specimens, spanning 139 species and 15 passerine families (see Appendix Table 2 for individuals included in the pruned phylogeny). For modern samples, DNA was extracted from tissue samples (heart, liver, muscle) or blood using standard salt extraction techniques or the DNeasy Blood and Tissue kit (Qiagen) and resuspended in 1X low TE (10mM Tris, 0.1 mM EDTA). For historical samples, DNA was extracted from toepads using standard Phenol-Chloroform-Isoamyl extraction techniques, cleaned using SPRI (Sera-Mag) beads, and resuspended in 1X low TE buffer (10mM Tris, 0.1 mM EDTA).

We used 0.3-2 µg of DNA from modern samples as input for our libraries, which was diluted with 1x low TE buffer (10mM Tris, 0.1 mM EDTA) into a final volume of 110 µL in a sonicator tube. DNA was sonicated in a qSonic machine typically with the following conditions: pulse = 15 seconds on, 15 seconds off; amplitude = 40%; time = 2.5 minutes of sonication, after which the tube was vortexed and spun down, and then followed by two more rounds of 2.5 minutes more sonication (7.5 minutes total). For historical samples, a minimum of 0.15 µg of DNA was sonicated for 30-60 seconds (1-2 pulse cycles) if necessary, then treated with USER enzyme (New England Biolabs) following the protocol in Briggs and Heyn (2012) prior to library preparation. USER enzyme is a mix of Uracil DNA glycosylase (UDG) and the DNA glycosylase-lyase Endonuclease VIII; UDG removes uracil from degraded DNA and the endonuclease VIII repairs the gap left by the removal of uracil (Briggs et al. 2010). Then for all samples, we performed a doubled sided bead cleanup (first 0.5x and then 0.65x beads) with low-ratio formula SPRI (Sera-Mag) beads to preferentially select for DNA within the size range of 300-500 bp.

Libraries were constructed with a KAPA HyperPrep Kit (Roche) using a one quarter reaction size. The libraries were dual indexed with 8-bp indexes. We standardized the libraries to a common concentration (15 ng/µL) and then combined 150 ng of each indexed library into a pool of 102-104 samples for the modern samples and 66 samples for the historic samples (five pools total). We mixed 2 µg of DNA from a particular pool with 20 µg of chicken Hybloc DNA (Applied Genetics Laboratories). We hybridized the resulting mix to the capture probes for 20 hours and then performed the capture reaction according to the KAPA HyperCap Kit (Roche). We verified that the captures enriched our targeted DNA using qPCR on two positive and two negative control loci. The captured DNA was sequenced on two Illumina NovaSeq S4 150 PE lanes at the California Institute for Quantitative Biosciences at the University of California, Berkeley

(QB3-Berkeley), with each of the four modern sample pools receiving 19% of the sequencing effort and the pool of historical samples receiving 24% of the sequencing effort.

2.2.2 Data assembly

The program HTStream v 1.3.3 (<https://github.com/s4hts/HTStream>; (HTStream version 1.3.3 n.d.) was used to: 1) remove phiX sequences, 2) remove PCR duplicates, 3) remove adapter sequences and merge any overlapping reads, 4) screen out any remaining adapter contamination using a file containing the Illumina adapter sequences, 5) remove low quality bases with a window size of 10 bp and a quality threshold of 20, 6) trim off sequences containing N characters, 7) remove any reads that were smaller than 20 bp, and 8) calculate statistics about the reads and data quality. The cleaned reads were mapped to our target file of sequences using the BWA-MEM 0.7.17-r1188 mapper (H. Li and Durbin 2009). Samtools was used to merge, sort, and index the bam files of the mapped reads (Danecek et al. 2021). Picard v. 2.23.4 (<http://broadinstitute.github.io/picard/>) was used to add read groups and GATK v3.6 (McKenna et al. 2010) was used to realign the reads surrounding indels. ANGSD v. 0.933-79-gda26ba4 (Korneliussen et al. 2014) was used to export a consensus sequence in Fasta format for the phylogenetic loci (165 RELEC loci; Karin et al. 2019) and the candidate beak loci (Table 2.1) with a minimum base quality of 20, a minimum mapping quality of 30, a minimum depth of coverage of 5, a maximum depth of coverage of 10000, and an iupacRatio of 0.2. The consensus sequences for the loci were all aligned with each other after they were exported, because for each individual a base call is made across all of the positions in the reference sequence that was used for mapping the reads. We assessed the quality of the unmapped reads with FastQC v 0.11.8 (Andrews 2010), assessed the mapped reads with Qualimap v.2.2.1 (Okonechnikov et al. 2016), and used MultiQC version 1.1 (Ewels et al. 2016) to summarize the results of these programs.

2.2.3 Phylogeny

We used RAxML version 8.2.12 (Stamatakis 2014) with the GTRCAT model, 100 alternative runs on different starting trees, and rapid bootstrapping to infer maximum likelihood gene trees for each locus with corresponding bootstrap and bipartition information. We used ASTRAL-Hyb (Zhang and Mirarab 2022) to infer a species tree from the RAxML gene trees. The ASTRAL-Hyb method improves the accuracy of species tree inference by down-weighting relationships with poor support or with long terminal branches (Zhang and Mirarab 2022).

2.2.4 Ancestral state reconstruction of bill size

To reconstruct the ancestral states of bill size, continuous traits (length at nares, length at culmen, width, and depth) were downloaded from the AVONET dataset (Tobias et al. 2022) and log10-transformed for each species in our sunbird phylogeny. Surface area was calculated as a proxy for beak size using a formula for the lateral surface area of a cone (Benham and Bowie 2021; Miller et al. 2018) and also log10-transformed. These continuous traits were then analyzed in R version 4.3.2 (R Core Team 2023) using the reduced 139 taxa phylogeny and the ‘fastAnc’ function in the *phytools* package, which makes use of Maximum Likelihood to estimate ancestral states at internal nodes in a phylogeny (Revell 2024).

2.2.5 Sequence alignment of beak candidate genes

The following steps were performed in Geneious Prime 2023.0.1 (<https://www.geneious.com>). Sequences were aligned to the corresponding gene for Zebra Finch (*Taeniopygia guttata*). Exons were then extracted, concatenated, and exported in FASTA format. We wanted to determine whether sites under selection are located near functionally important sites. To do this, we downloaded annotated homologs from NCBI (<https://www.ncbi.nlm.nih.gov/protein>) which were then aligned to sunbird sequences using MUSCLE version 5.1 (Edgar 2022). We used protein-coding genes from humans (*Homo sapiens*), Zebra Finches (*Taeniopygia guttata*), Chicken (*Gallus gallus*), and Medium Ground Finches (*Geospiza fortis*). Missing data and ambiguities from polymorphism in the sequence data were kept in the alignment (see Results and Table 2.1 for more details). No premature stop codons were detected in any alignment.

The dataset and the phylogeny were pruned to one representative per species (139 individuals) using the *ape* package (Paradis and Schliep 2019) in R version 4.3.2 (R Core Team 2023). The bill gene sequence data used for analyses had 100% correspondence to the individual birds used in the phylogeny. If the bill locus data was incomplete for a particular representative individual of a species, it was substituted with a different individual with more complete data and the phylogeny pruned accordingly. In other words, analyses had different individuals and differently pruned phylogenies depending on the gene. These intraspecific substitutions were not an issue as samples formed monophyletic groups in the unpruned phylogeny.

2.2.6 Test for positive selection

Mutations in protein-coding genes that result in changes in amino acid sequence may underlie shifts in bill development and lead to variation in bill size and shape. The ratio of non-synonymous to synonymous substitutions, omega ($\omega = dN/dS$), is a commonly used metric in studies testing for positive selection in protein-coding genes (e.g., Nielsen and Yang 1998). Substitutions in a nucleotide sequence that result in an amino acid change are referred to as non-synonymous mutations (dN), whereas those that result in no amino acid changes are synonymous mutations (dS); and a ratio of greater than one ($\omega > 1$) indicates positive (adaptive or diversifying) selection, an equal ratio indicates neutral evolution, and a ratio of less than one ($\omega < 1$) is suggestive of negative (purifying) selection (Jeffares et al. 2015). Omega (ω) can be calculated for individual sites, branches, or both using a variety of statistical analyses developed to infer selection (see below). Evaluation of protein-coding genes at the site level could identify positions within loci that are potentially driving adaptive protein evolution. Identification of positive selection at the branch level would provide clues as to whether selection occurred on an ancestral branch or whether it varies across a clade of taxa.

Two tests were performed to test for positive selection: Mixed Effects Model of Evolution (MEME) and Adaptive Branch Site Random Effects Likelihood (aBSREL). MEME is used to test whether a site is evolving under episodic positive selection versus negative or neutral selection for a proportion of branches, and can detect lineage-to-lineage variation in ω for a given site in a protein (Murrell et al. 2012). aBSREL is used to test whether a proportion of sites on a branch evolved under positive selection (Smith et al. 2015). To test specific evolutionary hypotheses, branches from a phylogeny can be specified a priori to test whether a proportion of sites on the ‘foreground’ branches have undergone higher rates of evolution than ‘background’ branches

across the phylogeny (Smith et al. 2015). We tested whether our focal beak genes were under positive selection in sunbirds by specifying the branch at the base of the sunbird clade as the “foreground”. Additional aBSREL analyses were run with all branches selected to explore whether selection could be detected anywhere on the tree, but these analyses have lower power given the number of tests and should be considered as exploratory in nature (Smith et al. 2015). Both analyses were performed on the Datamonkey Adaptive Evolution Server (Weaver et al. 2018) using a phylogeny with 139 terminal taxa (see above). P-value thresholds were 0.1 for MEME and 0.05 for aBSREL according to defaults on the Datamonkey web server (<http://www.datamonkey.org/>) (Weaver et al. 2018) and the HyPhy package documentation (<http://hyphy.org/>) (Pond et al. 2019). The threshold for MEME was set to a less stringent value so as to detect more potential sites under selection.

A subset of the protein-coding region of the genes *LPP* and *RNASEH2B* were visualized using the function “msaplot” in the *ggtree* package in R (Yu et al. 2017) to show several sites under selection (Figures 2.2 and 2.3). The ancestral state distribution of one site was mapped across the tree as an example of the distribution of character states (Figure 2.4) using the “Trace Character History” function and the “Parsimony Ancestral States” option for ancestral state reconstruction methods in Mesquite (Maddison and Maddison 2021).

2.3 Results

2.3.1 Ancestral state reconstruction

Ancestral state reconstructions of bill size were performed using a phylogeny derived from the analysis of the RELEC loci for 139 taxa and constructed using a species tree approach. Ancestral state reconstruction of the log-transformed bill dimensions suggest that ancestral sunbirds had moderately sized bills. Subsequent modifications primarily occurred on the axis of length (Figure 2.1A), though width and depth necessarily changed as well (Figure 2.1B-C), likely to provide structural support at the base of the bill. No major change was observed on the ancestral branch of sunbirds. There are several instances of shorter bills independently evolving within sunbirds, such as the genera *Euchloridia* and *Hedydipna*, and longer bills in *Nectarinia*, *Arachnothera*, and *Anthodiaeta thomensis*. Reconstruction of bill surface area largely recapitulates the results observed for bill length (Figure 2.1D).

2.3.2 Sequencing results

Our sequence data for the candidate genes was complete for most individuals, especially for the ingroup taxa, including the ingroup historical samples. Missing exons are detailed in Table 2.1. The outgroup species *Turdus migratorius* had the most missing data, followed by other outgroup species such as *Empidonax hammondi* and *Basileuterus belli*. Sunbird sequences were complete except for *Anthreptes simplex* (missing part of *LPP* exon 1, all of *RNASEH2B* exon 1, and all of *SHH* exons 1-3) and all *Leptocoma* species, all *Aethopyga* species, and *Euchloridia pallidigaster* (missing all of *RNASEH2B* exon 1; Table 2.1). The coverage of candidate loci ranged from 135 to 271x and the average coverage was 186x (Table 2.1). The age of these samples ranged from three years to 100+ years old at the time of sample preparation, with the oldest specimen having been collected in 1909 (MVZ:Bird:108656).

The general features of the bill morphology genes chosen for this study and the results from MEME and aBSREL are summarized in Table 2.1. All the exons in each locus were

successfully sequenced except for *CALM2*, *LPP*, *RDH14*, and *RNASEH2B* which were each missing an exon. The ratio of variant codons (not including ambiguous codons) to total codons for a given gene ranged from 0% variant (both *CALM* genes) to 33.2% (*RNASEH2B*).

2.3.3 MEME

MEME recovered evidence of episodic positive selection at a total of 28 sites across five proteins: *ALX1*, *BMP4*, *LPP*, *RNASEH2B*, and *SHH* (Table 2.1). Of these 28 sites, 15 fall within annotated regions when compared to the proteins of the passerine species Medium Ground Finch and Zebra Finch, the non-passerine Chicken (*Gallus gallus*), and Humans (*Homo sapiens*; Table 2.2). The genes with the highest number of sites under selection were *LPP* with 14 (Table 2.2, Figure 2.2), followed by *RNASEH2B* with seven (Table 2.2, Figure 2.3). In particular, the eighth codon of *LPP* has a high density of sites under selection (Figure 2.2). Patterns in the selected amino acid positions differed and at least one was mostly restricted to sunbirds (*LPP* codon 566). To illustrate the evolutionary history of one of the selected sites, we reconstructed the ancestral states and character changes for codon 544 (Figure 2.4). A majority of the flowerpecker clade appears to have either reverted to or retained the ancestral state of Glutamine at codon 544, observed in *Empidonax hammondi*, while the *Lamprothreptes* clade of sunbirds evolved a derived state of Leucine (colored backgrounds in Figure 2.4).

2.3.4 aBSREL

aBSREL did not detect any statistically significant, elevated rates of evolution at the base of sunbirds using a threshold of $p \leq 0.05$. When all branches were selected, aBSREL did not detect any elevated rates of evolution for the ingroup (sunbirds) relative to either the flowerpeckers or other outgroup taxa. When all branches were selected and p-values were corrected for multiple comparisons, aBSREL did detect elevated rates for one gene, *ALX1*, in which node 29 leading to the outgroups (*Dacnis cayana*, (*Pipilo maculatus*, *Basileuterus belli*)) may have undergone episodic diversifying selection ($p = 0.0229$; Table 2.1).

When all branches were selected and p-values were not corrected for multiple comparisons, aBSREL detected elevated rates for *RNASEH2B* in the ingroup and *LPP* in the outgroup. *RNASEH2B* node 141 leading to *Nectarinia famosa* and *N. johnstoni* had a raw p-value of 0.0103. *LPP* nodes 184 leading to (*Cinnyris bifasciatus*, (*C. mariquensis*, *C. shelleyi*)) and 193 leading to (*C. mariquensis*, *C. shelleyi*) had raw p-values of 0.0054 and 0.0091 respectively. *LPP* nodes 27 leading to (*Serinus burtoni*, (*Dacnis cayana*, (*Pipilo maculatus*, *Basileuterus belli*))) and 29 leading to (*Dacnis cayana*, (*Pipilo maculatus*, *Basileuterus belli*)) had raw p-values of 0.0081 and 0.0284 respectively. No other branches had statistically significant, elevated rates of evolution, raw or corrected.

2.4 Discussion

Due to the bill's functional role in foraging and our current understanding of plant-sunbird pollination interactions (Hořák and Janeček 2021; Janeček et al. 2020), bill morphology is likely under selective pressure in sunbirds. We observed that changes in bill size in sunbirds were primarily driven by changes in bill length (Figure 2.1A-D). However, there was not a major shift in bill length at the base of sunbirds based on the ancestral state reconstructions. Rather, major changes occurred in several clades within sunbirds and at the base of flowerpeckers. I then

evaluated whether candidate genes for bill morphology had signals of positive selection because it was likely that sunbirds experienced natural selection on bill size during their evolutionary history. I tested for selection at the site level using MEME and at the branch level using aBSREL. I found 28 codons to be under positive selection using MEME. However, the branch at the base of sunbirds was not found to have elevated rates of selection relative to the outgroup taxa as hypothesized. Interpretations of these results are discussed below.

The MEME results suggest that there has been some selection on the candidate bill genes across the species represented in our study. Several sites matched the criteria for selection according to MEME. MEME identified one codon in *ALX1*, two codons in *BMP4*, four codons in *SHH*, seven codons in *RNASEH2B*, and 14 codons in *LPP* to be under selection (Table 2.1). The genes *LPP* and *RNASEH2B* have the highest number of inferred sites under selection in this study. Intriguingly, elevated rates for *RNASEH2B* and *LPP* were detected by aBSREL when no multiple testing corrections were done, suggesting that these genes may have had higher rates of evolution on some branches. Both of these genes were found within larger genetic regions associated with major effects on bill morphology in Galápagos Finches (Figure 3A and Supplementary Note 3 in Enbody et al. 2023). However, many of the sites identified by Enbody et al. (2023) were in intronic or intergenic regions and are thus not directly comparable with our analyses of protein coding regions (this was the case for *LPP* and *RNASEH2B* genes). A mechanism for *LPP* and *RNASEH2B* to affect bill morphology is currently not known (Enbody et al. 2023). Nevertheless, our results suggest these two genes have undergone selection in the radiation of sunbirds, a group of birds in which there have been several changes in bill size (Figure 2.1). Further testing is needed to determine whether the observed amino acid changes are associated with changes in bill morphology, but *LPP* and *RNASEH2B* might be particularly interesting candidate genes to consider for further work on the genetics of bill morphology.

None of the candidate genes for bill morphology were associated with elevated rates of selection at the ancestral branch of sunbirds in our study. aBSREL did not detect elevated levels of diversification for sunbirds relative to either the flowerpeckers or other outgroup taxa. Thus, there was no evidence for rapid protein evolution at the base of sunbirds within our chosen genes as initially hypothesized. When all branches were selected, aBSREL did identify one branch where all three tips share the same amino acid substitution within *ALX1* that was identified as under selection using MEME (Table 2.1) – the outgroup clade (Spotted Towhee (*Pipilo maculatus*), Golden-browed Warbler (*Basileuterus belli*), and Turquoise Honeycreeper (*Dacnis cayana*)). These three species are part of the nine-primaried oscines, a group to which Galápagos Finches also belong. However, this particular site was not noted to be associated with changes in bill shape when *ALX1* was compared across two different Galápagos Finch bill morphs and diverse vertebrate taxa (Lamichhaney et al. 2015). Collectively, it may be that bill morphology is too labile in sunbirds to detect selection using branch-specific models as discussed below.

When comparing the ratio of variant codons to total codons (Table 2.1), several of the genes in this study are more conserved than others. This is exemplified by the genes in the Calmodulin family (e.g., *CALM1* and *CALM2*) that play a role in mediating the Ca^{2+} signaling pathway and are known to be expressed at higher levels in species of Galápagos Finches with elongated beaks (Abzhanov et al. 2006). However, no amino acid changes were detected in the exons of either *CALM1* or *CALM2* in our study (Table 2.1). In contrast, *LPP* had only 66.3% sites that were identical across all the birds in the study and selection was detected at 14 codons using

MEME. Figure 2.4 shows how variable one site in the *LPP* gene was across the phylogeny. *Empidonax hammondi* at the base of the tree exhibits the ancestral condition Glutamine. All ingroups relative to *E. hammondi* possess the derived condition of Arginine, Leucine, or Proline, except for the flowerpeckers (Dicaeidae) which have reverted or retained the ancestral condition.

Ancestral state reconstructions did not support our initial hypothesis of bill evolution occurring at the base sunbirds, thus the absence of any selection on that branch as inferred by aBSREL was not unexpected. These candidate genes may have had a general lack of signal in aBSREL analyses due to a combination of gene conservation and gene expression. Because the proportion of sites under selection was generally low across many of the genes (i.e., few amino acid changes), it may be that selection was weak on the ancestral branch to sunbirds and thus not detectable by aBSREL. Another possibility is that selection is acting on levels of gene expression rather than on protein-coding regions. Recent studies found signatures of selection in non-coding regions, suggesting that regulatory changes play a significant role in the evolution of bill morphology (Enbody et al. 2023; Yusuf et al. 2020). Amino acid changes that affect protein structure and function may be disadvantageous, leading to the conservation of coding regions and selection occurring in non-coding, regulatory regions. Alternatively, if adaptive evolution of sunbird bills only involved very few non-synonymous changes, then selection might not be detected on a long branch where many synonymous changes are expected to occur because of neutral selection. Conserved genes may still affect bill phenotype by way of other genes, such as the synergistic interactions between *SHH* and *FGF8* (Abzhanov and Tabin 2004) or changes in the timing and location of gene expression for a given developmental mechanism (Mallarino et al. 2011, 2012).

Although curvature was not assessed here, it is presumably also under genetic influence. The bills of sunbirds vary, from nearly straight to strongly decurved (Cheke and Mann 2001). However, study of the genomic basis of bill shape has primarily focused on species with straight conical beaks (e.g., Galápagos Finch, Zebra Finch); few studies have investigated the genomic and developmental basis of bill curvature. Quantitative Trait Loci studies in the Domesticated Pigeon (Columbiformes: Columbidae: *Columba livia*) have identified several genomic regions associated with bill curvature and general craniofacial variation, though it is not known whether these regions are relevant across birds (Boer et al. 2021). Models of beak morphogenesis developed from the crania of Galápagos Finches and Hawaiian Honeycreepers suggest that beak curvature is a result of developmental factors such as gradients in cell proliferation rates (Mosleh et al. 2023). It remains to be tested what mechanisms may contribute to bill curvature in sunbirds.

A natural question arising from a study like this one is whether and how the selected amino acids affect protein function. To begin to address this question, I determined how many of the selected sites fell into protein positions with functional annotations, which are areas that seem likely to affect protein structure. Of the 28 sites identified by MEME, 15 fell within annotated regions of proteins in Medium Ground Finch (*Geospiza fortis*), Zebra Finch (*Taeniopygia guttata*), Chicken (*Gallus gallus*), and Human (*Homo sapiens*; Table 2.2). For *LPP*, eight codons fall in or around putative binding sites as identified by annotations in Zebra Finch, six of which occur within the same exon (Figure 2.1, Table 2.2). Further functional testing is needed to determine their significance in bill evolution in sunbirds. In summary, some evidence of selection on these genes was detected with MEME and results for aBSREL analyses without multiple testing correction are weak evidence of rapid evolution of these genes within sunbirds.

A caveat to any candidate gene study is that the chosen genes may not be the actual ones under selection. In our case, sunbird bills may have evolved via a different suite of genes related to bill morphology than those included in this study. In addition, it is quite possible that sunbird bills evolved because of changes in many genes of small effect on bill size and shape which would make selection on any single gene difficult to detect. Nevertheless, it is worth noting that sunbirds, Galápagos Finches, and Zebra Finches are members of the oscine superclade within passerine birds, a clade that has a remarkable degree of variation in beak morphology and ecology. Although the roles of the *LPP* and *RNASEH2B* genes in craniofacial development are unknown, these genes were both recovered to be under selection in sunbirds and Galápagos Finches. This suggests that a common genetic pathway may underlie some of the variation observed in the beak morphology of oscine passerines.

2.5 Conclusions

This study and the many studies cited herein reinforce the complex nature of the genetic architecture underlying bill morphology. I explored the genetics of bill size and shape within a family of birds with long bills in a field of study that has primarily focused on species with short- to medium-sized bills. No single gene has been consistently implicated across studies of bill morphology, and the results of this study further corroborate this trend. Future work is needed to test the correlation between genotype and phenotype, particularly in highly variable genes like *LPP* and *RNASEH2B*. More developmental studies are needed across a broader range of species to better understand the diversity of signaling pathways that have evolved across birds.

2.6 Tables and Figures

Table 2.1: Putative genes associated with bill morphology analyzed in this study. Average coverage across loci was calculated by averaging coverage of a given exon across all 139 libraries, then calculating the average coverage across all exons that were successfully captured for a locus. p-value is indicated in parentheses for the codon/branch under positive selection. P-value thresholds were 0.10 for MEME and 0.05 for aBSREL. For the number of variant codons, i.e., non-synonymous amino acid changes, the first value includes ambiguous codons, and the second value does not. Ambiguous codons are those positions where a single amino acid could not be inferred because of genetic polymorphism within the individual bird that led to two different amino acids. Bold text indicates results where positive selection was detected. and Percent pairwise identity is a measure of identical sites across all individuals in the alignment, including gap versus non-gap sites but excluding gap versus gap sites; first percentage excludes missing data, second percentage includes missing data; *exon 1 is one codon in length; 1 = missing last exon; 2 = missing last exon 9; 3 = missing first exon; 4 = missing 10th exon.

Gene	Name	Taxon	Association	Average coverage across gene	# of exons	# of codons	% pairwise identity ^a	# of variant codons	MEME		aBSREL		Missing data
									codon under positive selection (p-value)	node under positive selection (p-value)			
ALX1	ALX homeobox 1	<i>Geospiza</i> spp.	Blunt bills (short, tall; Lamichhaney et al., 2015)	202.08	4	328	92.4%, 50.9%	33, 18	76 (0.00)	29 (0.0229)	● 102 gaps (exon 2) in <i>Promerops gurneyi</i> ● 43 gaps (exon 3) in <i>Turdus migratorius</i> , <i>Empidonax hammondii</i> , <i>Basileuterus belli</i> , and <i>Chlorocichla</i> sp.		
BMP4	Bone morphogenic protein 4	<i>Geospiza</i> spp.	Beak depth and width (Abzhanov et al., 2004, 2006)	271.45	2	403	94.50%, -	21, 12	170 (0.04) 213 (0.10)	none			
CALM1	Calmodulin 1	<i>Geospiza</i> spp.	Associated with elongated beaks (Abzhanov et al., 2004, 2006; Lamichhaney et al., 2015)	141.97	6 ^{1*}	139	100%, 32.4%	0, 0	0	none	● 94 gaps in <i>Turdus migratorius</i> (exons 1-4) ● 48 (exon 3) gaps in <i>Empidonax hammondii</i> ● 10 gaps (exon 1-2) in Muscicapidae sp.		
CALM2	Calmodulin 2	<i>Geospiza</i> spp.	Elongated beaks (Abzhanov et al., 2004, 2006; Lamichhaney et al., 2015)	170.75	6 [*]	149	100%, 36.2%	0, 0	0	none	● 94 gaps (exons 1-4) in <i>Turdus migratorius</i>		
FGF8	Fibroblast growth factor 8	<i>Geospiza</i> spp., <i>Gallus gallus</i>	Deep and broad beaks, expressed during embryonic development of the premaxilla (Abzhanov & Tabin, 2004; Bhullar et al., 2015; Wu et al., 2004)	205.29	5	214	93.9%, 77.6%	13, 6	0	none	● 36 gaps (exon 4) in <i>Dicaeum</i> sp.		
FGF10	Fibroblast growth factor 10	<i>Geospiza</i> spp.	Beak size, unknown role (Lamichhaney et al., 2015)	136.35	3	212	92.0%, 47.9%	16, 9	0	none	● 101 gaps (exons 2-3) in Muscicapidae sp. and <i>Turdus migratorius</i>		
HMG2	High mobility AT-hook 2	<i>Geospiza</i> spp.	Overall beak size (sum of bill length, width, and depth; Chaves et al., 2016; Lamichhaney et al., 2016)	134.77	5	109	83.5%, 65.1%	16, 14	0	none	● 20 gaps (first 10 amino acids of exons 2 and 5) in <i>Turdus migratorius</i>		
LPP	LIM Domain Containing Preferred Translocation Partner In Lipoma	<i>Geospiza</i> spp.	Beak size, unknown role (Enbody et al., 2023)	204.87	9 ²	567	66.3%, 62.1%	206, 135	20 (0.03) 68 (0.10) 98 (0.02) 155 (0.07) 275 (0.02) 331 (0.00) 369 (0.06) 531 (0.01) 536 (0.07) 544 (0.04) 553 (0.03) 557 (0.07) 565 (0.07) 566 (0.00)	none	● 46 gaps (part of exon 1) in <i>Anthreptes simplex</i>		

Gene	Name	Taxon	Association	Average coverage across gene		# of exons	# of codons	% pairwise identity ^a	# of variant codons	MEME		aBSREL		Missing data
				# of exons	% identity					codon under positive selection (p-value)	node under positive selection (p-value)			
<i>RDH14</i>	Retinol dehydrogenase e 14	<i>Geospiza</i> spp.	Beak development (Lamichhaney et al., 2015; Rowe et al., 1991)	183.18	2 ³	205	88.8%, 88.8%	22, 15	0	none	none			
<i>RNASEH2B isoform X1</i>	Ribonuclease H2 subunit B isoform X1	<i>Geospiza</i> spp.	Beak size, unknown role (Enbody et al., 2023)	139.52	11 ⁴	286	59.4%, 18.9%	116, 95	35 (0.02) 42 (0.05) 76 (0.04) 132 (0.08) 150 (0.05) 200 (0.08) 246 (0.00)	none	27 gaps (exon 1) <i>Muscicapidae</i> sp., <i>Irena</i> spp., <i>Chlorocichla</i> sp., <i>Basileuterus belli</i>, <i>Pipilo maculatus</i>, sunbirds (<i>Anthreptes simplex</i>, <i>Aethopyga siparaja</i>, <i>A. mysticallis</i>, <i>Leptocoma brasiliana</i>, <i>L. calcostetha</i>, <i>L. zeylonica</i>, <i>Euchloridia pallidigaster</i>) 190 (exons 1, 4-6, 8-9, 11) in <i>Empidonax hammondi</i> 128 gaps (exon 4, 6, 8-9, 11) in <i>Turdus migratorius</i> 87 gaps (exon 2) in <i>Pipilo maculatus</i>, <i>Turdus migratorius</i> 337 gaps (part of exon 1, exons 2-3) of <i>Anthreptes simplex</i>			
<i>SHH</i>	Sonic hedgehog	<i>Gallus gallus</i>	Expressed during embryonic development (Abzhanov & Tabin, 2004)	260.48	3	425	82.8%, 20.6%	71, 52	266 (0.08) 313 (0.07) 372 (0.04) 374 (0.04)	none				

Table 2.2: Amino acid changes within or near annotated protein regions of Medium Ground Finch (*Geospiza fortis*), Zebra Finch (*Taeniopygia guttata*), Chicken (*Gallus gallus*), and Human (*Homo sapiens*).

Gene	Codon position under selection	P value	MEME # branches under selection	Amino acid change	Comparison to annotated protein of other species
<i>ALX1</i>	76	0.00	2	Ser76Thr Ser76Asn	6 codons away from a phosphorylation site in <i>Homo sapiens</i>
<i>BMP4</i>	170	0.04	8	Ala170Ser Ala170Gln	In TGFb propeptide region of <i>Geospiza fortis</i>
<i>BMP4</i>	213	0.10	2	Thr213Ser	In TGFb propeptide region of <i>Geospiza fortis</i>
<i>SHH</i>	372	0.04	1	Gln372Thr	In hint module of <i>Taeniopygia guttata</i> and <i>Gallus gallus</i>
<i>SHH</i>	374	0.04	5	Val374Leu Val374Met Val374Ala	In hint module of <i>Taeniopygia guttata</i> and <i>Gallus gallus</i>
<i>SHH</i>	313	0.07	5	Arg313Gln Arg313Lys	In hint module of <i>Geospiza fortis</i> , <i>Taeniopygia guttata</i> , and <i>Gallus gallus</i>
<i>SHH</i>	266	0.08	2	Arg266Gln	In hint module of <i>Geospiza fortis</i> , <i>Taeniopygia guttata</i> , and <i>Gallus gallus</i>
<i>LPP</i>	369	0.06	4	Gly369Ser Gly369Val	In putative homodimer interface (polypeptide binding) of <i>Geospiza fortis</i>
<i>LPP</i>	531	0.01	5	Arg531Gln Arg531Pro	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Adjacent to start of Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	536	0.07	4	Val536Ala	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Within Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	544	0.04	6	Arg544Gln Arg544Leu Arg544Pro	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Within Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	553	0.03	3	Leu553Ser	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Within Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	557	0.07	4	Val557Ala	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Within Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	565	0.07	5	Gly565Ala Gly565Asp	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> Adjacent to end of Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>
<i>LPP</i>	566	0.00	9	Ala566Val	In a homodimer interface (polypeptide binding) of <i>Geospiza fortis</i> At end of Zn binding site in <i>Gallus gallus</i> and <i>Homo sapiens</i>

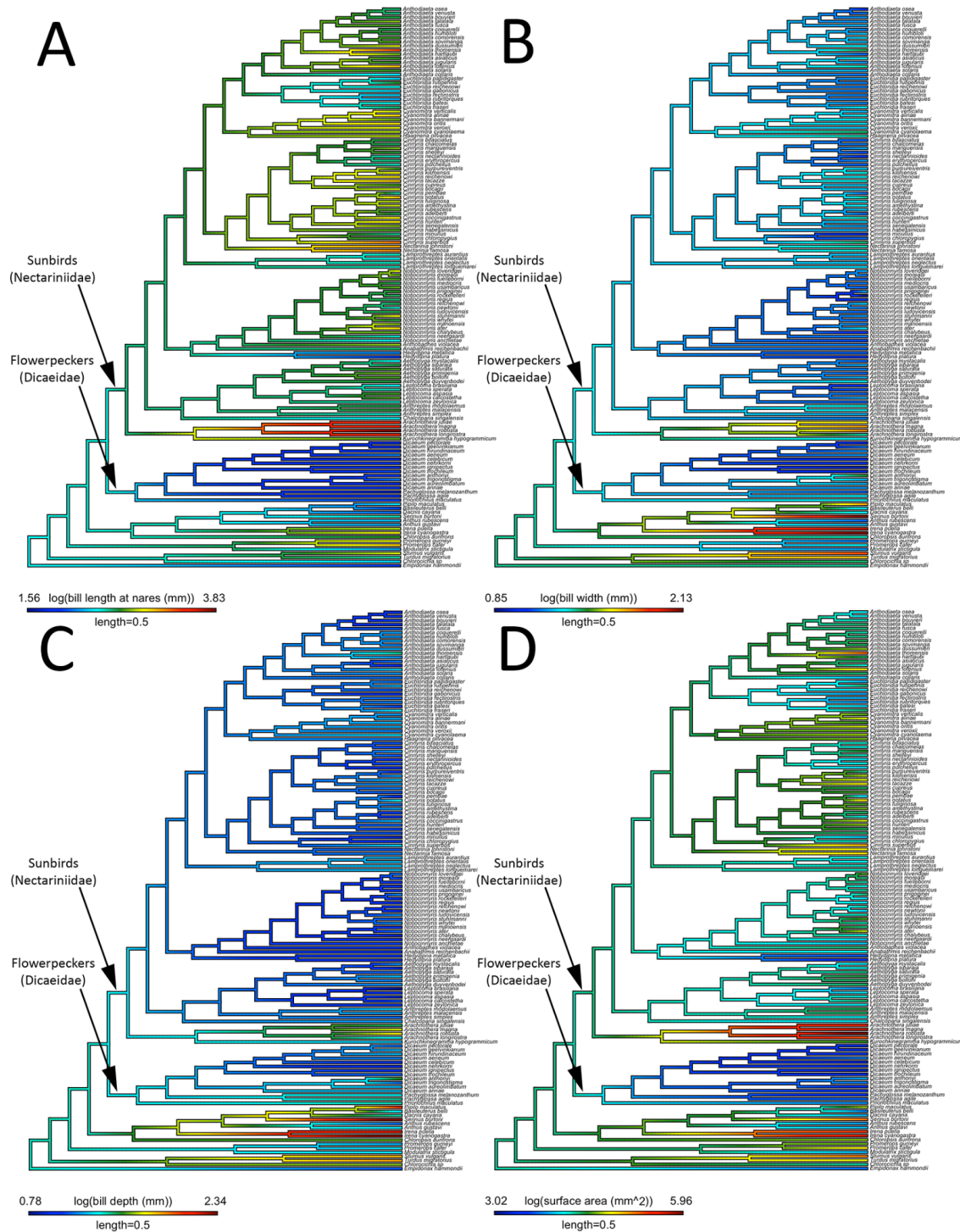
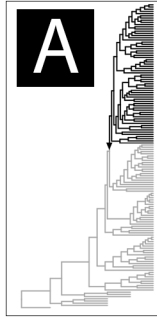
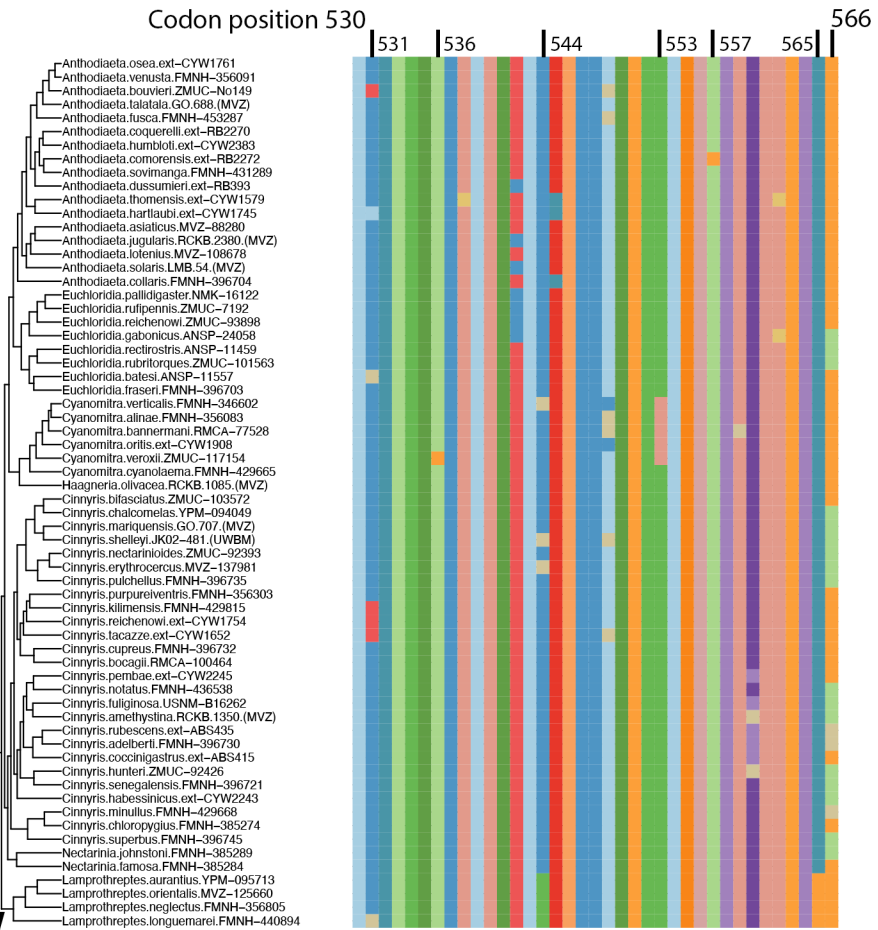


Figure 2.1: Evolution of bill size across the Nectariniidae and Dicaeidae phylogeny. Ancestral state reconstruction (maximum likelihood) of: (A) log(bill length at nares), (B) log(bill width), (C) log(bill depth), and (D) log(bill lateral surface area). Evolutionary transitions towards longer measurements are depicted by warmer colors (yellow to orange to red), while transitions towards shorter measurements are represented by cooler colors (cyan to dark blue to black).



Sunbirds
(Nectariniidae)
continued



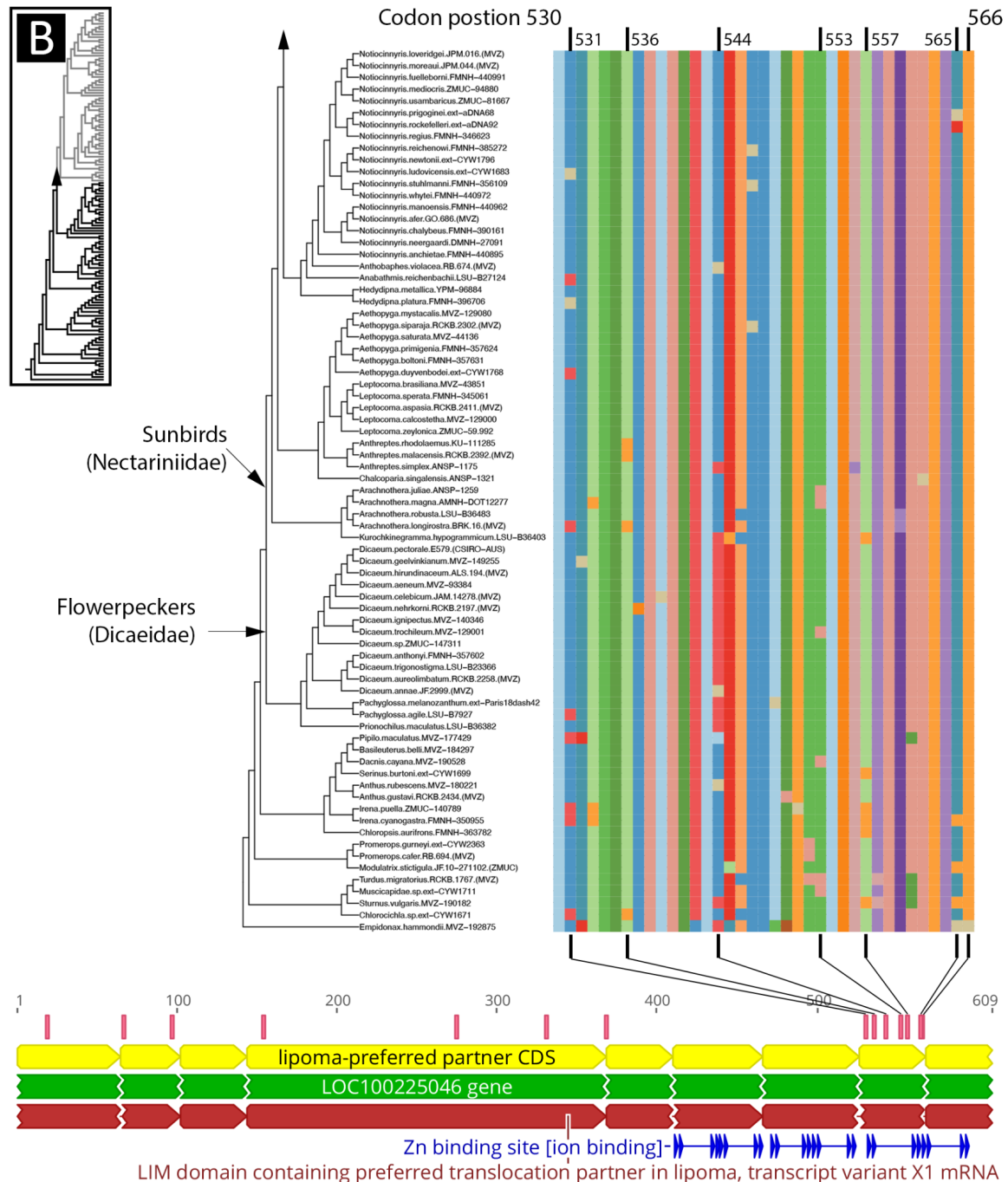
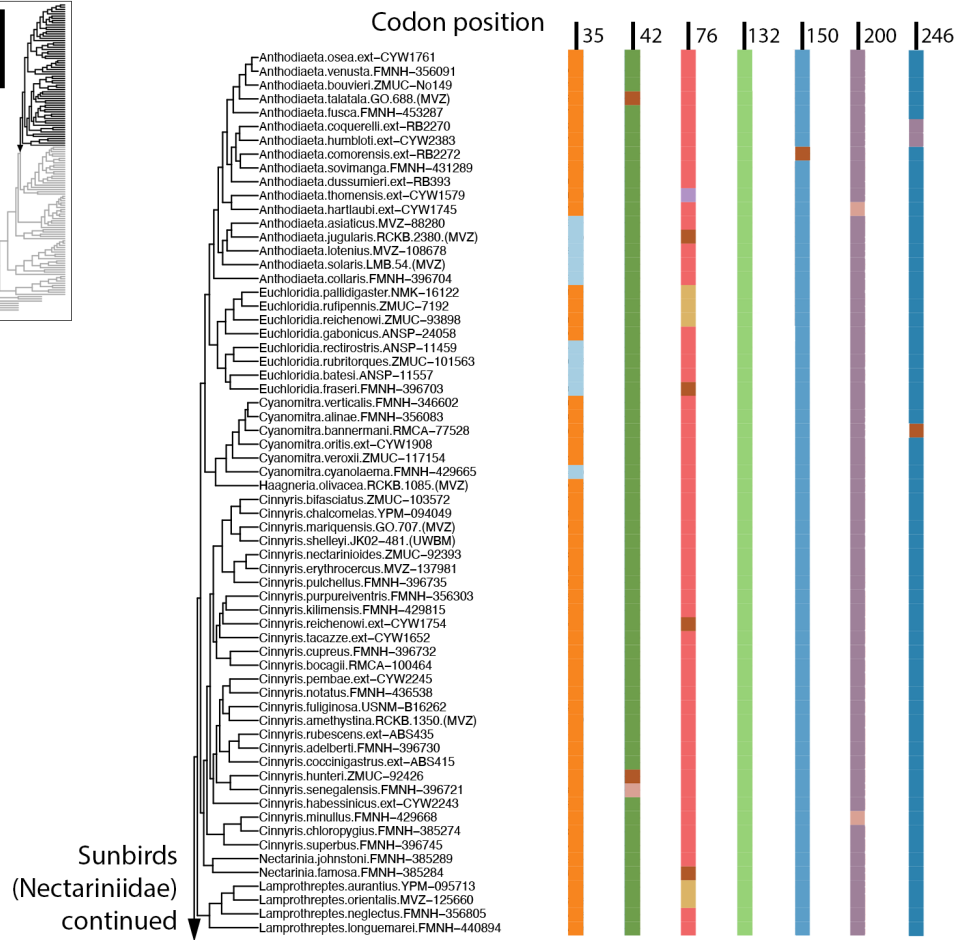
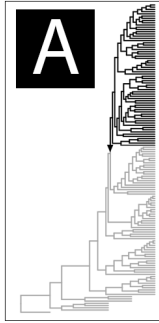


Figure 2.2A-B: Codons in the 8th exon of the *LPP* gene, including sites inferred to be under selection as detected by MEME relative to their position in the gene. The species identity and phylogenetic placement of the alignments are indicated on the phylogenetic tree depicted on the left-hand side of the figure. The colors in each codon position represent different amino acid character states. The black vertical bars indicate the sites under selection in the 8th exon. At the bottom of the figure, the red vertical bars indicate the sites under selection in the annotated protein. The diagonal lines connect the black vertical bars to the corresponding red vertical bars to indicate its relative position in the annotated protein. Exon 9 is missing from all alignments.



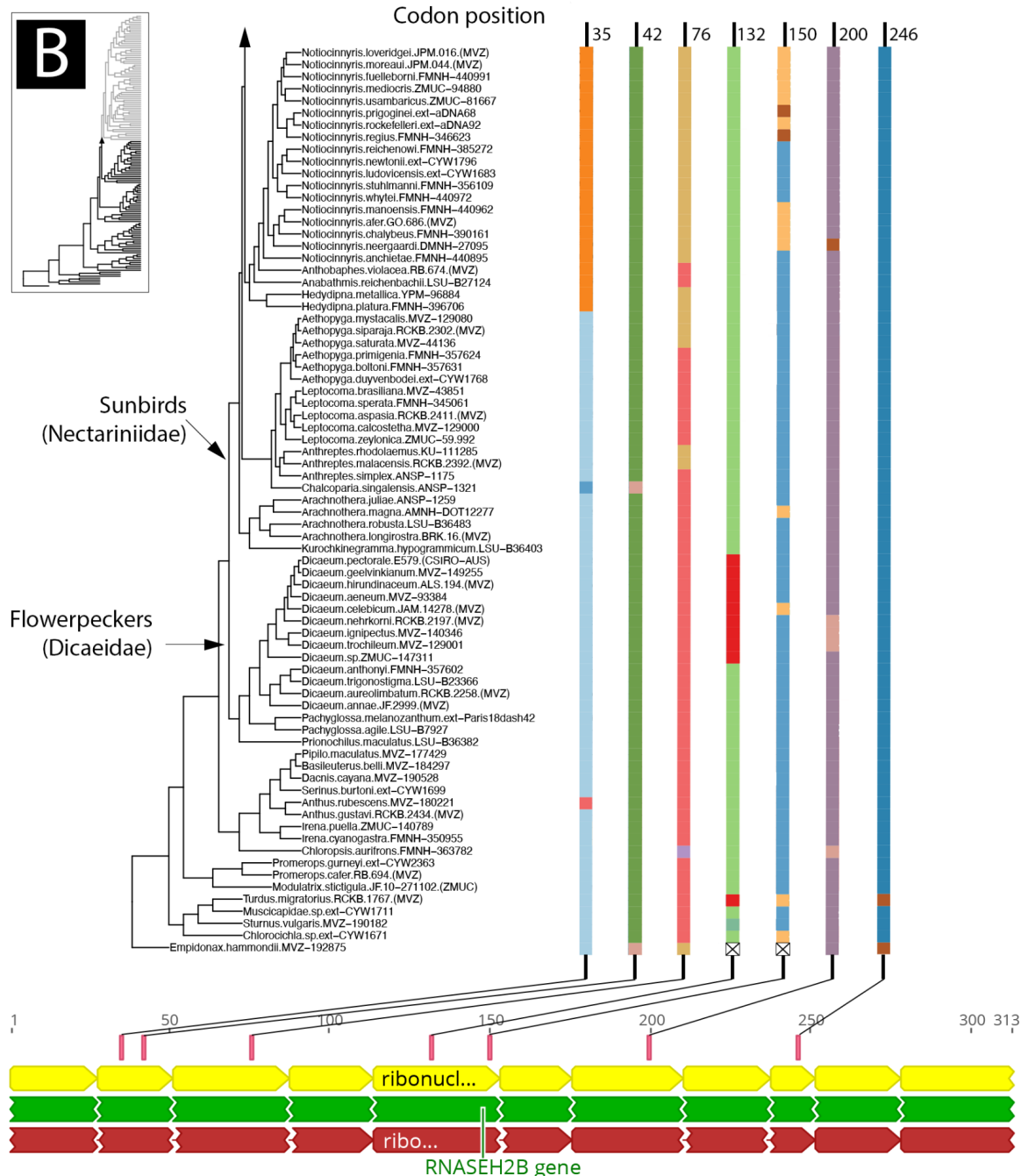


Figure 2.3A-B: Codons in the *RNASEH2B* gene inferred to be under selection as detected by MEME, relative to their position within the gene. The species identity and phylogenetic placement of the alignments are indicated on the phylogenetic tree depicted on the left-hand side of the figure. The colors in each codon position represent different amino acid character states. The two white positions with an X represent codons missing in *Empidonax hammondi*. At the bottom of the figure, the red vertical bars indicate the sites under selection in the annotated protein. The diagonal lines connect the black vertical bars to the corresponding red vertical bars to indicate its relative position in the annotated protein.

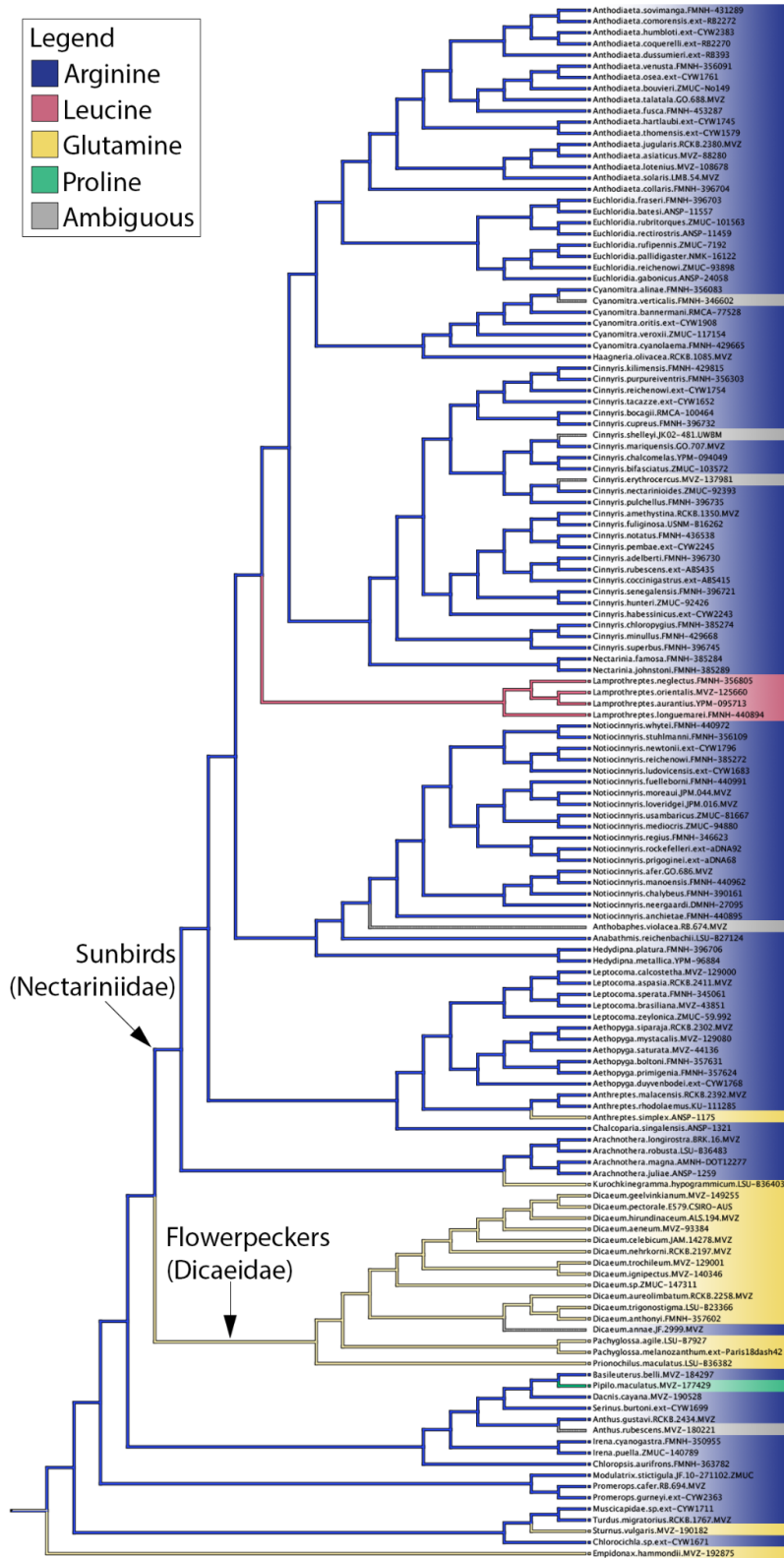


Figure 2.4: Character history of amino acid 544 in the LPP gene. Branch color and background color of taxon names indicate the amino acid present at site 544.

Chapter 3

The proof is in the plumage: dietary ethanol exposure in birds revealed by evaluating ethyl glucuronide levels in feathers

3.1 Introduction

Ethanol may be widespread in both fruit and nectar consumed by vertebrates given the fermentative activities of yeast on the sugars found in these foods (Dudley 2014). Recent work in hominid (Carrigan et al. 2015) and other mammalian taxa (Janiak et al. 2020) suggests that dietary ethanol exposure is correlated with positive selection on genes associated with its catabolism. Ethanol exposure may thus be broadly characteristic of all nectarivores and frugivores. Whereas ethanol sensing and consumption may be adaptive relative to net caloric gain (Dudley 2014), its overconsumption may also result in costs. Inebriation may reduce survival and reproductive success, including but not limited to increased predation risk due to impaired flight performance (Sánchez et al. 2010), injury or death due to accidental collisions during flight (Kinde et al. 2012; Stephen and Walley 2000), and altered singing behavior (in Zebra Finches, *Taeniopygia guttata*; Olson et al. 2014).

The prevalence of ethanol exposure and its role in the evolution of vertebrate diets have largely been unexplored. Wild chimpanzees (*Pan troglodytes verus*) have been observed opportunistically to consume fermenting raffia palm (*Raphia hookeri*) sap as it accumulates in tree-top buckets placed by local people (Hockings et al. 2015). Nectar- and sap-feeding species such as Pen-tailed Treeshrews (*Ptilocercus lowii*) and Slow Loris (*Nycticebus coucang*) consume fermented nectar of the bertam palm (*Eugeissona tristis*; Wiens et al. 2008), and captive Aye-aye (*Daubentonia madagascariensis*) and Slow Loris prefer to consume higher ethanol concentrations when multiple nectar-simulating solutions are concurrently available (Gochman et al. 2016).

Ethanol concentrations in fruit and nectar can be challenging to measure in the field, and natural rates of dietary ingestion are difficult to determine. However, methods have been developed to measure secondary metabolites within animal tissues that obligately derive from ethanol consumption (or rarely, endogenous production). Ethyl glucuronide (ethyl-b-D-6 glucuronic acid, or EtG) is a minor but direct metabolite of ethanol, and is a commonly used biomarker in studies of alcohol consumption by humans (Biondi et al. 2019). In humans, EtG offers a retrospective view of alcohol consumption depending on the biological material at hand, ranging from hours to days in urine and blood, and from weeks to months in scalp hair. In non-human taxa, EtG has been identified in hair, urine, and/or blood from various mammalian species, ranging from small-bodied taxa such as the Pen-tailed Treeshrew, Plantain Squirrel (*Callosciurus notatus*), Common Treeshrew (*Tupaia glis*), Gray Tree Rat (*Lenothrix canus*; Wiens et al. 2008) and Brown Rat (*Rattus norvegicus*; Kharbouche et al. 2010), to the relatively large Geoffroy's Spider Monkey (*Ateles geoffroyi*; Campbell et al. 2022; see Table S3.1).

Given their extensive dietary diversity (Rico-Guevara et al. 2019), birds represent a rich assemblage with which to test ethanol exposure across dietary categories. Documentation of the interaction between birds and ethanol-containing nutritional substrates lags behind that for

mammals, being primarily anecdotal in wild birds and inconclusive in captive populations. A wide variety of avian taxa have been observed ingesting human-sourced ethanol when provided the opportunity; review of the scientific literature and YouTube videos suggests that at least 55 species of birds will opportunistically feed on anthropogenic alcoholic beverages (Tryjanowski et al. 2020). Strong circumstantial evidence also supports naturally occurring ethanol toxicosis in the Cedar Waxwing (*Bombycilla cedrorum*; Fitzgerald et al. 1990; Kinde et al. 2012), Bohemian Waxwing (*Bombycilla garrulus*; Stephen and Walley 2000), Pine Grosbeak (*Pinicola enucleator*; Stephen and Walley 2000), and European Starling (*Sturnus vulgaris*; “Alcohol poisoning, not avian flu, killed Romanian birds” 2011). Experimental studies on captive birds, however, suggest variable behavioral responses to ethanol; ethanol odors did not affect food preferences in the frugivorous Yellow-vented Bulbul (*Pycnonotus xanthopygos*; Mazeh et al. 2008), and no preference was found when the choice between a 1% ethanol diet and a control diet with no ethanol was offered to the subtropical frugivorous Cape White-eye (*Zosterops virens*), Speckled Mousebird (*Colius striatus*), and Red-winged Starling (*Onychognathus morio*; Zungu and Downs 2017). To date, no large-scale ecological study has been conducted on ethanol concentrations within either fruits or nectars consumed by birds, and avian communities worldwide are likely adapted to broad ranges of dietary ethanol availability.

A recent study of hummingbirds evaluated dietary ethanol preference at fairly low and realistic naturally occurring concentrations within artificial nectars (Choi et al. 2023). Captive Anna’s Hummingbirds (*Calypte anna*) did not discriminate between 0% and 1% ethanol solutions in 20% sucrose but consumed 2% ethanol solutions at substantially lower rates (Choi et al. 2023). In addition, the ethanol concentrations within hummingbird feeders were monitored to evaluate the long-held suspicion that artificial nectar ferments when left outdoors for extended periods of time. Observed changes in ethanol concentrations were positively correlated with average weekly air temperature and peaked at ~0.045% v/v (Choi et al. 2023). These findings, in conjunction with earlier work on nectarivorous mammals (Wiens et al. 2008), lend further credence to the hypothesis that fermenting nectar can be a significant source of dietary ethanol.

Here, we tested for evidence of ethanol consumption by birds through indirect study of bird feather and liver samples, using EtG detection protocols already established for human tissues. As both feathers and hair are keratinized structures (Di-Poï and Milinkovitch 2016), we hypothesized that chronic exposure would be at sufficient levels such that the alcohol metabolite EtG would be detectable. More specifically, we hypothesized that species with diets containing large proportions of fermentable sugars, such as floral nectar and fruits, would be the most likely to ingest ethanol and therefore would be likely accumulate EtG in their feathers and livers (FEtG and LEtG, respectively). We primarily focused our sampling efforts on Anna’s Hummingbird (*Calypte anna*), but also included other taxa based on tissue availability. We then analyzed relationships between EtG concentrations and broad categories of avian ecological diversity (Tobias et al. 2022).

3.2 Materials and Methods

3.2.1 Sample collection and processing

Feathers were obtained from 64 archived specimens at the Museum of Vertebrate Zoology (University of California, Berkeley) from the western USA (California, Nevada, Arizona, Oregon), and from New Jersey. Birds were stored at -20 °C until sample collection and were

weighed after thawing. We experimented with several feather sampling strategies before ultimately deciding to use whole body feathers, and these results were included in the final dataset (see Supporting Text, available online). Body feathers from the torso, neck, and head were placed into 2.0 mL reinforced screw cap tubes and kept at -20 °C until processing. Because previous studies have found that EtG can be lost in the initial washing process that is standard protocol for human hair assays (Müller and Iwersen-Bergmann 2020), feathers were not washed prior to pulverizing in order to maximize our ability to test for EtG.

Feathers were processed as follows: a minimum of 20 mg of feathers were placed into 2.0 mL reinforced screw cap tubes containing three 2 mm stainless steel balls. Samples were then pulverized using a Powerlyzer Bead Beater (Mo Bio Laboratories, Inc.) for two rounds at the following settings: 2900 rpm, 2 cycles at 3 minutes, dwell 30 seconds. Additional rounds were performed as needed until the sample was close to a powder-like consistency. Tubes were then stored at -20 °C until analysis. Livers were also collected from 26 individuals during dissection (liver samples ranged in mass from 0.09-0.55 g) and were kept at -20 °C until analysis. Pulverized feathers and frozen liver samples were sent to the United States Drug Testing Laboratories (USDTL, Des Plaines, IL, USA) for extraction and quantification of EtG.

3.2.2 Sample extraction and EtG quantification

Feather samples were analyzed using a previously published method (Jones, Jones, Plate, Lewis, et al. 2012). Briefly, EtG was detected in aqueous extracts of powdered feather specimens (10-50 mg) using a Sciex 5500 liquid chromatography-tandem mass spectrometry (LC-MS/MS) system following a solid phase extraction. Tissue specimens were analyzed using a separate published method (Jones, Jones, Plate, and Lewis 2012). Tissue specimens were first homogenized, and the analytes were then isolated using a solid phase extraction procedure. EtG was detected in the extracts using electro-spray ionization (ESI) in the negative mode with a Sciex Triple Quad™ 5500 tandem mass spectrometer (Foster City, CA, USA; see Supplemental Text).

3.2.3 Data analyses

Results from both the feather and tissue samples were categorized in the following manner: readings were labeled as “inconclusive” if there were an apparent signal but did not satisfy all the identification criteria (e.g., “low on column”, “failed mass ratio”, or “RRT”), and were eliminated from most analyses. For individuals with two positive FEtG readings, the mean sample weight and FEtG concentration were included in the dataset regardless of feather sampling strategy. In cases with one conclusive and one inconclusive reading, only the conclusive result was used. Negative and positive FEtG concentrations were log-transformed (with the addition of 1.0 across all samples to compensate for zero values) so as to normalize data prior to analysis. For analyses that excluded FEtG-absent samples, all values for FEtG concentration were log-transformed without modification.

All statistical analyses were performed using R v4.2.2 (R Core Team 2023). Evolutionary relationships were mapped onto a phylogeny (Figure 3.1) generated using TimeTree 5 (Kumar et al. 2022). If a species was not present in the TimeTree database, then the parent taxon was used as a reference lineage. The branch lengths in Figure 3.1 are not represented proportionally to time, but branch lengths were included in the phylogenetically controlled analyses. Analyses were then conducted to determine patterns in FEtG presence among species and ecological

categories, with and without controls for phylogenetic relationships. We ran a one-way ANOVA to determine whether FEtG concentrations (excluding inconclusive results) differed among all species. An additional one-way ANOVA was conducted to determine whether FEtG concentrations differed among only those species that conclusively tested positive for FEtG. Binomial logistic regression was used to predict the probability of FEtG presence based on multiple ecological categories when including all individuals and excluding inconclusive results.

Finally, we used a phylogenetic linear mixed model with the `pglmm` function from the R package *phyr* v1.1.0 (D. Li et al. 2020) and the phylogeny from TimeTree (Figure 3.1) to account for phylogenetic relationships among species. The dependent variable was the log-transformed average FEtG concentration for each species. The independent variables were two ecological categories (trophic level, trophic niche) obtained from the AVONET dataset (Pigot et al. 2020; Tobias et al. 2022). As defined in Pigot et al. (2020), trophic level refers to the organism's position in a food web, with herbivores being primary consumers, carnivores as secondary and tertiary consumers, and omnivores falling in between with roughly half of their food items falling in each category. Trophic niche broadly encompasses the major foods utilized, with specialists being defined as having over 60% of their diet falling into one food resource (e.g., nectar, fruit, vertebrates, etc). The model was fitted using a Gaussian distribution for continuous values (D. Li et al. 2020). In addition to using the original trophic niche assigned by AVONET, we also conducted analyses of FEtG-present and FEtG-absent species with the niches simplified to two categories (nectarivore versus non-nectarivore) and three categories ([nectarivore+frugivore], granivore, and all other niches) given limited numbers of species per niche. However, use of simplified niches did not change the conclusions of this study (see Results).

To provide context for our use of a method designed and calibrated for human samples on birds, the figures include a literature-established human hair EtG threshold which suggests that a concentration of ≥ 30 pg/mg EtG is indicative of chronic excessive alcohol consumption in humans (Kintz 2015). However, as little is known about the dynamics of alcohol metabolism by birds, we caution against directly comparing these measurements for avian EtG with reported data on human EtG levels.

3.3 Results

3.3.1 Feather EtG

A total of 40 out of 64 individual birds conclusively tested positive for the presence of FEtG, 13 individuals were noted as inconclusive for FEtG, and FEtG was not found in 11 individuals (Table A2, available online). FEtG was consistently detected in all 14 Anna's Hummingbirds, the species for which we had the most samples (Table A2, available online). Of 17 species surveyed, FEtG was conclusively measured in ten species, inconclusively so in four species, and was not detected in three species (Fig. A3, available online). Concentrations from the conclusive FEtG results ranged from 11.8 pg/mg to 89.63 pg/mg. No clear trend was detected among the different feather types (Figure S3.1), and results were accordingly pooled among all feathers for statistical analyses.

We found support for the hypothesis that nectarivores are exposed to ethanol, because we observed FEtG in two nectarivorous species, Anna's Hummingbird ($n = 14$) and Black-chinned Hummingbird ($n = 1$). However, FEtG was not detected in one of our nectarivorous species – Ruby-throated Hummingbird ($n = 5$) – and determination was inconclusive in another – Costa's

Hummingbird (n = 1). We also found evidence of ethanol exposure in a variety of species from other trophic niches (Figures 3.1 and 3.2). We detected FEtG in three granivorous species, three invertivorous species, one omnivorous species, and one vertivorous species. The three species in which FEtG was not detected included two invertivores and one frugivore.

A one-way ANOVA of all FEtG concentrations across all species revealed that there was a statistically significant difference in FEtG concentration among species ($F_{14, 36} = 9.539$, $p < 0.01$; Figure 3.2). One-way ANOVA for only individuals that conclusively tested positive for FEtG revealed no statistically significant difference in FEtG concentration ($F_{9, 30} = 1.115$, $p = 0.382$; Fig. 2). Results of a binomial logistic regression showed that trophic level alone influenced the probability of EtG presence; categorization as an herbivore increased the chance of EtG presence by 18.6% (95% confidence interval [2.90, 119]) relative to carnivore categorization, and omnivore categorization by 1.6% relative to carnivore categorization (95% confidence interval [.237, 10.8]). Results of analyses run with simplified niches and other ecological categories did not change these conclusions and thus are not shown.

The phylogenetic generalized linear mixed model found significant interactions between trophic level alone and log-transformed average FEtG concentrations for species testing positive for FEtG, with omnivores having lower concentrations compared to herbivores and carnivores (slope = -0.35504 ± 0.10156 , P-value = 0.0004726, AIC = 2.592).

3.3.2 Liver EtG

Of the 26 analyzed individuals, Liver EtG (LEtG) was conclusively detected in four Anna's Hummingbirds and two American Robins, with concentrations ranging from 10.66 ng/g to 45.37 ng/g. FEtG was also detected in 17 of 26 individuals tested for LEtG (Table S3.2). Of the six individuals with LEtG present, all but one American Robin also exhibited FEtG (Figure S3.2). Among the four Anna's Hummingbirds exhibiting both FEtG and LEtG, FEtG concentration was higher than LEtG concentration in three of the four samples (Figure S3.2). An additional Anna's Hummingbird and two American Robins were inconclusive for LEtG presence (Table S3.2).

3.4 Discussion

3.4.1 Presence of EtG in bird feathers and livers

We found that many birds are likely to have been exposed to dietary ethanol. Using a method initially developed for human hair, we showed that bird feathers (and livers to a lesser degree) are a suitable matrix for EtG accumulation, and for determination. The presence of EtG is widespread across the avian tree (Figure 3.1) and is found in both bird feathers (10 out of 17 sampled species) and livers (two out of five sampled species). EtG was found in nectarivores as originally hypothesized, which supports the idea that the consumption of foods containing sugar can lead to ethanol exposure (Dudley 2014; Wiens et al. 2008). However, FEtG was not detected in one hummingbird, its determination was inconclusive in another hummingbird, and it was absent from the single frugivore in our dataset (Cedar Waxwing, *Bombycilia cedrorum*). Thus, detection of FEtG is not a foregone conclusion for species that consume sugary foods. We also found FEtG in many additional species from a wide variety of other trophic niches (Figures 3.1 and 3.2), which suggests that exposure to ethanol is not restricted to species with diets specialized on sugary foods. Therefore, exposure of animals to ethanol could be much more widespread than has been previously recognized.

A clear difference was observed between FEtG presence or absence among species (Figure 3.2). However, there were no significant differences in FEtG concentration among positively characterized species, in part due to overlapping variation in concentrations across many species (Figure 3.2). EtG presence in livers was inconsistent even within Anna's Hummingbird (*Calypte anna*), the most nectarivorous and intensively sampled of species. Overall, assays of EtG have the potential to expand our understanding of the breadth of avian foraging ecology, and pose new questions as to the physiology of dietary ethanol exposure and its subsequent catabolism across diverse taxa.

We were successful in our main objectives of: 1) testing avian tissues using EtG assays, 2) detecting EtG in many different bird species, and 3) observing evidence of ethanol exposure in nectarivores. These data pose many additional questions about EtG in birds, such as whether birds in different trophic niches exhibit different exposure to ethanol, and whether patterns in exposure could be explained in part by evolutionary history. A binomial logistic regression of trophic level found that herbivores have a higher probability of FEtG being present than do carnivores or omnivores. When considering average FEtG concentrations for the FEtG-positive species, a statistical model accounting for phylogenetic relationships among species found omnivores to have lower metabolite levels relative to carnivores and herbivores. An increased and more diverse sampling across independent origins of trophic niches would provide more evolutionary insight into patterns of FEtG accumulation in birds. For example, hummingbirds were the sole such representative of nectarivory in our dataset (Figure 3.1), but nectarivory has independently evolved at least 24 times (Hewes et al. 2022). We predict that other nectarivorous species will likely be exposed to ethanol and will exhibit EtG in their feathers.

These results indicate that birds are indeed metabolizing dietary ethanol (and/or producing ethanol endogenously). Assays of feathers performed better than of livers, although the sample size for livers is an important caveat. However, FEtG and LEtG occurrence (and by implication, ethanol ingestion), is likely more complex than initially supposed. Inconsistency in FEtG presence across a taxon or trophic niche within our dataset points to substantial gaps in our knowledge of the foraging biology of birds (e.g., Figures 3.1 and 3.2). The kinetics of EtG deposition and elimination in birds have also yet to be explored. Below, we explore some of the complexities that underlie EtG accumulation and ethanol exposure as associated with particular ecological and evolutionary contexts of birds.

3.4.2 Ethanol and FEtG in the context of avian ecology: molt patterns, seasonality in food availability and avian migration, and endogenous fermentation

We here present alternative hypotheses for the absence or presence of FEtG in birds relative to certain trophic niches and trophic levels (see Pigot et al. 2020 and Tobias et al. 2020 for relevant categorizations), along with potential factors underlying the wide intraspecific variation seen in some taxa. We also discuss why FEtG may offer but a narrow retrospective on ethanol exposure, based on our current understanding of human EtG kinetics, as well as knowledge of the ontogenetic development of feathers.

Molt patterns and EtG incorporation

What is the process by which EtG is incorporated into feathers? In humans, EtG is assumed to be incorporated into hair shafts via sweat, sebum, and/or through the bloodstream

into the developing hair follicle (Schröder et al. 2012). Thus, EtG presence in actively growing hair indicates sustained dietary exposure. EtG can be isolated in sweat alone due to its hydrophilic properties (Schummer et al. 2008). In contrast, sebum is nonpolar and unlikely to be a mechanism for EtG incorporation (Kharbouche et al. 2010). Birds lack sweat glands, but their skin does produce a sebaceous secretion as well as oils used for preening in the uropygial gland (A. M. Lucas and Stettenheim 1972; Stettenheim 2000). It has also been proposed that because rats also lack sweat glands, EtG incorporation during active hair growth is the only accumulation pathway for this species (Kharbouche et al. 2010). Therefore, we hypothesize that the primary path of EtG incorporation in bird feathers is via the bloodstream in the developing feather follicle.

How then does the intersection of EtG incorporation and feather development shape the significance of our findings? For assays of human hair, proximal scalp hair (0 – 6 cm) is typically used on the assumption that it captures alcohol consumption in the few months prior to sample collection (Oppolzer et al. 2017). Human hair grows continuously during the growth phase, which can last from two to six years (Braun-Falco et al. 2000). By contrast, typical feather growth is constrained to a much narrower window of time (i.e., weeks to months) depending on the type of feather and physiological circumstances. Molt is defined by one complete cycle of feather loss and replacement, and has often evolved so as to avoid overlap with other metabolically taxing periods such as reproduction (Stettenheim 2000). Feathers develop within follicles, the basal region of which is supplied by blood during active growth (Stettenheim 2000). When a feather stops growing, the blood vessels are then resorbed (Stettenheim 2000), with the feather becoming inert. The phenology, extent of molt (either partial or complete), molt strategies, number of molts per year, and duration of molt all vary interspecifically (Johnson et al. 2012). Feather growth rate can also vary depending on the feather type, size of the feather, and size of the bird (Jenni et al. 2020). In our study, the entire feather was used for analysis, presumably capturing any exposure during feather growth. Hypothetically, FEtG levels would then only be informative of the period during which molt occurred, and would not represent the time between molt completion and sampling (Figures 3.4A-B).

In addition to the aforementioned dynamics of FEtG incorporation, seasonality in exposure to environmental ethanol will be difficult to infer given diverse natural histories among birds. Many behavioral and ecological factors may influence EtG levels, including but not limited to shifts in diet composition and fermentability of food items throughout the year, and the timing of migration with respect to the timing of molt (see below). Additionally, because feathers sampled in this study were obtained from wild birds, we cannot exclude the possibility of human-mediated alcohol exposure. Bird enthusiasts speculate that artificial hummingbird feeders may be a source of dietary ethanol if feeder contents are not changed regularly and fermentation occurs (see Choi et al. 2023).

Historically, animals have been suggested to be exposed to lower concentrations and lower total dosage of ethanol in the wild, as compared to human consumption of alcoholic beverages (Dudley 2004). In humans, alcohol metabolism rates vary with body mass (Crunelle et al. 2016), and the connection between EtG thresholds and the extent of alcohol consumption has been established based on self-reported consumption data. Categorization as “abstinent” refers to the complete abstinence from alcoholic beverages, “moderate” as consuming an occasional drink during several days per week (around 60 g of ethanol per day, ≥ 7 pg/mg EtG), and “excessive” as routine consumption or binge drinking habits (around 120 g of ethanol per day,

≥30 pg/mg EtG; (Kintz 2015). However, in a recent study of hummingbird feeding preferences and ethanol exposure via artificial hummingbird feeders, it was estimated that if Anna's Hummingbirds were to feed from feeders containing 0.02% ethanol while also meeting their daily energetic needs, their total incidental consumption of ethanol would be 2 mg (0.04% of body mass of a 4.48 g hummingbird; Choi et al. 2023). By comparison, this dosage for an 80 kg human would be more than the equivalent of consuming one standard drink of 12 g of ethanol per day (i.e., 0.015% body mass; Choi et al. 2023). These observations concur with our findings of elevated FEtG in Anna's Hummingbirds. It is possible that their high FEtG is partially due to nectar fermentation in hummingbird feeders because sampled individuals were primarily found in residential neighborhoods. Of the 40 birds that tested positive for FEtG (ranging in body mass from 2.4 g to 392 g, as weighed postmortem after thawing and prior to sample collection), 22 individuals exceeded feather EtG concentrations of 30 pg/mg (i.e., the human threshold of chronic excessive alcohol consumption), and ten of these were Anna's Hummingbirds (Figure 3.2). Such high levels of FEtG are strongly suggestive of high chronic intake of alcohol. These results highlight future avenues of research to assess natural as well as anthropogenically sourced ethanol consumption, along with EtG formation and accumulation in birds.

Whereas our study focused on EtG presence, it is important to highlight other factors affecting EtG adsorption, accumulation, and loss. In humans, EtG is known to decrease in concentration from washing of samples in vitro (Luginbühl et al. 2019; Müller and Iwersen-Bergmann 2020). A similar decrease of FEtG through wear seems likely since birds retain their feathers until the next molt cycle, a process that commonly occurs on a semi-annual or annual basis. Variation in feather microstructures due to coloration (pigment and structural) may also affect interactions between EtG and feather microstructures. Birds possess a wide variety of pigments involved in feather coloration, as compared to the melanins of humans. Depending on the group in question, birds can utilize melanins, carotenoids, porphyrins, and other synthesized pigments (or a combination thereof), in addition to structural color (Terrill and Shultz 2023). Whereas some drugs have a strong affinity for melanin in human hair and rat fur (Green and Wilson 1996; Hubbard et al. 2000; Rollins et al. 2003), this effect does not extend to EtG in human hair (Appenzeller et al. 2007) or rat fur (Kharbouche et al. 2010). More research is needed to determine the pharmacokinetics of EtG to non-melanin pigments, particularly in non-human taxa. In conclusion, we posit that levels of EtG within feathers may not necessarily correspond linearly to the cumulative extent of dietary ethanol consumption given the kinetics of EtG gain and loss.

Seasonality in food availability and migration

Seasonal and geographic variation in food resources may further complicate our current understanding of ethanol exposure in birds. Levels of fermentation will depend on local temperature and the amount of sugary food locally available, so whether an individual migrates, its molt location, the degree of molt completion and dietary variation, could all lead to varying degrees of EtG incorporation into feathers.

We focus on our most nectarivorous group, the hummingbirds (Caprimulgiformes: Trochilidae), to illustrate potential factors influencing variability in EtG levels both across a family and within a species. Populations of Anna's Hummingbirds (*Calypte anna*) in California exhibit partial migration where some individuals are year-round residents while others migrate to more northern latitudes (Clark and Russell 2020). Also, higher air temperatures led to more ethanol

production in hummingbird feeders (Choi et al. 2023). Therefore, fluctuations in air temperature in temperate zones such as the San Francisco Bay Area may strongly influence rates of nectar fermentation, both naturally in flowers and artificially in anthropogenic food sources, thereby affecting the production of ethanol. In other parts of the world, such as in the epicenter of hummingbird diversity in the Neotropics, effects of diurnal temperature variation may be offset by increased population densities of hummingbirds such that nectar may be consumed prior to extensive fermentation. In contrast, FETG was not detected in three Ruby-throated Hummingbirds (*Archilochus colubris*) from central New Jersey and inconclusive for another two. However, all individuals were found within a two-week period in November 2018 (Figure S3.4A), which coincides with their southward winter migration. Prioritization of accumulating fat and protein reserves through consumption of arthropods may have reduced the proportion of nectar in the diet, and lower air temperatures may also have precluded substantial nectar fermentation.

Our results underscore the continued importance of natural history observations such as analysis of avian stomach contents to characterize diet, along with documentation of seasonal and geographical variation in diet within species. Further exploration is needed to contextualize these results in terms of avian physiology and health, and greater sampling is needed to establish the extent of fermented foods in the diets of different species and how much dietary variation there is within species.

Endogenous ethanol production

One non-obvious source of ethanol in birds is via endogenous fermentation within the digestive tract of the bird itself, or even of its prey. Although rarely diagnosed, auto-brewery syndrome is known to occur in humans when ingested carbohydrates are fermented to alcohol by fungi in the gut microbiome (Malik et al. 2019). It is therefore plausible that fermentation in the digestive tract may explain why granivorous and omnivorous birds with diets high in carbohydrates and sugars (e.g., seeds and nuts) tested positive for FETG. Endogenously-produced ethanol has previously been found in the crops of chickens (*Gallus gallus domesticus*; (Bolton 1965) and caecae of Willow Ptarmigans (*Lagopus lagopus*; McBee and West 1969). For House Wrens (*Troglodytes aedon*), with a diet primarily composed of arthropods (Johnson 2020), endogenous fermentation could be occurring in the digestive tract of their herbivorous prey. Caterpillars, for example, typically consume leaves which are then digested by microbial symbionts, possibly producing ethanol as a byproduct. It is plausible, given sufficient fermentation and rates of prey consumption, that a voracious bird such as a wren could accumulate substantial ethanol metabolites within its tissues.

Similarly, predators such as White-tailed Kites (*Elanus leucurus*) may experience endogenous fermentation within prey or post-mortem fermentation of prey, but associated ethanol concentrations would be unlikely to match that of nectar or fruits. At first glance, it is not immediately clear how White-tailed Kites, a vertivorous species, could be exposed to ethanol in its dietary ecology. White-tailed Kites primarily feed on small mammals such as voles (Dunk 2020), and meadow voles (*Microtus* spp.) were found in the digestive tracts of two specimens (MVZ:Bird:196046 and MVZ:Bird:196047; W.-C., pers. obs.). As with the House Wren, it is possible that a diet of endogenously fermenting, herbivorous prey could result in measurable levels of FETG in White-tailed Kites. This phenomenon may be less relevant for Common Murres (*Uria aalge*), an aquatic predator of fish and crustaceans (Ainley et al. 2021), for which the assay

nonetheless yielded inconclusive results. Further testing is needed to determine whether the inconclusive results are noisy results that obscure more conclusive presence or absence assessments of EtG.

3.4.3 Limitations and future directions

Our results suggest that measuring EtG holds promise for studying ethanol exposure in animals, but given the unusual, non-human input material of this assay it is worthwhile to highlight several limitations. In addition, our study was designed to gather a first look at EtG detection in birds. Future studies should assess how additional taxonomic and ecological sampling will be most fruitful for achieving a better understanding of ethanol exposure.

Our data set was based upon avian species which we could opportunistically sample so as to gauge how widespread EtG is distributed among birds. Consequently, the sampling lacks sufficient independent instances of the evolution of trophic niches (e.g., nectarivores are represented only by hummingbirds, and omnivores represented by one sparrow species) and trophic levels (e.g., omnivores represented by one hummingbird and two thrush species; Figure 3.1), as well as sufficient sampling within each species to fully assess relationships between ecology, phylogeny, and exposure to ethanol.

Within the context of our study, trophic niche proved to be less informative than expected, as there was substantial overlap in FEtG levels among FEtG-possessing species. This study is a humble reminder that animals often eat a diversity of dietary items that defy simple categorization. For example, the eponymous Acorn Woodpecker (*Melanerpes formicivorus*), here categorized as a granivore (Figure 3.1, Table S3.2), consumes more than just acorns. In addition to insects and the occasional vertebrate, this species also visits hummingbird feeders and feeds on sap, nectar, and fruit (Koenig et al. 2020), which could explain the EtG observed in their feathers. The same may be true for the Band-tailed Pigeon (*Patagioenas fasciata*), which is categorized as a granivore but frequently consumes fruit and various inflorescences (Keppie and Braun 2020). Yet another example are invertivores, among which FEtG presence here was inconsistent. FEtG was detected in the American Robin (*Turdus migratorius*), Hermit Thrush (*Catharus guttatus*), and House Wren (*Troglodytes aedon*) but not in the Barn Swallow (*Hirundo rustica*), Yellow-rumped Warbler (*Setophaga coronata*), and Bewick's Wren (*Thryomanes bewickii*). Within our sampling, "invertivore" covers a broad dietary spectrum, with the Barn Swallow and wrens being the most invertivorous and the American Robin and Hermit Thrush the least. As a case in point, fruit can constitute over 90% of the diet of American Robins (Vanderhoff et al. 2020), and up to 60% of the diet of Hermit Thrushes (Dellinger et al. 2020), depending on the time of year.

Milling efficiency of samples has been found to have a significant effect on EtG extraction efficiency (Albermann et al. 2012; Mönch et al. 2013). Over the course of this study, we found it necessary to repeat the bead beating protocol for certain samples to achieve as close to a powder-like consistency as possible, likely because of structural differences in feathers across the sampled species (and particularly in the proportions of the quill shaft/rachis to the vane). Feathers from larger species have a thicker rachis that was especially resistant to repeated bead beating cycles. Whereas inconsistency in grinding levels, and consequently in the extractability of EtG, may complicate the interpretation of EtG across species and time, we nonetheless were successful in establishing the non-trivial occurrence of EtG in feathers. Refinement, calibration,

and standardization of the EtG assay for feathers will improve the consistency of such measurements across taxonomic groups.

Additionally, it is important to note that the assay itself was designed and calibrated for detecting EtG in human hair and tissues. FEtG readings from 12 out of the 64 birds (18.75%) were inconclusive, and FEtG was absent in some individuals even though conspecifics tested positive (Table S3.2). The amount of input material into the assay and the material type could have played a role in the detection of EtG in our samples (see Supporting Text). For several individuals a second assay with increased feather input was done when the first assay yielded inconclusive results. Increased starting material resulted in a conclusive reading for some individuals and a second inconclusive reading for others. Thus, at least some of the inconclusive results were due to inadequate amounts of starting material in the first assay. Initial experimentation of testing flight versus body feathers and [rachis+calamus] versus barbs also yielded inconsistent results. For example, both components tested positive for EtG in some, both tested inconclusive for others, or inconclusive for one component and positive or absent for the other component. In addition, liver EtG assays were different from FEtG, because absent or inconclusive results for liver EtG were obtained for 20 out of the 26 individuals tested, despite some of the same individuals testing positive for feather EtG. This may be because EtG in tissue potentially offers a shorter retrospective view of ethanol consumption and reflective of that individual's diet in the weeks before death as opposed to during its most recent molting period. Thus, refinement of this assay is needed to quantify samples below the minimum threshold, as well as to resolve issues surrounding identification criteria leading to inconclusive results. Inconclusive results could include false absence of both low and high EtG concentrations, which would add noise to our dataset. Therefore, we have confidence in positive results, but suggest that the absent or inconclusive results should be interpreted with caution unless the diet is well known.

We propose several comparative studies to further explore alcohol metabolism in birds. Additional context is needed to establish biologically meaningful thresholds in birds, and to establish the connection between degree of alcohol exposure, the pathways involving alcohol metabolism, varying extent of nutrient assimilation, effects of body mass, and alcohol metabolite accumulation. In addition to including a second assay to detect ethanol consumption at a different temporal scale (see Supporting Text), controlled feeding of ethanol to captive birds and testing for FEtG will be necessary to establish baselines for ethanol-metabolizing ability, and to test how EtG accumulates in feathers in relation to circannual molt cycles, wear, and uropygial gland secretions used in preening. In addition to increasing sample size, future exploration of the evolution of ethanol metabolism in birds should take phylogeny into account. To tackle the question of whether certain trophic niches are exposed to significantly more dietary alcohol (e.g., nectarivores and frugivores), and as a result could be more likely to have higher FEtG concentrations, greater replicates per species and broader sampling is needed across the avian phylogeny. Hummingbirds represent just one of many independent origins of nectarivory in birds (Hewes et al. 2022), and inclusion of more nectarivores would inform our understanding of the factors influencing the evolution of ethanol metabolism. Comparative analyses of avian alcohol metabolism across trophic niches, especially nectarivores, are also needed to establish whether certain feeding ecologies are associated with different levels of ethanol metabolism and tolerance relative to EtG formation. A comparison of ethanol metabolism in four passerine species representing three trophic niches found that rates of alcohol metabolism partially

depended on the relative quantity of ethanol-containing food in their diet (Eriksson and Nummi 1982). The Bohemian Waxwing (*Bombycilla garrulus*; frugivore) was most efficient at metabolizing alcohol, followed by the European Starling (*Sturnus vulgaris*; omnivore) and Eurasian Bullfinch (*Pyrrhula pyrrhula*; omnivore), and finally by the European Greenfinch (*Chloris chloris*; granivore; Eriksson and Nummi 1982). Studies tracking behavioral and physiological changes with alcohol consumption would further inform our knowledge of whether alcohol catabolism is incidental or adaptive in different organisms (Wiens et al. 2008), as would investigations into the metabolic pathways that eliminate alcohol. Finally, inclusion of additional trophic niches across birds (and also among mammals) would enable us to better contextualize evolutionary adaptations relating to dietary ethanol exposure in vertebrates as a whole.

3.5 Conclusions

We have shown direct evidence that birds are exposed to dietary ethanol given that ethyl glucuronide was conclusively detected in avian feathers. Levels of FEtG varied both within and between species and was detected in species spanning many trophic niches. Levels of FEtG for samples with conclusive results will provide a baseline range for future detailed studies of relative feather concentrations among avian taxa, albeit with certain methodological constraints. Further research is needed to establish associated consequences of ethanol exposure, including physiological or behavioral risks, kinetics of ethanol catabolism in birds as compared to mammals, and whether dietary ethanol exposure has influenced the evolution of the genes underlying ethanol metabolism in different lineages of birds. This study broadens our understanding of avian feeding ecology, establishes the occurrence of ethanol in the nutritional landscape, and emphasizes the need to survey a broader diversity of species relative to dietary ethanol exposure. The discovery of an ethanol metabolite in bird feathers begs the question as to whether tolerance has evolved in birds, and whether the excessive alcohol consumption characterized in some humans can similarly occur in birds.

3.6 Tables and Figures

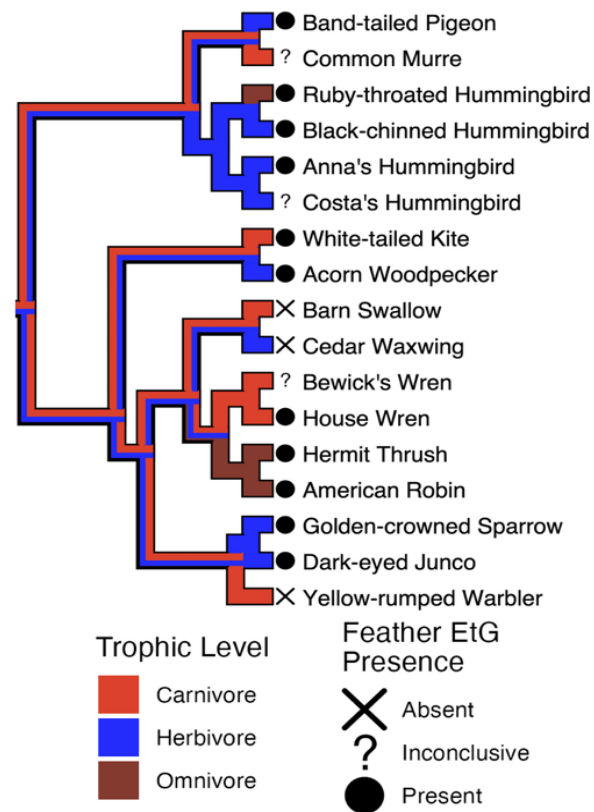


Figure 3.1: An unrooted phylogeny generated using TimeTree for the species included in the study, and the corresponding FEtG presence and trophic level. The phylogeny depicts only relationships among species because branch lengths are not proportional to time. Trophic levels are derived from the AVONET dataset (Tobias et al. 2022).



Figure 3.2: Distribution of log₁₀ transformed feather EtG concentrations of each species. The individual data points for each sample are plotted as points over the boxplots, with absent and inconclusive results depicted as 0 pg/mg. The boxplot demonstrates the median and the first and third quartile. The whiskers indicate the minimum and maximum values. The red dashed line indicates a threshold of 30 pg/mg; concentrations at and above this threshold are suggestive of chronic excessive alcohol consumption in humans. For each species, the mean and standard deviation (SD) are given to the right and the sample size (n) provided to the left. Colored font indicates trophic niche: pink, nectarivore; yellow, granivore; blue; aquatic predator; red, vertivore; purple, frugivore; brown, omnivore; grey, invertivore.

3.7 Supporting Text

3.7.1 Supplementary Methods

Additional feather sampling strategies

Several sample collection methods were evaluated during our study. Initially we attempted to separate the calamus and rachis (the quill and central shaft) from the vane (the flat surface of interlocked barbs) of flight feathers (wing primaries and secondaries and tail rectrices). The vane was pooled with body feathers, then [vane+body] feathers and [calamus+rachis] were processed and submitted for analysis (Figure S3.1A). However, sufficient calamus and rachis (minimum 20 mg) could not be collected from most species. Intact flight feathers (including calamus and rachis) and body feathers (torso, neck, head) were also analyzed separately for a subset of samples (Figure S3.1B). Subsequent sampling focused on body feathers for feasibility and consistency. Both growing and fully grown feathers were included.

Detailed methods for EtG testing of feather at USDTL

Specimens were analyzed at the United States Drug Testing Laboratories, Inc. (Des Plaines, IL, USA) using a previously published method (Jones, Jones, Plate, Lewis, et al. 2012). Weighed portions (10-50 mg) of powdered feather were placed in 2 mL polypropylene vials. The ETG was extracted from the powdered specimens by adding 1mL DI water to the vials and incubating in a 40 °C sonicating water bath and 50 L ETG-*d*₅ (20 ng/mL) was added as the internal standard. After centrifugation, the decanted extracts were subjected to solid phase extraction (SPE) using a quaternary amine with chloride counter ion SPE cartridge with a 200 mg bed in a 10 mL reservoir (CUQAX12Z, United Chemical Technologies, Bristol, PA, USA). The extracts were loaded onto the SPE columns after the columns were conditioned with methanol (2 mL) and DI water and rinsed with 2 mL DI water and 2 mL methanol. The ETG was eluted from the SPE cartridges with 2 mL of 2% formic acid in methanol following a rinse step of 2 mL of methanol with 2% formic acid. The eluates were evaporated under a stream of nitrogen at 40 °C and reconstituted in 0.1 mL of DI water. Separation was achieved on an Agilent 1200 HPLC system fitted with a Synergi Polar RP (50 mm × 2.0 mm, 2.5 μm particle size) C-18 column (Phenomenex, Torrance, CA, USA) held at 30 °C.

The detector was a Sciex 5500 liquid chromatography-tandem mass spectrometry (LC-MS/MS) using electro-spray in the negative mode. The internal standard (ETG-*d*₅) was monitored using the m/z 226.1 > 74.9 (quantification ion) transition and the m/z 226.1 > 85.2 (qualifying ion) transition. The m/z 221.1 > 74.9 (quantification ion) and m/z 221.1 > 85.0 (qualifying ion) transitions were used to monitor EtG. The limit of detection was 4 pg/mg, the limit of quantitation was 8 pg/mg, and the assay was linear up to 200 ng/mg.

The identification criteria used for this procedure included four components: retention time, signal to noise, baseline resolution and relative ion intensity. The retention time of each analyte was required to be within 0.2 min of the calibrator. A signal to noise of greater than 3:1 was required of each ion chromatogram. A minimum of 90% return to baseline was required to consider a peak to be adequately resolved from a co-eluting peak. The relative ion intensity of the product ions for each analyte (mass ratio) was required to be within 20% of the corresponding relative ion intensity of the calibrator. This assay was developed to analyze human hair specimens.

There were specimens where there was an apparent signal but did not satisfy all the identification criteria. These results were noted as inconclusive.

Phosphatidylethanol (PEth) and considerations for its application to bird tissue

The inclusion of a second metabolite assay would allow for multiple perspectives on the dynamics of ethanol metabolism within a single individual. EtG is only one of several metabolites currently in use for monitoring alcohol usage in humans. Phosphatidylethanol (PEth) is a direct alcohol biomarker that can be detected in either whole or dried blood from a few days up to weeks (Luginbühl et al. 2021). Because PEth provides more immediate and narrower temporal evidence of ethanol consumption, while circumventing issues associated with using feathers, regular sampling of blood from a population throughout a year could yield insights not possible with FEtG alone. One must take into consideration the numerous stresses of blood sampling when establishing ethical, species-specific blood sampling parameters. A minimum of 40 µL of blood as a dried blood spot is needed to assay for PEth in humans (Jones, pers. comm.). A general rule of thumb for sampling blood from birds is no more than <1% of body mass (in volume) per sampling event, and no more than 2% in a 14-day period - see (Fair et al. 2010) and (Voss et al. 2010). For example, 40-45 µL could be taken from Anna's Hummingbirds (*Calypte anna*) which typically weigh 4-4.5 g (Clark and Russell 2020). However, hummingbirds have high energetic needs and thus may be more susceptible to the effects of blood loss. Current recommendations advise a more conservative limit of 0.5% to 1% total lean body mass (Tell et al. 2021).

3.7.2 Supplementary Tables

Table S3.1. Overview of studies that have tested for Ethyl glucuronide in non-human species.

Reference	Species	Biological Material Tested
Wiens et al. (2008)	Pen-tailed Treeshrew (<i>Ptilocercus lowii</i>)	Hair
	Plantain Squirrel (<i>Callosciurus notatus</i>)	
	Common Treeshrew (<i>Tupaia glis</i>)	
	Gray Tree Rat (<i>Lenothrix canus</i>)	
Kharbouche et al. (2010)	Brown Rat (<i>Rattus norvegicus</i>)	Hair (pigmented versus non-pigmented)
Wright and Ferslew (2012)	Brown Rat (<i>Rattus norvegicus</i>)	Blood, urine
Małkowska et al. (2012)	Brown Rat (<i>Rattus norvegicus</i>)	Hair
Campbell et al. (2022)	Spider Monkey (<i>Ateles geoffroyi</i>)	Urine

Table S3.2: Sample numbers for EtG assay of feathers and livers. Inconclusive samples were specimens where there was an apparent signal, but that did not satisfy all the identification criteria (see Supporting Text). Trophic niche was taken from Tobias et al. (2022).

Order	Family	Species	Trophic Level	Trophic Niche	Number of individuals assayed for feather EtG			Number of individuals assayed for liver EtG		
					Positive	Inconclusive	Total	Positive	Inconclusive	Total
Caprimulgiformes	Trochilidae	Ruby-throated Hummingbird (<i>Archilochus colubris</i>)	Omnivore	Nectarivore	0	2	5	0		5
		Black-chinned Hummingbird (<i>Archilochus alexandri</i>)	Herbivore	Nectarivore	1		1			
		Anna's Hummingbird (<i>Calypte anna</i>)	Herbivore	Nectarivore	14		14	4	1	10
		Costa's Hummingbird (<i>Calypte costae</i>)	Herbivore	Nectarivore	0	1	1			
Columbiformes	Columbidae	Band-tailed Pigeon (<i>Patagioenas fasciata</i>)	Herbivore	Granivore	4		5			
Accipitriformes	Accipitridae	White-tailed Kite (<i>Elanus leucurus</i>)	Carnivore	Vertivore	3	1	5			
Charadriiformes	Alcidae	Common Murre (<i>Uria aalge</i>)	Carnivore	Aquatic predator	0	2	5			
Piciformes	Picidae	Acorn Woodpecker (<i>Melanerpes formicivorus</i>)	Herbivore	Granivore	4	1	5			
Passeriformes	Hirundinidae	Barn Swallow (<i>Hirundo rustica</i>)	Carnivore	Invertivore	0		1			
	Bombycillidae	Cedar Waxwing (<i>Bombycilla cedrorum</i>)	Herbivore	Frugivore	0		1			
	Turdidae	Hermit Thrush (<i>Catharus guttatus</i>)	Omnivore	Invertivore	1		1			
		American Robin (<i>Turdus migratorius</i>)	Omnivore	Invertivore	3	2	5	2	2	5
Troglodytidae		Bewick's Wren (<i>Thryomanes bewickii</i>)	Carnivore	Invertivore	0	2	2			
		House Wren (<i>Troglodytes aedon</i>)	Carnivore	Invertivore	2		2	0		1
	Passerellidae	Dark-eyed Junco (<i>Junco hyemalis</i>)	Herbivore	Granivore	5		5	0		5
		Golden-crowned Sparrow (<i>Zonotrichia atricapilla</i>)	Herbivore	Omnivore	3	2	5			
Parulidae		Yellow-rumped warbler (<i>Dendroica coronata</i>)	Carnivore	Invertivore	0		1			
Grand Total					40	13	64	6	3	26

Table S3.3: Catalog numbers of vouchered specimens. Specimens that lack institutional catalog numbers (CC and CYWC numbers) are currently awaiting cataloging at the Museum of Vertebrate Zoology (MVZ). FEtG = Feather EtG, LEtG = liver EtG, + = present, - = absent, ? = inconclusive, * = data from the AVONET dataset (Tobias et al. 2022).

Order			MVZ:Bird				Trophic	
Family	Species	Catalog No.	Date Collected	FtEG	LEtG	Level*	Trophic Niche*	
Accipitriformes								
Accipitridae	<i>Elanus leucurus</i>	196045	2-Apr-2017	-		Carnivore	Vertivore	
	<i>Elanus leucurus</i>	196046	26-Jun-2019	+		Carnivore	Vertivore	
	<i>Elanus leucurus</i>	196047	14-Aug-2019	?		Carnivore	Vertivore	
	<i>Elanus leucurus</i>	196048	10-Apr-2019	+		Carnivore	Vertivore	
	<i>Elanus leucurus</i>	196049	26-Sep-2015	+		Carnivore	Vertivore	
Caprimulgiformes								
Trochilidae	<i>Calypte anna</i>	184818	21-Jun-2018	+		Herbivore	Nectarivore	
	<i>Calypte anna</i>	195875	29-Apr-2014	+	-	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196028	18-Apr-2020	+		Herbivore	Nectarivore	
	<i>Calypte anna</i>	196029	1-Jan-2019	+	+	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196030	20-Apr-2018	+		Herbivore	Nectarivore	
	<i>Calypte anna</i>	196031	1-Jan-2019	+	+	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196032	29-Oct-2018	+	-	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196033	14-Nov-2018	+	-	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196034	6-Nov-2018	+	-	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196035	19-Nov-2018	+	?	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196036	4-Oct-2018	+	+	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196037	9-Dec-2018	+	-	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196038	7-Nov-2018	+	+	Herbivore	Nectarivore	
	<i>Calypte anna</i>	196039	23-Apr-2018	+		Herbivore	Nectarivore	
	<i>Calypte costae</i>	CYWC204	5-Apr-2021	?		Herbivore	Nectarivore	
	<i>Archilochus alexandri</i>	CYWC205	15-May-2021	+		Herbivore	Nectarivore	
	<i>Archilochus colubris</i>	196023	12-Sep-2018	?	-	Omnivore	Nectarivore	
	<i>Archilochus colubris</i>	196024	15-Sep-2018	-	-	Omnivore	Nectarivore	
	<i>Archilochus colubris</i>	196025	6-Sep-2018	-	-	Omnivore	Nectarivore	
	<i>Archilochus colubris</i>	196026	16-Sep-2018	?	-	Omnivore	Nectarivore	
	<i>Archilochus colubris</i>	196027	27-Sep-2018	-	-	Omnivore	Nectarivore	
Charadriiformes								
Alcidae	<i>Uria aalge</i>	196040	1-Sep-2014	-		Carnivore	Aquatic predator	
	<i>Uria aalge</i>	196041	27-Oct-2014	?		Carnivore	Aquatic predator	
	<i>Uria aalge</i>	196042	8-Aug-2016	?		Carnivore	Aquatic predator	
	<i>Uria aalge</i>	196043	14-Aug-2015	-		Carnivore	Aquatic predator	
	<i>Uria aalge</i>	196044	19-Aug-2014	-		Carnivore	Aquatic predator	
Columbiformes								
Columbidae	<i>Patagioenas fasciata</i>	196018	28-Mar-2015	+		Herbivore	Granivore	
	<i>Patagioenas fasciata</i>	196019	11-Feb-2015	-		Herbivore	Granivore	
	<i>Patagioenas fasciata</i>	196020	19-Jan-2015	+		Herbivore	Granivore	
	<i>Patagioenas fasciata</i>	196021	19-Jan-2015	+		Herbivore	Granivore	
	<i>Patagioenas fasciata</i>	196022	13-Jan-2015	+		Herbivore	Granivore	
Passeriformes								
Bombycillidae	<i>Bombycilla cedrorum</i>	196051	17-Jan-2019	-		Herbivore	Frugivore	
Hirundinidae	<i>Hirundo rustica</i>	196050	1-Jun-2020	-		Carnivore	Invertivore	
Parulidae	<i>Dendroica coronata</i>	196069	15-Jan-2019	-		Carnivore	Invertivore	
Passerellidae	<i>Junco hyemalis</i>	196060	5-Nov-2018	+	-	Herbivore	Granivore	
	<i>Junco hyemalis</i>	196061	29-Jan-2019	+	-	Herbivore	Granivore	
	<i>Junco hyemalis</i>	196062	20-Oct-2018	+	-	Herbivore	Granivore	
	<i>Junco hyemalis</i>	196063	16-Nov-2016	+	-	Herbivore	Granivore	
	<i>Junco hyemalis</i>	196064	2-Nov-2018	+	-	Herbivore	Granivore	
	<i>Zonotrichia atricapilla</i>	184213	21-Nov-2019	?		Herbivore	Omnivore	
	<i>Zonotrichia atricapilla</i>	196065	14-Jan-2019	+		Herbivore	Omnivore	
	<i>Zonotrichia atricapilla</i>	196066	10-Dec-2019	?		Herbivore	Omnivore	
	<i>Zonotrichia atricapilla</i>	196067	27-Oct-2019	+		Herbivore	Omnivore	
	<i>Zonotrichia atricapilla</i>	196068	8-Feb-2020	+		Herbivore	Omnivore	
	Troglodytidae	<i>Thryomanes bewickii</i>	196070	13-May-2016	?		Carnivore	Invertivore
		<i>Thryomanes bewickii</i>	196071	9-Oct-2018	?		Carnivore	Invertivore
		<i>Troglodytes aedon</i>	196052	14-Oct-2014	+	-	Carnivore	Invertivore
		<i>Troglodytes aedon</i>	196053	20-Jul-2018	+		Carnivore	Invertivore

Order	MVZ:Bird					Trophic	
Family	Species	Catalog No.	Date Collected	FtEG	LEtG	Level*	Trophic Niche*
Turdidae	<i>Turdus migratorius</i>	196055	20-Oct-2018	?	+	Omnivore	Invertivore
	<i>Turdus migratorius</i>	196056	12-Nov-2018	+	+	Omnivore	Invertivore
	<i>Turdus migratorius</i>	196057	25-Jan-2019	+	-	Omnivore	Invertivore
	<i>Turdus migratorius</i>	196058	8-Dec-2020	+	?	Omnivore	Invertivore
	<i>Turdus migratorius</i>	196059	14-Nov-2018	?	?	Omnivore	Invertivore
	<i>Catharus guttatus</i>	196054	23-Dec-2018	+		Omnivore	Invertivore
Piciformes							
Picidae	<i>Melanerpes formicivorus</i>	193321	29-Jun-2021	+		Herbivore	Granivore
	<i>Melanerpes formicivorus</i>	193322	29-Jun-2021	+		Herbivore	Granivore
	<i>Melanerpes formicivorus</i>	CYWC141	20-Jul-2021	+		Herbivore	Granivore
	<i>Melanerpes formicivorus</i>	CC4608	12-Oct-2021	?		Herbivore	Granivore
	<i>Melanerpes formicivorus</i>	CC4607	12-Oct-2021	+		Herbivore	Granivore

3.7.3 Supplementary Figures

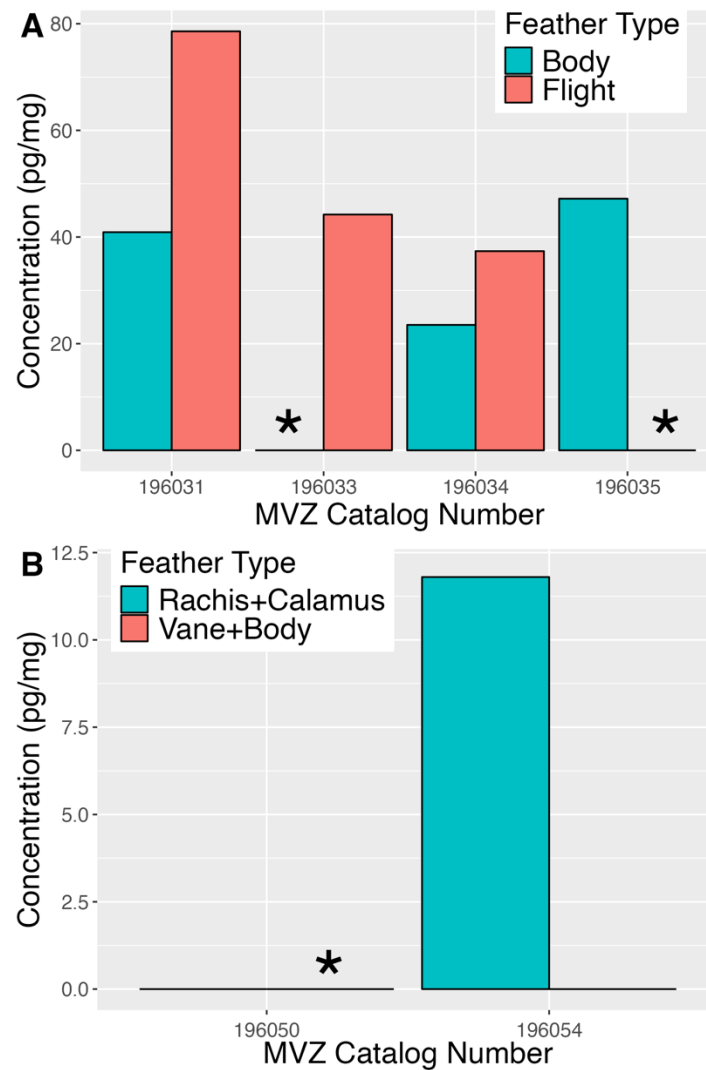


Figure S3.1: Bar plot comparing feather EtG concentrations from (A) flight feathers and body feathers of Anna's Hummingbirds and (B) [rachis+calamus] versus [vane+body feathers]. An asterisk denotes inconclusive FETG results for which there was an apparent signal that did not however satisfy all identification criteria. Lines along the X-axis without an asterisk are zero values.

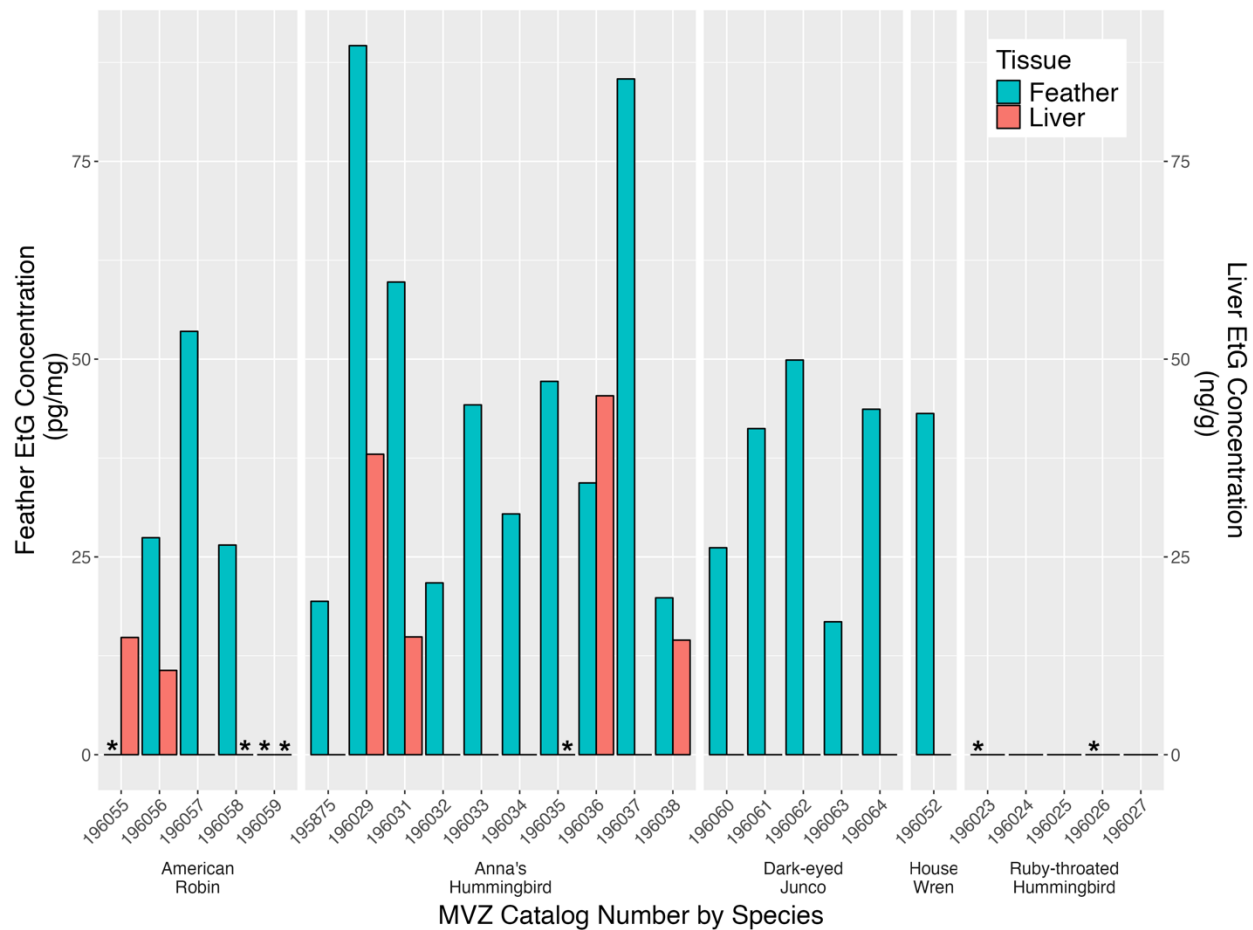


Figure S3.2: Intra-individual comparison of all feather EtG and liver EtG concentrations. Absent and inconclusive results are depicted as zeros. An asterisk indicates an inconclusive EtG result. Lines along the X-axis without an asterisk are zero values.

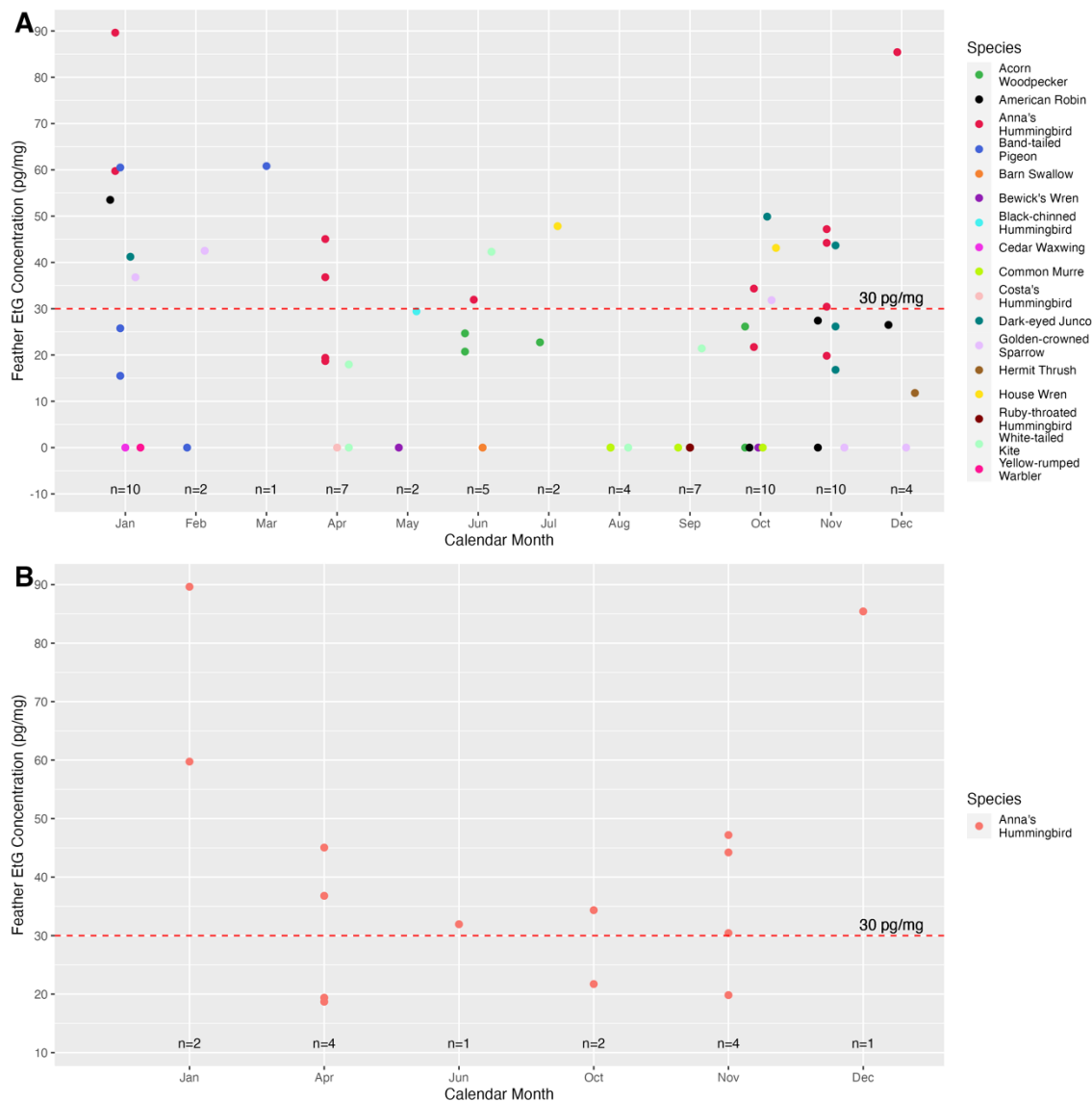


Figure S3.4: Feather EtG concentrations by calendar month (A) for all species with conclusive and inconclusive results and (B) for Anna's Hummingbirds (*Calypete anna*). The plotted date is the date of death; points are staggered to improve visibility.

Conclusion

In this study, I assessed three previously underexplored aspects of nectivorous birds to contextualize the findings within the framework of nectarivory as a syndrome. I shed light on the many ways that ecology, phylogenetic history, and functional morphology have shaped bird evolution. The results reinforce the position that the degree of convergence in traits associated with adaptation to a nectivorous lifestyle is highly variable across birds. My dissertation provides a foundation for future research in the areas of functional tongue morphology (Chapter 1), the genetic basis of long bills (Chapter 2), and the ecology of dietary ethanol in birds (Chapter 3).

Chapter 1 advances our understanding of the morphology of the sunbird tongue and underscores the significance of internal anatomy in informing functional hypotheses of how birds drink nectar. Quantification of variation in the sunbird tongue's skeletal elements revealed patterns associated with ecology and phylogeny. Additionally, through revealing structures that facilitate the seal against the upper palate, the morphology of the sunbird tongue apparatus supports the hypothesis of nectar transport via hermetic suction feeding. These findings as well as experimental feeding studies (D. Cuban unpubl. data) refute the previous hypothesis of nectar transport via capillary action and highlights the importance of testing hypotheses of adaptation. The existence of variable basihyal-urohyal shapes across sunbird lineages with similar trends in bill size, tongue apparatus size, and tongue shape suggest a complex relationship between diet, feeding behavior, and phylogeny. This can be observed in morphospace of the basihyal-urohyal shapes, particularly in how the nectarivorous Cape Sugarbird (*Promerops cafer*) clusters towards sunbirds, while the insectivorous Fraser's Sunbird (*Euchloridia fraseri*) is an outlier among sunbirds and clusters more closely with insectivorous outgroups. Although sunbird tongues are adapted to feed on nectar, it is crucial to acknowledge that the tongue is a multipurpose tool capable of feeding on more than just nectar, such as arthropods, for which the feeding biomechanics have not been extensively studied. Studies of how birds feed on solid foods are needed to fully understand the diverse factors shaping tongue morphology within nectarivorous lineages of birds.

In Chapter 2, I tested for signatures of rapid evolution in sunbirds and their relatives within genes previously implicated to underlie variation in bill size and shape. Using a newly developed multilocus phylogeny of sunbirds as our framework, positive selection was detected for a subset of loci for several genes, but no non-synonymous mutations were exclusive to Nectariniidae. The genetic changes underlying bill morphology may be too labile in sunbirds to detect as there was no evidence of rapid evolution at the ancestral branch of sunbirds in any of the candidate genes. It may be that mechanisms other than amino acid changes play a larger role in shaping the bill, such as regulatory changes in gene expression. In addition to conducting similar surveys of other lineages of nectar-feeding passerines, developmental studies of long-billed species would provide insight into the genetic architecture of bill morphology beyond that of seed-eating birds which have been the focus of such studies to date.

Chapter 3 demonstrated the prevalence of dietary ethanol (either through direct ingestion or endogenous production) across birds representing different diet categories. A novel substrate, bird feathers, was used in place of human hair to measure ethyl glucuronide, a commonly used biomarker and direct metabolite of ethanol. Feather EtG was found to be present across a surprising number of diet categories surveyed, though within a given species its presence

and concentration was inconsistent. Although the sampling for liver EtG was less extensive, the results nevertheless corroborate that of feather EtG. Thus, ethanol is not a molecule exclusive to the diet of nectarivorous species. Anna's Hummingbird exhibited the highest concentrations of EtG in both their feathers and livers, which may be the result of high exposure to ethanol in their diet. This adds greater dimension to our understanding of the nutritional landscape in birds and lays the groundwork for future exploration of the role of ethanol in the evolution of birds.

Both sunbirds and hummingbirds are good exemplar systems in which to make advances on our understanding of nectarivory. In particular, the results of Chapter 1 highlighted the need to test long-held assumptions regarding nectar-feeding adaptations in the context of nectarivory as a syndrome. Whether these traits are truly adaptive and how they function will inform the degree to which disparate lineages are convergent. However, this work will be challenging as fundamental aspects of the ecology of many bird species remain poorly understood or are at present unknown. Much more natural history information, such as diet composition and foraging behavior, are needed to improve our ability to infer patterns in how and when these traits evolved in the phylogeny of a clade. In turn, knowledge of adaptive evolution in the genetics of these traits would also help elucidate the relationship between genotype, phenotype, and function through the integration of the genetic basis of traits, traditional anatomical studies, and feeding experiments. Future comparative studies would benefit from studying how birds have specialized on other groups of foods, ultimately culminating in a holistic view of the evolution of feeding morphology in birds.

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Appendices

Appendix Table 1: CT scan voucher sampling. Fluid-preserved bird specimens used in Chapter 1 are listed with the revised taxonomy from Bowie and Fjeldså (2020), museum catalog number, and MorphoSource DOI. The original species and subspecies identification from the museum catalog was included if different from the species according to eBird. Institutional abbreviations for catalog numbers are as follows: ANSP = Academy of Natural Sciences of Drexel University (Philadelphia, Pennsylvania), FMNH = Field Museum of Natural History (Chicago, Illinois), KU = University of Kansas Biodiversity Institute (Lawrence, Kansas), MVZ = Museum of Vertebrate Zoology at the University of California, Berkeley (Berkeley, California), NHMUK = Natural History Museum (London, United Kingdom), UMMZ = University of Michigan Museum of Zoology (Ann Arbor, Michigan), UWBM = University of Washington Burke Museum (Seattle, Washington), YPM = Yale Peabody Museum (New Haven, Connecticut). Numbers in parentheses are field numbers, which either lack institutional catalog numbers (i.e., I-jz specimens from Zeyl et al. 2022 or are currently awaiting cataloging at their respective museums at the UWBM and MVZ.

Family	Revised Taxonomy	Species according to eBird	Original Species ID	Catalog Number	MorphoSource DOI	In phylo geny	In analys es
Trochilidae		<i>Calypte anna</i>		UWBM uncatalogued	Cuban et al. (in prep)		
Meliphagidae		<i>Acanthorhynchus tenuirostris</i>		nhmuk:1964.17.104	ark:/87602/m4/M125748		
Modulatricidae	<i>Modulatrix stictigula pressa</i>	<i>Modulatrix stictigula pressa</i>		FMNH:birds:437611	ark:/87602/m4/M85074	Y	Y
Chloropseidae	<i>Chloropsis hardwickii</i>	<i>Chloropsis hardwickii</i>		FMNH:birds:288746	ark:/87602/m4/M78331	Y - Genus	Y
Promeropidae	<i>Promerops cafer</i>	<i>Promerops cafer</i>		YPM:orn:112687	ark:/87602/m4/531835	Y	Y
Irenidae	<i>Irena cyanogastra</i>	<i>Irena cyanogastra</i>	<i>Irena cyanogaster cyanogaster</i>	FMNH:birds:462007	ark:/87602/m4/M57165	Y	Y
Irenidae	<i>Irena puella</i>	<i>Irena puella</i>		nhmuk:A/1969.3.32	ark:/87602/m4/M125828	Y	Y
Modulatricidae	<i>Arcanator orostruthus</i>	<i>Arcanator orostruthus</i>	<i>Phyllastrephus orostruthus</i>	FMNH:birds:438573	ark:/87602/m4/522708		
Modulatricidae	<i>Kakamega poliothorax</i>	<i>Kakamega poliothorax</i>	<i>Trichastoma poliothorax</i>	FMNH:birds:511259	ark:/87602/m4/M73414		
Dicaeidae	<i>Prionoglossa olivaceus</i>	<i>Prionochilus olivaceus parsoni</i>		FMNH:birds:480630	ark:/87602/m4/529678	Y - Genus	Y
Dicaeidae	<i>Dicaeum aeruginosum</i>	<i>Dicaeum agile aeruginosum</i>	<i>Dicaeum aeruginosum aeruginosum</i>	FMNH:birds:503806	ark:/87602/m4/415115	Y - Genus	Y
Dicaeidae	<i>Dicaeum anthonyi</i>	<i>Dicaeum anthonyi anthonyi</i>		FMNH:birds:437593	ark:/87602/m4/M72798	Y	Y
Dicaeidae	<i>Dicaeum australe</i>	<i>Dicaeum australe australe</i>		FMNH:birds:478848	ark:/87602/m4/M114367		Y
Dicaeidae	<i>Dicaeum trigonostigma</i>	<i>Dicaeum trigonostigma</i>		nhmuk:A/1970.19.10	ark:/87602/m4/M92003	Y	Y
Nectariniidae	<i>Aethopyga flagrans</i>	<i>Aethopyga flagrans</i>		FMNH:birds:449199	ark:/87602/m4/415125		Y
Nectariniidae	<i>Aethopyga linaraborae</i>	<i>Aethopyga linaraborae</i>		FMNH:birds:472727	ark:/87602/m4/368967		Y
Nectariniidae	<i>Aethopyga siparaja</i>	<i>Aethopyga siparaja mangini</i>		FMNH:birds:97195	ark:/87602/m4/528388	Y	Y
Nectariniidae	<i>Anthobaphes violacea</i>	<i>Anthobaphes violacea</i>		I-jz:JZ:47 (field number)	ark:/87602/m4/M167289	Y	Y
Nectariniidae	<i>Anthodiaeta collaris</i>	<i>Hedydipna collaris zambesiana</i>	<i>Anthreptes collaris zambesianus</i>	FMNH:birds:438619	ark:/87602/m4/372759	Y	Y

Family	Revised Taxonomy	Species according to eBird	Original Species ID	Catalog Number	MorphoSource DOI	In phylo geny	In analys es
Nectariniidae	<i>Anthodiaeta hartlaubii</i>	<i>Anabathmis hartlaubii</i>		UMMZ:birds:226236	ark:/87602/m4/M82735	Y	Y
Nectariniidae	<i>Anthodiaeta jugularis</i>	<i>Cinnyris jugularis</i>	<i>Nectarinia jugularis jugularis</i>	FMNH:birds:427573	ark:/87602/m4/436379	Y	Y
Nectariniidae	<i>Anthodiaeta sovimanga</i>	<i>Cinnyris sovimanga sovimanga</i>	<i>Nectarinia souimanga souimanga</i>	FMNH:birds:363864	rk:/87602/m4/436404	Y	Y
Nectariniidae	<i>Anthreptes malacensis</i>	<i>Anthreptes malacensis chlorigaster</i>		FMNH:birds:358541	ark:/87602/m4/M97704	Y	Y
Nectariniidae	<i>Arachnothera longirostra</i>	<i>Arachnothera longirostra dilutior</i>		FMNH:birds:455080	ark:/87602/m4/528544	Y	Y
Nectariniidae	<i>Arachnothera magna</i>	<i>Arachnothera magna magna</i>		FMNH:birds:289026	ark:/87602/m4/M97689	Y	Y
Nectariniidae	<i>Cinnyris adelberti</i>	<i>Chalcomitra adelberti</i>	<i>Nectarinia adelberti adelberti</i>	FMNH:birds:288249	ark:/87602/m4/525095	Y	Y
Nectariniidae	<i>Cinnyris amethystina</i>	<i>Chalcomitra amethystina</i>	<i>Nectarinia amethystina amethystina</i>	FMNH:birds:390164	ark:/87602/m4/415171	Y	Y
Nectariniidae	<i>Cinnyris chloropygius</i>	<i>Cinnyris chloropygius orphogaster</i>	<i>Nectarinia chloropygia orphogaster</i>	FMNH:birds:346615	ark:/87602/m4/436386	Y	Y
Nectariniidae	<i>Cinnyris cupreus</i>	<i>Cinnyris cupreus</i>	<i>Nectarinia cuprea cuprea</i>	FMNH:birds:288208	ark:/87602/m4/436373	Y	Y
Nectariniidae	<i>Cinnyris erythrocerus</i>	<i>Cinnyris erythrocerus</i>	<i>Nectarinia erythrocerca</i>	FMNH:birds:496763	ark:/87602/m4/415164	Y	Y
Nectariniidae	<i>Cinnyris notatus</i>	<i>Cinnyris notatus notata</i>	<i>Nectarinia notata notata</i>	FMNH:Birds:345976	ark:/87602/m4/528561	Y	Y
Nectariniidae	<i>Cinnyris pulchellus</i>	<i>Cinnyris pulchellus pulchellus</i>	<i>Nectarinia pulchella pulchella</i>	FMNH:birds:320105	ark:/87602/m4/415150	Y	Y
Nectariniidae	<i>Cinnyris reichenowi</i>	<i>Cinnyris reichenowi preussi</i>	<i>Nectarinia preussi preussi</i>	FMNH:birds:511326	ark:/87602/m4/436411	Y	Y
Nectariniidae	<i>Cinnyris stuhlmanni</i>	<i>Cinnyris stuhlmanni</i>	<i>Nectarinia afra chapini</i>	FMNH:birds:484123	ark:/87602/m4/436477	Y	Y
Nectariniidae	<i>Cinnyris ursulae</i>	<i>Cinnyris ursulae</i>	<i>Nectarinia ursulae</i>	FMNH:birds:511332	ark:/87602/m4/M73437		Y
Nectariniidae	<i>Cyanomitra alinae</i>	<i>Cyanomitra alinae tanganjicae</i>	<i>Nectarinia alinae tanganjicae</i>	FMNH:birds:364065	ark:/87602/m4/482583	Y	Y
Nectariniidae	<i>Cyanomitra verticalis</i>	<i>Cyanomitra verticalis verticalis</i>	<i>Nectarinia verticalis verticalis</i>	FMNH:birds:107964	ark:/87602/m4/436471	Y	Y
Nectariniidae	<i>Euchloridia fraseri</i>	<i>Deleornis fraseri</i>		KU:birds:113213	ark:/87602/m4/M77493		
Nectariniidae	<i>Euchloridia fraseri</i>	<i>Deleornis fraseri</i>	<i>Anthreptes fraseri cameroonensis</i>	FMNH:birds:396377	ark:/87602/m4/554721	Y	Y
Nectariniidae	<i>Haagneria olivacea</i>	<i>Cyanomitra olivacea ragazzii</i>	<i>Nectarinia olivacea ragazzii</i>	FMNH:birds:496758	ark:/87602/m4/436417	Y	
Nectariniidae	<i>Haagneria olivacea</i>	<i>Cyanomitra olivacea</i>		FMNH:birds:496758	ark:/87602/m4/436417		Y
Nectariniidae	<i>Lamprothreptes longuemarei</i>	<i>Anthreptes longuemarei nyassae</i>		FMNH:birds:438618	ark:/87602/m4/415157	Y	Y
Nectariniidae	<i>Leptocoma sperata</i>	<i>Leptocoma sperata</i>		ANSP:birds:199661	ark:/87602/m4/M90669		
Nectariniidae	<i>Leptocoma sperata</i>	<i>Leptocoma sperata sperata</i>	<i>Nectarinia sperata sperata</i>	FMNH:birds:345062	ark:/87602/m4/436392	Y	Y
Nectariniidae	<i>Notiocinnyris chalybeus</i>	<i>Cinnyris chalybeus</i>		(Field number:I-jz:JZ:46)	ark:/87602/m4/M167288	Y	Y
Nectariniidae	<i>Notiocinnyris manoensis</i>	<i>Cinnyris manoensis pinto</i>	<i>Nectarinia manoensis pinto</i>	FMNH:birds:444455	ark:/87602/m4/415128	Y	Y

Family	Revised Taxonomy	Species according to eBird	Original Species ID	Catalog Number	MorphoSource DOI	In phylo geny	In analys es
Nectariniidae	<i>Notiocinnyris mediocris</i>	<i>Cinnyris mediocris bensoni</i>	<i>Nectarinia mediocris bensoni</i>	FMNH:birds:438631	ark:/87602/m4/436434	Y	Y
Nectariniidae	<i>Notiocinnyris regius</i>	<i>Cinnyris regius kivuensis</i>	<i>Nectarinia regia kivuensis</i>	FMNH:birds:350907	ark:/87602/m4/436423	Y	Y

Appendix Table 2: Genetic voucher sampling. Samples used in the phylogenetic analyses for Chapters 1 and 2 are listed with the updated taxonomy from Bowie and Fjeldså (2020) and Bowie et al. (in prep) and museum catalog number, if available. Samples lacking museum catalog numbers are indicated by the extraction number or their field number followed by the museum. Institutional abbreviations for catalog numbers are as follows: AMNH = American Museum of Natural History (New York City, New York), ANSP = Academy of Natural Sciences of Drexel University (Philadelphia, Pennsylvania), CSIRO-AUS = Commonwealth Scientific and Industrial Research Organization (Canberra, Australia), DMNH = Durban Natural Science Museum (Durban, South Africa), FMNH = Field Museum of Natural History (Chicago, Illinois), KU = University of Kansas Biodiversity Institute (Lawrence, Kansas), LSU = Louisiana State University Museum of Natural Science (Baton Rouge, Louisiana), MVZ = Museum of Vertebrate Zoology at the University of California Berkeley (Berkeley, California), NMK = National Museums of Kenya (Nairobi, Kenya), RMCA = Royal Museum for Central Africa (Tervuren, Belgium), UCLA = University of California Los Angeles (Los Angeles, California), UMMZ = University of Michigan Museum of Zoology (Ann Arbor, Michigan), USNM = National Museum of Natural History, Smithsonian Institution (Washington, District of Columbia), UWBM = University of Washington Burke Museum (Seattle, Washington), YPM = Yale Peabody Museum (New Haven, Connecticut), ZMUC = Zoological Museum, Natural History Museum of Denmark (Copenhagen, Denmark). ALS, BRK, EAA, GO, GOUW, JAM, JBK, JCW, JF, JK, JPM, LMB, and RCKB specimens lacking institutional catalog numbers are currently awaiting cataloging at the CSIRO-AUS, MVZ, UWBM, or ZMUC.

Family	Species	Museum Catalog Number	Source Material	Note
Chloropseidae	<i>Chloropsis aurifrons</i>	FMNH:Birds:363782	Modern	
Dicaeidae	<i>Dicaeum aeneum</i>	MVZ:Bird:93384	Historical	
Dicaeidae	<i>Dicaeum annae</i>	JF 2999 (MVZ)	Modern	
Dicaeidae	<i>Dicaeum anthonyi</i>	FMNH:Birds:357602	Modern	
Dicaeidae	<i>Dicaeum aureolimbatus</i>	RCKB 2258 (MVZ)	Modern	
Dicaeidae	<i>Dicaeum celebicum</i>	JAM 14278 (MVZ)	Modern	
Dicaeidae	<i>Dicaeum geelvinkianum</i>	MVZ:Bird:149255	Historical	
Dicaeidae	<i>Dicaeum hirundinaceum</i>	ALS 194 (MVZ)	Modern	
Dicaeidae	<i>Dicaeum ignipectus</i>	MVZ:Bird:140346	Historical	
Dicaeidae	<i>Dicaeum nehrkorni</i>	RCKB 2197 (MVZ)	Modern	
Dicaeidae	<i>Dicaeum pectorale</i>	E579 (CSIRO-AUS)	Modern	
Dicaeidae	<i>Dicaeum sp.</i>	ZMUC:AVES-147311	Modern	
Dicaeidae	<i>Dicaeum trigonostigma</i>	LSU:BirdTissues:B23366	Modern	
Dicaeidae	<i>Dicaeum trochileum</i>	MVZ:Bird:129001	Historical	
Dicaeidae	<i>Pachyglossa agile</i>	LSU:BirdTissues:B7927	Modern	
Dicaeidae	<i>Pachyglossa melanozanthum</i>	ext-Paris18dash42	Unknown	
Dicaeidae	<i>Prionochilus maculatus</i>	LSU:BirdTissues:B36382	Modern	
Fringillidae	<i>Serinus burtoni</i>	ext-CYW1699	Modern	
Irenidae	<i>Irena cyanogastra</i>	FMNH:Birds:350955	Modern	
Irenidae	<i>Irena puella</i>	ZMUC:AVES-140789	Modern	
Modulatricidae	<i>Modulatrix stictigula</i>	JF 10-271102 (ZMUC)	Modern	
Motacillidae	<i>Anthus gustavi</i>	RCKB 2434 (MVZ)	Modern	
Motacillidae	<i>Anthus rubescens</i>	MVZ:Bird:180221	Modern	
Nectariniidae	<i>Aethopyga boltoni</i>	FMNH:Birds:357631	Modern	
Nectariniidae	<i>Aethopyga duyvenbodei</i>	ext-CYW1768	Modern	
Nectariniidae	<i>Aethopyga mystacalis</i>	MVZ:Bird:129080	Historical	
Nectariniidae	<i>Aethopyga primigenia</i>	FMNH:Birds:357624	Modern	
Nectariniidae	<i>Aethopyga saturata</i>	MVZ:Bird:44136	Historical	
Nectariniidae	<i>Aethopyga siparaja</i>	RCKB 2302 (MVZ)	Modern	
Nectariniidae	<i>Anabathmis reichenbachii</i>	LSU:BirdTissues:B27124	Modern	
Nectariniidae	<i>Anthobaphes violacea</i>	RB 674 (MVZ)	Modern	
Nectariniidae	<i>Anthodiaeta asiaticus</i>	MVZ:Bird:88280	Historical	
Nectariniidae	<i>Anthodiaeta collaris</i>	FMNH:Birds:396704	Modern	
Nectariniidae	<i>Anthodiaeta comorensis</i>	ext-RB2271	Unknown	*Representative for RNASEH2B

Family	Species	Museum Catalog Number	Source Material	Note
Nectariniidae	<i>Anthodiaeta comorensis</i>	ext-RB2272	Unknown	
Nectariniidae	<i>Anthodiaeta coquerelli</i>	ext-RB2270	Unknown	
Nectariniidae	<i>Anthodiaeta dussumieri</i>	ext-RB393	Unknown	
Nectariniidae	<i>Anthodiaeta fusca</i>	FMNH:Birds:453287	Modern	
Nectariniidae	<i>Anthodiaeta hartlaubi</i>	ext-CYW1745	Modern	
Nectariniidae	<i>Anthodiaeta humbloti</i>	ext-CYW1825	Modern	*Representative for RNASEH2B
Nectariniidae	<i>Anthodiaeta humbloti</i>	ext-CYW2383	Modern	
Nectariniidae	<i>Anthodiaeta jugularis</i>	RCKB 2380 (MVZ)	Modern	
Nectariniidae	<i>Anthodiaeta lotenius</i>	MVZ:Bird:108678	Historical	
Nectariniidae	<i>Anthodiaeta osea</i>	ext-CYW1761	Modern	
Nectariniidae	<i>Anthodiaeta solaris</i>	LMB 54 (MVZ)	Modern	
Nectariniidae	<i>Anthodiaeta sovimanga</i>	FMNH:Birds:431289	Modern	
Nectariniidae	<i>Anthodiaeta talatala</i>	GO 688 (MVZ)	Modern	
Nectariniidae	<i>Anthodiaeta thomensis</i>	ext-CYW1579	Modern	
Nectariniidae	<i>Anthodiaeta venusta</i>	FMNH:Birds:356091	Modern	
Nectariniidae	<i>Anthreptes malacensis</i>	RCKB 2392 (MVZ)	Modern	
Nectariniidae	<i>Anthreptes rhodolaemus</i>	KU:Birds:111285	Unknown	
Nectariniidae	<i>Anthreptes simplex</i>	ANSP:ORN:1175	Modern	
Nectariniidae	<i>Arachnothera juliae</i>	ANSP:ORN:1259	Modern	
Nectariniidae	<i>Arachnothera longirostra</i>	BRK 16 (MVZ)	Modern	
Nectariniidae	<i>Arachnothera magna</i>	AMNH:Birds:DOT-12277	Modern	
Nectariniidae	<i>Arachnothera robusta</i>	LSU:BirdTissues:B36483	Modern	
Nectariniidae	<i>Chalcoparia singalensis</i>	ANSP:ORN:1321	Modern	
Nectariniidae	<i>Chlorocichla sp.</i>	ext-CYW1671	Modern	
Nectariniidae	<i>Cinnyris adelberti</i>	FMNH:Birds:396730	Modern	
Nectariniidae	<i>Cinnyris amethystina</i>	RCKB 1350 (MVZ)	Modern	
Nectariniidae	<i>Cinnyris bifasciatus</i>	ZMUC:AVES-103572	Modern	
Nectariniidae	<i>Cinnyris bocagii</i>	RMCA-100464	Historical	
Nectariniidae	<i>Cinnyris bouvieri</i>	ZMUC:AVES-No149	Historical	
Nectariniidae	<i>Cinnyris chalcornelas</i>	YPM:ORN:094049	Historical	
Nectariniidae	<i>Cinnyris chloropygius</i>	FMNH:Birds:385274	Modern	
Nectariniidae	<i>Cinnyris coccinigastrus</i>	ext-ABS415 / ext-CYW1891	Unknown	
Nectariniidae	<i>Cinnyris cupreus</i>	FMNH:Birds:396732	Modern	
Nectariniidae	<i>Cinnyris erythrocerus</i>	MVZ:Bird:137981	Historical	
Nectariniidae	<i>Cinnyris fuliginosa</i>	USNM:BIRDS:16262	Modern	
Nectariniidae	<i>Cinnyris habessinicus</i>	ext-CYW2243	Historical	
Nectariniidae	<i>Cinnyris habessinicus</i>	ext-CYW2244	Historical	*Representative for RNASEH2B
Nectariniidae	<i>Cinnyris hunteri</i>	ZMUC:AVES-92426	Historical	
Nectariniidae	<i>Cinnyris kilimensis</i>	FMNH:Birds:429815	Modern	
Nectariniidae	<i>Cinnyris mariquensis</i>	GO 707 (MVZ)	Modern	
Nectariniidae	<i>Cinnyris minullus</i>	FMNH:Birds:429668	Modern	
Nectariniidae	<i>Cinnyris nectarinioides</i>	ZMUC:AVES-92393	Historical	
Nectariniidae	<i>Cinnyris notatus</i>	FMNH:Birds:436538	Modern	
Nectariniidae	<i>Cinnyris pembae</i>	ext-CYW2245	Historical	
Nectariniidae	<i>Cinnyris pulchellus</i>	FMNH:Birds:396735	Modern	
Nectariniidae	<i>Cinnyris purpureiventris</i>	FMNH:Birds:356303	Modern	
Nectariniidae	<i>Cinnyris reichenowi</i>	ext-CYW1754	Modern	
Nectariniidae	<i>Cinnyris rubescens</i>	ext-ABS435	Unknown	
Nectariniidae	<i>Cinnyris senegalensis</i>	FMNH:Birds:396721	Modern	
Nectariniidae	<i>Cinnyris shelleyi</i>	JK02-481 (UWBM)	Modern	
Nectariniidae	<i>Cinnyris superbus</i>	FMNH:Birds:396745	Modern	
Nectariniidae	<i>Cinnyris tacaze</i>	ext-CYW1652	Modern	
Nectariniidae	<i>Cyanomitra alinae</i>	FMNH:Birds:356083	Modern	
Nectariniidae	<i>Cyanomitra bannermani</i>	ext-CYW1762	Modern	*Representative for RNASEH2B
Nectariniidae	<i>Cyanomitra bannermani</i>	RMCA-77528	Historical	
Nectariniidae	<i>Cyanomitra cyanolaema</i>	FMNH:Birds:429665	Modern	
Nectariniidae	<i>Cyanomitra oritis</i>	ext-CYW1908	Modern	
Nectariniidae	<i>Cyanomitra veronii</i>	ZMUC:AVES-117154	Modern	
Nectariniidae	<i>Cyanomitra verticalis</i>	ext-CYW2361	Modern	*Representative for RNASEH2B
Nectariniidae	<i>Cyanomitra verticalis</i>	FMNH:Birds:346602	Modern	
Nectariniidae	<i>Euchloridia batesi</i>	ANSP:ORN:11557	Modern	
Nectariniidae	<i>Euchloridia fraseri</i>	FMNH:Birds:396703	Modern	
Nectariniidae	<i>Euchloridia gabonicus</i>	ANSP:ORN:24058	Modern	
Nectariniidae	<i>Euchloridia pallidigaster</i>	NMK16122	Historical	

Family	Species	Museum Catalog Number	Source Material	Note
Nectariniidae	<i>Euchloridia rectirostris</i>	ANSP:ORN:11459	Modern	
Nectariniidae	<i>Euchloridia reichenowi</i>	ZMUC:AVES-93898	Historical	
Nectariniidae	<i>Euchloridia rubritorques</i>	ZMUC:AVES-101563	Modern	
Nectariniidae	<i>Euchloridia rufipennis</i>	ZMUC:AVES-7192	Modern	
Nectariniidae	<i>Haagneria olivacea</i>	RCKB 1085 (MVZ)	Modern	
Nectariniidae	<i>Hedydipna metallica</i>	YPM:ORN:96884	Historical	
Nectariniidae	<i>Hedydipna platara</i>	FMNH:Birds:396706	Modern	
Nectariniidae	<i>Kurochkinogramma hypogrammicum</i>	LSU:BirdTissues:B36403	Modern	
Nectariniidae	<i>Lamprothreptes aurantius</i>	YPM:ORN:095713	Historical	
Nectariniidae	<i>Lamprothreptes longuemareii</i>	FMNH:Birds:440894	Modern	
Nectariniidae	<i>Lamprothreptes neglectus</i>	FMNH:Birds:356805	Modern	
Nectariniidae	<i>Lamprothreptes orientalis</i>	MVZ:Bird:125660	Historical	
Nectariniidae	<i>Leptocoma aspasia</i>	RCKB 2411 (MVZ)	Modern	
Nectariniidae	<i>Leptocoma brasiliensis</i>	MVZ:Bird:43851	Historical	
Nectariniidae	<i>Leptocoma calcostetha</i>	MVZ:Bird:129000	Historical	
Nectariniidae	<i>Leptocoma sperata</i>	FMNH:Birds:345061	Modern	
Nectariniidae	<i>Leptocoma zeylonica</i>	ZMUC:AVES-59.992	Historical	
Nectariniidae	<i>Muscicapidae sp.</i>	ext-CYW1711	Modern	
Nectariniidae	<i>Nectarinia famosa</i>	FMNH:Birds:385284	Modern	
Nectariniidae	<i>Nectarinia famosa</i>	FMNH:Birds:440960	Modern	*Representative for RNASEH2B
Nectariniidae	<i>Nectarinia johnstoni</i>	FMNH:Birds:385289	Modern	
Nectariniidae	<i>Notiocinnyris afer</i>	GO 686 (MVZ)	Modern	
Nectariniidae	<i>Notiocinnyris anchietae</i>	FMNH:Birds:440895	Modern	
Nectariniidae	<i>Notiocinnyris chalybeus</i>	FMNH:Birds:390161	Modern	
Nectariniidae	<i>Notiocinnyris fuelleborni</i>	FMNH:Birds:440991	Modern	
Nectariniidae	<i>Notiocinnyris loveridgei</i>	JPM 016 (MVZ)	Modern	
Nectariniidae	<i>Notiocinnyris ludovicensis</i>	ext-CYW1683	Modern	
Nectariniidae	<i>Notiocinnyris manoensis</i>	FMNH:Birds:440962	Modern	
Nectariniidae	<i>Notiocinnyris mediocris</i>	ZMUC:AVES-94880	Modern	
Nectariniidae	<i>Notiocinnyris moreau</i>	JPM 044 (MVZ)	Modern	
Nectariniidae	<i>Notiocinnyris neergaardi</i>	DMNH:Bird:27091	Historical	*Representative for RNASEH2B
Nectariniidae	<i>Notiocinnyris neergaardi</i>	DMNH:Bird:27095	Historical	
Nectariniidae	<i>Notiocinnyris newtonii</i>	ext-CYW1796	Modern	
Nectariniidae	<i>Notiocinnyris prigoginei</i>	ext-aDNA68	Historical	
Nectariniidae	<i>Notiocinnyris regius</i>	FMNH:Birds:346623	Modern	
Nectariniidae	<i>Notiocinnyris reichenowi</i>	FMNH:Birds:385272	Modern	
Nectariniidae	<i>Notiocinnyris rockefelleri</i>	ext-aDNA92	Historical	
Nectariniidae	<i>Notiocinnyris stuhlmanni</i>	FMNH:Birds:356109	Modern	
Nectariniidae	<i>Notiocinnyris usambaricus</i>	ZMUC:AVES-81667	Modern	
Nectariniidae	<i>Notiocinnyris whytei</i>	FMNH:Birds:440972	Modern	
Parulidae	<i>Basileuterus belli</i>	MVZ:Bird:184297	Modern	
Passerellidae	<i>Pipilo maculatus</i>	MVZ:Bird:177429	Modern	
Promeropidae	<i>Promerops cafer</i>	RB 694 (MVZ)	Modern	
Promeropidae	<i>Promerops gurneyi</i>	ext-CYW2363	Modern	
Sturnidae	<i>Sturnus vulgaris</i>	MVZ:Bird:190182	Modern	
Thraupidae	<i>Dacnis cayana</i>	MVZ:Bird:190528	Modern	
Turdidae	<i>Turdus migratorius</i>	RCKB 1767 (MVZ)	Modern	
Tyrannidae	<i>Empidonax hammondi</i>	MVZ:Bird:192875	Modern	