

Relating form to function in the hummingbird feeding apparatus (#17404)

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Relating form to function in the hummingbird feeding apparatus

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A complete understanding of the feeding structures is fundamental in order to study how animals survive. Some birds use long and protrusible tongues as the main tool to collect their central caloric source (e.g. woodpeckers and nectarivores). Hummingbirds are the oldest and most diverse clade of nectarivorous vertebrates, being a perfect subject to study tongue specializations. Their tongue functions to intraorally transport arthropods through their long bills and enables them to exploit the nectarivorous niche by collecting small amounts of liquid, therefore it is of vital importance to study its anatomy and structure at various scales. I focused on the portions of the hummingbird tongue that have been shown to be key for the understanding of their feeding mechanisms. I used histology, transmission and scanning electron microscopy, microCT, and *ex-vivo* experiments in order to advance our understanding of the morphology and functioning of the hummingbird feeding apparatus. I found that hummingbird tongues are composed mainly of thin cornified epithelium, lack papillae, and completely fill the internal cast of the rostral oropharyngeal cavity. This puzzle-piece match between bill and tongue will be determinant for the study of intraoral transport of nectar. Likewise, I found that the structural composition and tissue architecture of the tongue groove walls provide the rostral portion of the tongue with elastic properties that are central to the study of tongue-nectar interactions during the feeding process. Detailed studies on hummingbirds set the basis for comparisons with other nectar-feeding birds and contribute to comprehend the natural solutions to collecting liquids in the most efficient way possible.

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2 **Relating form to function in the hummingbird feeding apparatus**

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Abstract

A complete understanding of the feeding structures is fundamental in order to study how animals survive. Some birds use long and protrusible tongues as the main tool to collect their central caloric source (*e.g.* woodpeckers and nectarivores). Hummingbirds are the oldest and most diverse clade of nectarivorous vertebrates, being a perfect subject to study tongue specializations. Their tongue functions to intraorally transport arthropods through their long bills and enables them to exploit the nectarivorous niche by collecting small amounts of liquid, therefore it is of vital importance to study its anatomy and structure at various scales. I focused on the portions of the hummingbird tongue that have been shown to be key for the understanding of their feeding mechanisms. I used histology, transmission and scanning electron microscopy, microCT, and *ex-vivo* experiments in order to advance our understanding of the morphology and functioning of the hummingbird feeding apparatus. I found that hummingbird tongues are composed mainly of thin cornified epithelium, lack papillae, and completely fill the internal cast of the rostral oropharyngeal cavity. This puzzle-piece match between bill and tongue will be determinant for the study of intraoral transport of nectar. Likewise, I found that the structural composition and tissue architecture of the tongue groove walls provide the rostral portion of the tongue with elastic properties that are central to the study of tongue-nectar interactions during the feeding process. Detailed studies on hummingbirds set the basis for comparisons with other nectar-feeding birds and contribute to comprehend the natural solutions to collecting liquids in the most efficient way possible.

Keywords Anatomy – Bill – Computed tomography – Electron microscopy – Tongue

Introduction

A central challenge of biological studies is to describe the links among the structures (*e.g.* organismal morphology), underlying mechanisms (*e.g.* biomechanics), and emergent phenomena (*e.g.* performance, ecological and evolutionary patterns) in live organisms. Birds are an ideal subject to tackle this challenge since they have evolved the most morphologically diverse array of feeding structures among tetrapods (Rubega 2000). Our understanding of the form and function of the feeding structures is vital to grasp the functional constraints that steer the evolution of resource exploitation in animals. In birds, it has been recognized that bill shape is tightly correlated to diet (*cf.* Rubega 2000), therefore bill shape provides information about *which* type of food is consumed; as a complement, I hypothesize that tongue morphology could provide further information about *how* the food is consumed. Examples can be found in the extreme reduction of the tongue of cormorants (Jackowiak et al. 2006), the gigantic papillae of penguins (Kobayashi *et al.* 1998), and the numerous flexible projections of flamingo tongues (Zweers *et al.* 1995). Avian tongues present adaptations as extensive and varied as those of bird bills (Farner 1960). Unveiling the details of the morphology and coupling of the components of the feeding apparatus advances the understanding of its functioning and evolution.

Birds control the movement of their tongues with muscles attached to the hyobranchial apparatus (set of supporting bones); these ‘intrinsic hyolingual muscles’ (Homberger and Meyers 1989; Tomlinson 2000; but see Schwenk 2001) have their most rostral attachments on a paired bone called the *Paraglossum* (*cf.*, Weymouth *et al.* 1964; or *Os entoglossum*, Newton *et al.*

1896). Some birds, such as woodpeckers (Shufeldt 1900; Villard and Cuisin 2004) and nectar-feeding birds (Stiles 1981; Paton and Collins 1989), have to protrude their tongues to procure their food. Interestingly, woodpeckers have the ability to actively control their tongue tips (*cf.* Bock 1999), a capacity that is lacking in hummingbirds (Zusi 2013); the reason for this dissimilarity relies on the differential elongation of the tongue components, in woodpeckers the portion of the tongue supported by the *paraglossum* is not elongated while in hummingbirds this portion is greatly lengthened. In most birds, only the rostral third of the tongue is entirely free of musculature (review in Erdoğan and Iwasaki 2014), but in hummingbirds between half (Scharnke 1931; Weymouth *et al.* 1964) to three fourths (Rico-Guevara 2014) of the tongue lacks muscles, bone and/or cartilage support. Only a pair of cornified rods at the lingual tip (*cf.* Weymouth *et al.* 1964) provides rigidity to the rostral membranous tube-like grooves in hummingbird tongues (Fig. 1 in Rico-Guevara and Rubega 2011). It is puzzling that this highly specialized food collection tools lack active control, and it is important to understand how tissue organization and properties alone govern the tongue functioning in nectar collection.

In birds, diversity in feeding apparatus came with niche specialization; as one of the prime examples, primitive insectivorous hummingbirds entered the nectar-feeding niche and became one of the most specialized nectarivorous vertebrates (Stiles 1981; Fleming and Muchhala 2008; Baldwin *et al.* 2014). Hummingbirds still catch insects as their main source of protein, exhibiting a variety of hunting tactics (*e.g.* Stiles 1995; Rico-Guevara 2008) and using their tongues to drag prey they catch near their bill tips all the way to where it can be swallowed (*e.g.* Yanega 2007). Therefore, they use their tongue protrusion abilities for both arthropod intraoral transport and nectar collection (*e.g.* Rico-Guevara 2014). Although hummingbird

80 tongues have been studied for around two centuries (Martin 1833; Darwin 1841; Lucas 1891;
 81 Scharnke 1931; Weymouth *et al.* 1964; Hainsworth 1973), many aspects of their morphology
 82 and function still remain to be understood. The tongues of hummingbirds are forked at their tips
 83 (Martin 1833; Darwin 1841; Scharnke 1931; Hainsworth 1973), ending in two tube-like grooves
 84 with fringed edges (Lucas 1891). These grooves are exclusively rostral structures and the interior
 85 of the tongue base is not hollow (Scharnke 1931; Weymouth *et al.* 1964). There is only one
 86 study focusing on the morphology of the entire length of the tongue grooves (Hainsworth 1973),
 87 which unfortunately is lacking histological details. The most rostral cross section micrograph
 88 near the base of the tongue grooves (Weymouth *et al.* 1964), shows at least two distinct layers of
 89 tissue composing the dorsal and ventral surfaces of the grooves, which are not further described.
 90 Studies on nectar feeding in living birds suggest that the functional traits enabling hummingbird
 91 to extract liquid are related to the structural configuration of the tongue tip (Rico-Guevara and
 92 Rubega 2011; Rico-Guevara *et al.* 2015), rather than to active movements of their parts through
 93 muscle action. A deeper study of the entire length of hummingbird tongues is essential to
 94 understand the underlying architectural properties enabling the observed nectar extraction
 95 mechanisms. Because previous studies (*e.g.* Weymouth *et al.* 1964; Zusi 2013) have described in
 96 detail the hyobranchial apparatus, and the structure of the root, and body of the tongue (up to the
 97 bifurcation point) in hummingbirds, the present study presents only descriptions of the structures
 98 of the rostral portion of the tongue grooves, and in addition a description of the coupling between
 99 the bill and tongue. Understanding the morphology of the rostral portion of the grooves and the
 100 bill-tongue fit is crucial to understand the nectar-feeding mechanics in hummingbirds (*e.g.* Rico-
 101 Guevara 2014). Furthermore, since the proposed mechanism of nectar collection involves
 102 passive transformations of the tongue tips modulated by the interaction with the bill tips (Rico-

Guevara and Rubega 2011; Rico-Guevara *et al.* 2015), it is not enough to understand its morphology but also its functioning replicating such passive conditions.

The aims of this paper are 1) to provide a description of the coupling of the components of the feeding apparatus in hummingbirds –namely the bill-tongue three-dimensional fit, 2) to describe the tissue architecture and surfaces of the tongue tip, 3) to characterize and contextualize the gross and detailed morphology of the hummingbird feeding apparatus both in a comparative (among birds) and ecologically relevant (biomechanics) framework, and 4) to perform experiments ~~able~~ to reveal ~~to which~~ extent the feeding structures can passively transform to contribute in the nectar collection process (*i.e. post-mortem* experiments). I used histology, transmission and scanning electron microscopy, and high-resolution X-ray computed tomography (microCT) to describe larger anatomical features and the three-dimensional arrangement of the tongue inside the bill (Fig. 1, Video S1). There have been very few studies, like the one presented here, that merged microCT, light, and electron microscopy in order to examine morphological features by linking them across disparate spatial scales (Handsuh *et al.* 2013; Jung *et al.* 2016).

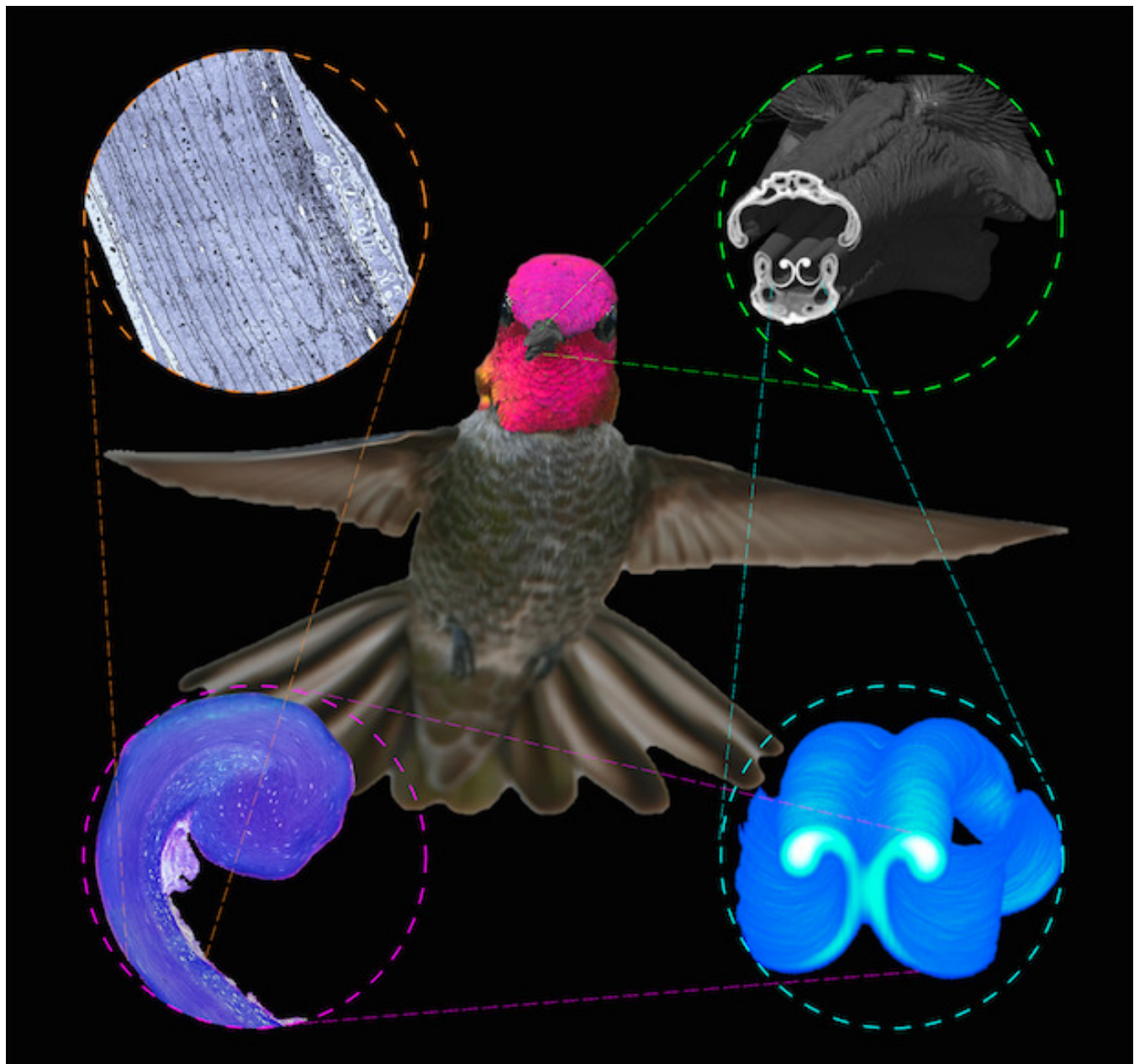


Figure 1. Depiction of the techniques used to study the hummingbird feeding apparatus. In the center, a photograph of an Anna's Hummingbird hovering (courtesy of Robert McQuade). Inside the upper right circle (green), a microCT scan coronal cutaway section portraying both the bill and tongue. Inside the lower right circle (blue), a microCT scan rendering portraying a section of the tongue. Inside the lower left circle (purple), a light microscopy photograph portraying a section of the tongue with the supporting rod at the top. And inside the upper left circle (orange), an electron microscopy photograph portraying a section of the tongue wall tissue to show its architecture.

Materials & Methods

I dissected five Ruby-throated Hummingbirds (*Archilochus colubris* Linnaeus, 1758), one Rufous Hummingbird (*Selasphorus rufus* Gmelin, 1788), one Anna's Hummingbird (*Calypte anna* Lesson, 1829), one Short-tailed Woodstar (*Myrmia micrura* Gould, 1854), one White-necked Jacobin (*Florisuga mellivora* Linnaeus, 1758), and one White-tipped Sicklebill (*Eutoxeres aquila* Bourcier, 1847). For a total of ten specimens from six hummingbird species, which were received as donations (e.g. dying birds that could not be rehabilitated) for the ornithological collections at the Department of Ecology and Evolutionary Biology of the University of Connecticut and at the Instituto de Ciencias Naturales of the National University of Colombia, between January 2012 and August 2013 and coming from several locations in the US, Colombia, and Ecuador. I only dissected (and processed as described below) recently deceased specimens ensuring that the tissues were fresh at the moment of each sample preparation. Once the investigation was concluded, the specimens were deposited in the freezer of the research laboratories at both universities (given the restrictions of the specimen preparations, see below) and are waiting for accession numbers and the development of specific collections for this kind of subjects. Electron microscopy specimens were deposited at the Bioscience Electron Microscopy Laboratory at the University of Connecticut. All activities in this study were reviewed and authorized by the Institutional Animal Care and Use Committee at the University of Connecticut; Institutional Animal Care and Use Committee Exemption Number E09-010. The anatomical nomenclature follows *Nomina Anatomica Avium* (Baumel *et al.* 1993).

High-resolution X-ray computed tomography (microCT)

I dissected three salvaged specimens, a Ruby-throated Hummingbird, an Anna's Hummingbird, and a Short-tailed Woodstar to scan their heads. Such dissections consisted in separating the head of the specimen from the rest of the body, which allowed a more expedited and low-cost staining procedure (see below) and a better positioning of the specimens for the scanning process (closer to the X-ray source to achieve higher resolution). In order to obtain detailed morphological data at the micrometric scale and visualize the tongue soft tissues, I employed a staining protocol with osmium tetroxide (OsO₄, *cf.* Metscher 2009) with the difference that I did not embedded my samples in resin, but instead placed them in small vials that could be positioned as close to the X-ray emitter as required for the desired resolution. I opted for osmium instead of iodine (*e.g.* Lautenschlager *et al.* 2014) because, although they both seem to bind to lipids (Bozzola and Russell 1999; Gignac and Kley 2014), osmium stabilizes tissue proteins, which then do not coagulate during dehydration with alcohol (Hayat 2000).

The heads were kept in 10% neutral buffered formalin and fixed with a solution containing 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) formaldehyde in 0.1 M sodium cacodylate trihydrate buffer (pH 7.4 adjusted with NaOH) for 8 h at 4°C. After two washes in distilled water, the heads were fixed/stained with 2% (wt/vol) OsO₄ in 0.1 M cacodylate buffer water for 4 h at 4°C. Samples were washed three times in distilled water (20 minutes apart at 4°C) and then dehydrated in a graded series of ethanol solutions. The specimens were stored in 100% ethanol at 4°C and scanned at The University of Texas High-Resolution X-ray Computed Tomography Facility. Scans were performed at 70 kV and 10W, with Xradia 0.5 and 4X objectives, and 1 mm SiO₂, or no filter. Specimens were scanned in three parts, scans were

stitched using Xradia plugins, and voxel size was between 15.5 and 5.2 μm . I obtained 16bit TIFF images that were reconstructed by Xradia Reconstructor, and the total number of slices per specimen was between 2223 and 2854, with scan times between 4 and 7 hours.

Histological preparations

I dissected two Ruby-throated Hummingbirds to extract their tongues, which were cut into ~3-mm long sections and fixed with 1.5% (wt/vol) glutaraldehyde - 1.5% (wt/vol) paraformaldehyde in standard buffer (0.1 M HEPES, 80 mM NaCl, 3 mM MgCl_2 , pH 7.4 adjusted with NaOH) for a total of 9h at 4°C with one change into fresh fixative after one hour. The sections were then fixed in a solution of 1% OsO_4 – 0.8% potassium ferricyanide – 0.1 M sodium cacodylate – 0.375 M NaCl for 2 h at 4°C and then washed in distilled water. The sections were dehydrated in a graded series of ethanol solutions, and embedded in epoxy resin (a mixture of Embed812, Araldite 502 and DDSA, blocks polymerized at 60°C for 48 hours). I obtained semi-thin cross sections (1 μm) that were stained with methylene blue/azure II (1:1) followed by counterstaining with fuchsin for light microscopy. Photomicrographs were captured using a JVC High Resolution CCTV digital camera on an Olympus BX51 compound microscope at different magnifications (up to 1,000x). I used Auto-Montage software (Syncroscopy Inc.) to compile images of multiple optical planes, thereby obtaining pseudo-planar fields of view with improved visualization of the tissue structures.

Transmission electron microscopy (TEM)

I used one Ruby-throated Hummingbird for TEM. Using some of the fixed and embedded sections (epoxy resin processed in a Microwave Tissue Processor, Pelco Biowave Pro) of the

tongue from the histological preparations, I obtained thin (80-nm) cross sections using a diamond knife on a Leica Ultracut UCT Ultramicrotome. The sections were put on Formvar support films for TEM and stained with either 2% uranyl acetate (UA) and lead citrate (LC, Reynolds, 1963), UA LC and RuO₄ vapors, or RuO₄ vapors only (Xue *et al.*, 1989). These sections were then imaged at the Bioscience Electron Microscopy Laboratory at the University of Connecticut, with a FEI Tecnai G2 Spirit BioTWIN transmission electron microscope at an accelerating voltage of 80 kV and at direct magnifications up to 120,000x.

Scanning electron microscopy (SEM)

I dissected two specimens, one Ruby-throated Hummingbird and one Rufous Hummingbird to extract their tongues. The tongues were flattened with microslides, and fixed with a solution containing 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M sodium cacodylate trihydrate buffer (pH 7.4 adjusted with NaOH) for 8 h at 4°C. After six washes (30 minutes apart) with the 0.1 M cacodylate buffer, the tongues were fixed/stained with 2% (wt/vol) OsO₄ (2.5 ml) in 0.1 M cacodylate buffer (1.7 ml) + distilled water (0.8 ml) for 8 h at 4°C. The tongues were cleaned by washing them three times in the cacodylate buffer and then dehydrated in a graded series of ethanol solutions. For all of these washes I used jets of fluid (using droppers immersed in the liquids) to ensure that the tongues were free of debris (and remaining nectar) in both dorsal and ventral surfaces; I did not scrap the tongue surfaces in order to keep them intact for posterior visualization. The first tongue was dried with a critical point dryer (Polaron E3000) for 2 h. Unfortunately, critical point drying (CPD) caused the edges of the tongue in the rostral region (where it forms the grooves) to spiral inward while drying, and only a small proportion of the dorsal surface of the tongue was visible after CPD. For the second

tongue, I opted to use nylon mesh biopsy capsules and tissue cassettes to keep the tissue from spiraling inward. I inserted the tissue between layers of filter paper (chemically stable and allows adequate fluid exchange) to prevent mechanical damage from the mesh. Using the SEM, I could visualize and photograph the regions of interest, including equal access to both dorsal and ventral surfaces.

After CPD, I sputter coated (Polaron E5100) the tongues with gold and palladium, and attached them to aluminum SEM stubs using double-sided carbon tape, coated the caudal ends of the tongues with silver paint, and connected them to the aluminum stubs in order to reduce charging effects. I imaged the tongues at the Bioscience Electron Microscopy Laboratory at the University of Connecticut, with a Zeiss DSM982 field emission scanning electron microscope operated at an accelerating voltage of 2 kV and at direct magnifications up to 50,000x.

Ex-vivo experiments

I dissected one Ruby-throated Hummingbird to examine tongue-nectar interactions *post-mortem*. Under an Olympus SZX-12 dissecting microscope, I attached a Micro-Manipulator Model FX-117 (Electron Microscopy Sciences®) *via* surgical micro clamps to the epibranchial bones of the hyobranchial apparatus (Fig. S2). I held the skull in place with articulating arms coupled to a soft “helmet” made out of a polyvinyl chloride sheet and an Irwin® Quick-Grip Mini Handi-Clamp with swiveling clamping pads provided with longitudinal and transversal furrows that matched the hummingbird’s bill basal diameter without compressing it. At the tip of the bill I positioned a Mitutoyo® Digimatic Digital Caliper connected to a laptop to compare the compression of the tongue by the bill tip in this artificial setting and match it with previous

estimates in living hummingbirds (Rico-Guevara *et al.* 2015). The end result was our ability to precisely control tongue flattening and protrusion (Video S2). I attached a second Micro-Manipulator to a reservoir filled with artificial nectar (18.6% sucrose concentration) in order to control the bill tip to nectar surface distance without moving the fixed head. Lastly, we filmed the tongue-nectar interactions by coupling a high-speed camera (TroubleShooter HR), running up to 1260 frames/s (1280 x 512 pixels), to the dissecting microscope.

Activities were reviewed and authorized by the Institutional Animal Care and Use Committee at the University of Connecticut; Exemption Number E13-001.

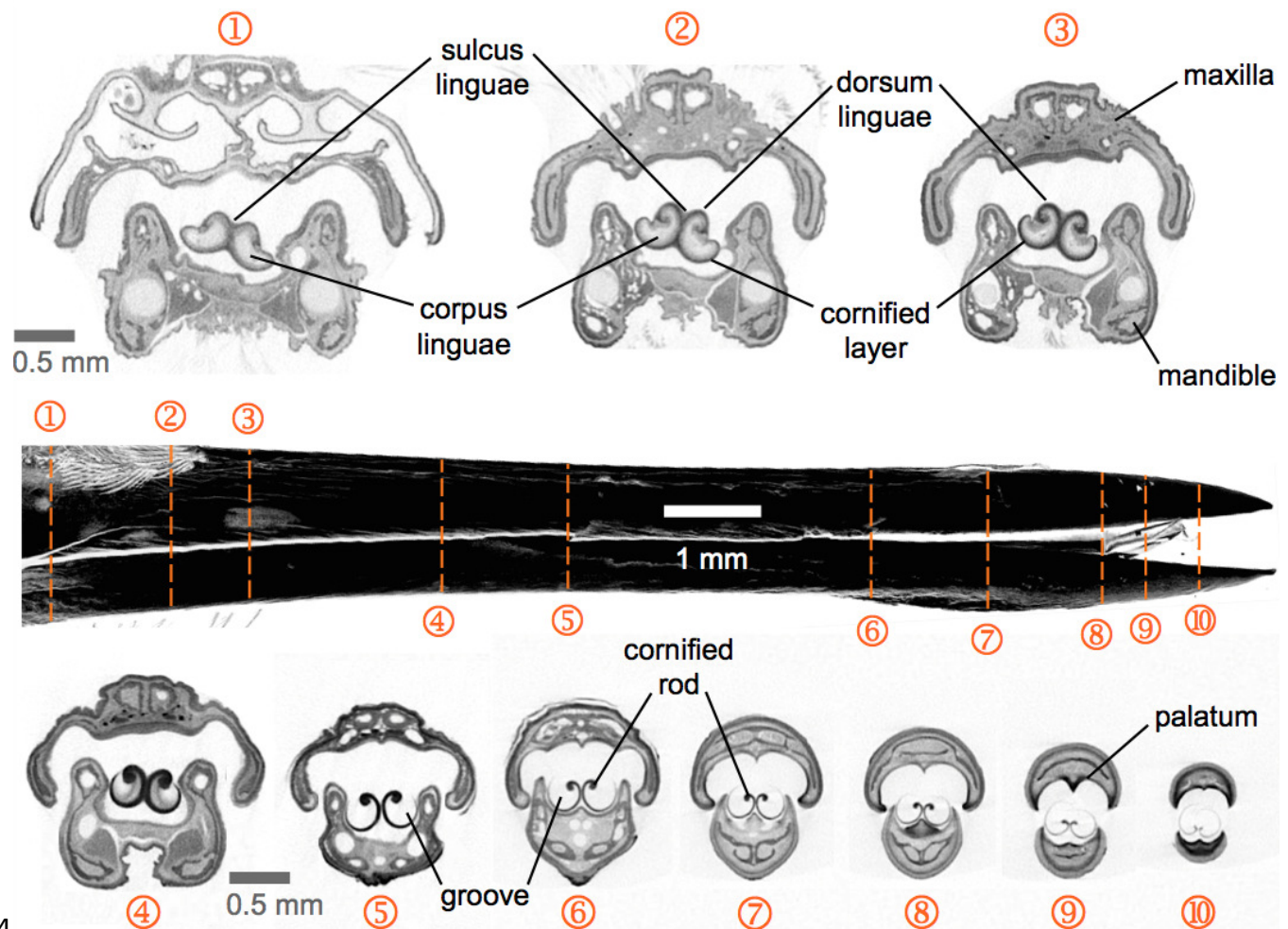
Results

High-resolution X-ray computed tomography (microCT)

I present the first complete cross-section series of a hummingbird feeding apparatus. I started with the most caudal section at the nasal operculum (Fig. 2, cross section [XS] 1) where the tongue is dorso-ventrally flattened, and the tongue body (*corpus linguae*) has started to divide medially due to an ingrowth (*sulcus linguae*) of the dorsal and ventral epithelia (Fig. 2, XS 1; *cf.* XS 11 in Weymouth *et al.* 1964). The tongue body in hummingbirds encompasses the tongue from a distinct base at the joint between the *basihyale* and the *paraglossum*, and until the rostral grooves. I do not present a description of the structure of the lingual body in this paper given that this has been detailed previously by Weymouth and collaborators (1964). At XS 2 there is a layer of cornified tissue (dark layer) almost completely surrounding the lingual body. Such layer becomes thicker at the ingrowth region and eventually connects, when moving rostrally through cross sections (Fig. 2, XS 2-5), effectively dividing the tongue body (*cf.* XS 13

270 in Weymouth *et al.* 1964) and giving rise to a bifid tongue. At XS 3 the semi-cylindrical
 271 configuration characteristic of the tongue grooves is already conspicuous (*cf.* XS 14 in
 272 Weymouth *et al.* 1964).

273



274

275

276 **Figure 2. Selected feeding apparatus cross sections (1-10) from a microCT scan of an**
 277 **Anna's Hummingbird.** Black structure in the middle of the figure is a lateral view of the bill
 278 from the reconstructed scan, and the dashed orange lines crossing it correspond to the numbered
 279 cross sections. Upper and lower bills (rhinotheca and gnathotheca are the keratinous sheaths of
 280 the maxillary and mandibular bones respectively) on each section appear separated but in a living
 281 hummingbird they can be fully coupled when the bill is shut, leaving virtually no space outside
 282 the tongue grooves in the rostral region. Relevant structures for understanding the feeding
 283 apparatus functioning are labeled (see text).

285

286 At XS 4 it is apparent that the tissue inside the lingual body chambers is thinner, leaving
 287 an empty space dorso-laterally (*cf.* XS 15-17 in Weymouth *et al.* 1964). At this section, the
 288 *dorsum linguae* is made of cornified tissue and it forms a pair of dorsal cornified rods of the
 289 lingual tip (*cf.* Weymouth *et al.* 1964). These dorsal rods become thicker and more robust when
 290 moving rostrally through cross sections (Fig. 2, XS 2-5), probably because they are the sole
 291 structural support of the rostral half of the tongue. By XS 5 there is no tissue inside the cornified
 292 semi-cylindrical grooves, and the two sides of the lingual body are completely separated (*i.e.*
 293 bifurcated tongue). There is almost no change between the tongue appearance and size between
 294 XS 5 and 6, which is about 3 mm corresponding to about half of the total groove length. From
 295 XS 6 to 8 there is no ostensible change in the tongue shape besides an overall reduction in size (~
 296 25%). The rostral portion of the tongue is characterized by a reduction of the rods and a thinning
 297 in the cornified tissue comprising the grooves (Fig. 2, XS 9-10). It is worth noting that from XS
 298 1 to 4 it is evident how the tongue fills the internal buccal spaces (when the bill is shut), leaving
 299 only a small space dorso-laterally. Such space matches the position of tongue base projections
 300 (Scharnke 1931; XS 2 in Weymouth *et al.* 1964). A reduction in the internal space outside the
 301 grooves and a tighter coupling between bill internal walls (oropharyngeal roof, or *palatum*, and
 302 oropharyngeal floor, or interramal region) and tongue shape is evident in the rostral portion of
 303 the feeding apparatus (Fig. 2, XS 5-10). A more in-depth description of the bill structures, such
 304 as the salivary ducts openings in the oropharyngeal floor (Fig. 2, XS 7), will be provided
 305 elsewhere.

306

308 *Histology and Electron Microscopy*

309 I focused on the rostral half of the tongue to complement the work of Weymouth *et al.*
 310 (1964) that focused on the caudal half. At its basal region, the tongue is a cylindrical structure
 311 containing bones, muscles, vessels, nerves, ~~etc.~~ all surrounded by stratified squamous epithelium
 312 (Weymouth *et al.* 1964). Moving rostrally, the tongue shape transitions into two distinct bean-
 313 shaped chambers running parallel to each other (Fig. 2, XS 1; Weymouth *et al.* 1964), the paired
 314 *paraglossum* becomes cartilaginous and thins until finally disappear, along with the muscles,
 315 vessels, nerves, ~~etc.~~, while the stratified squamous epithelium becomes thicker and a strongly
 316 cornified layer appears in between two layers of epithelium (analogous to the human nail matrix
 317 covered by the cuticle, Fig. 2, XS 2-3; Weymouth *et al.* 1964). In the rostral half of the tongue
 318 (Fig. 3A) all the connective tissue has disappeared, the bean-shaped chambers become hollow,
 319 and the remaining cornified epithelium (*stratum corneum*) is shaped like two extended ‘commas’
 320 mirroring each other and forming the paired grooves or semi-cylinders at the tongue tip (Figs. 2,
 321 XS 4-10, 3A; Weymouth *et al.* 1964; Ortiz-Crespo 2003).

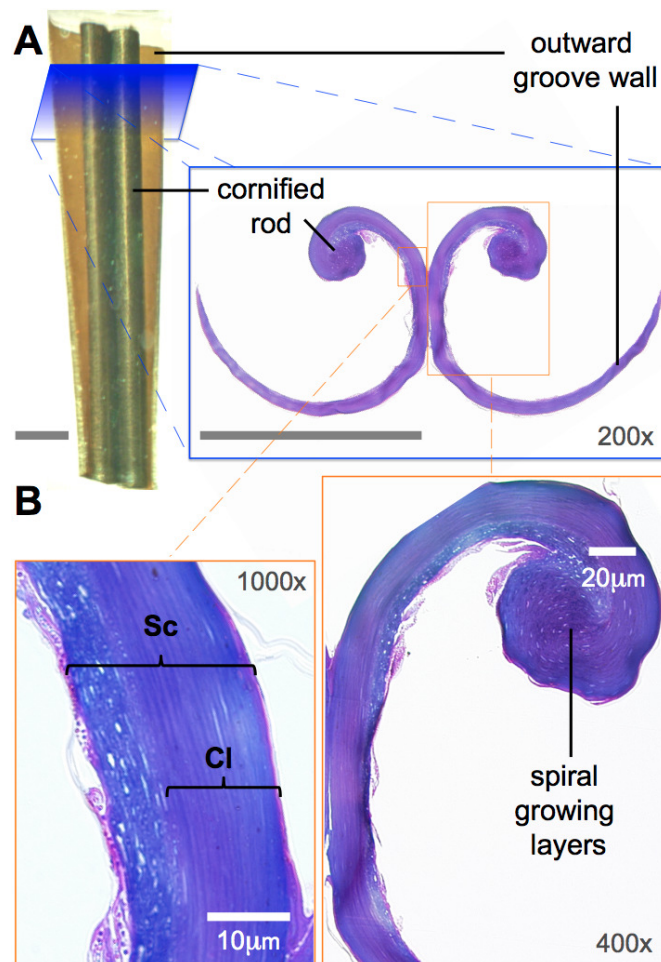


Figure 3. Low-magnification morphology of the rostral half (grooves) of a Ruby-throated Hummingbird tongue. (A) On the left, a section of the tongue embedded in resin; dorsal view oriented with the caudal end of the section at the top. On the right, a corresponding cross section (light microscope) showing the semi-cylindrical configuration of the grooves. The cornified rod of the lingual tip and the outward (lateral) groove wall are labeled for reference. Unlabeled scale bars = 250 μm . (B) Histological details of the groove wall (*left*), and the cornified rod (*right*), showing the *stratum corneum* (Sc), the strongly cornified layer (Cl), and the seemingly germinative layers remains at the dorsal rod.

I found elliptical-to-circular dark corpuscles distributed more evenly throughout the tongue tissue (black arrow head, Fig. 4A), which possibly are melanin granules (*e.g.* Dummet and Barends 1974). The cell boundaries are continuous lines of corneo-desmosomes (*e.g.* black arrow, Fig. 4B). I found structures of $\sim 35 \text{ \AA}$ diameter that possibly are microfibrils (*e.g.* white arrow, Fig. 4C); the ventral layers of cornified tissue are more similar to those found in feathers

(β -keratin) than to that of tissues with α -keratin (*cf.* Filshie and Rogers 1962). Specifically, the diameter of the putative microfibrils is within the range of other β -keratin tissue microarchitectures (Parakkal and Alexander 1972, p. 33), and almost a third of the diameter of α -keratin microfibrils (Filshie and Rogers 1962; Johnson and Sikorski 1965). Regarding the different staining methods, I found that staining with uranyl acetate and lead citrate provided the best imaging of the elliptical dark corpuscles and the most external layers of keratin, especially in the dorsal surface of the grooves (Fig. S1). However, vapor-staining with RuO_4 offered the best visualization of the corneo-desmosomes necessary to study the cell architecture (Fig. S1).

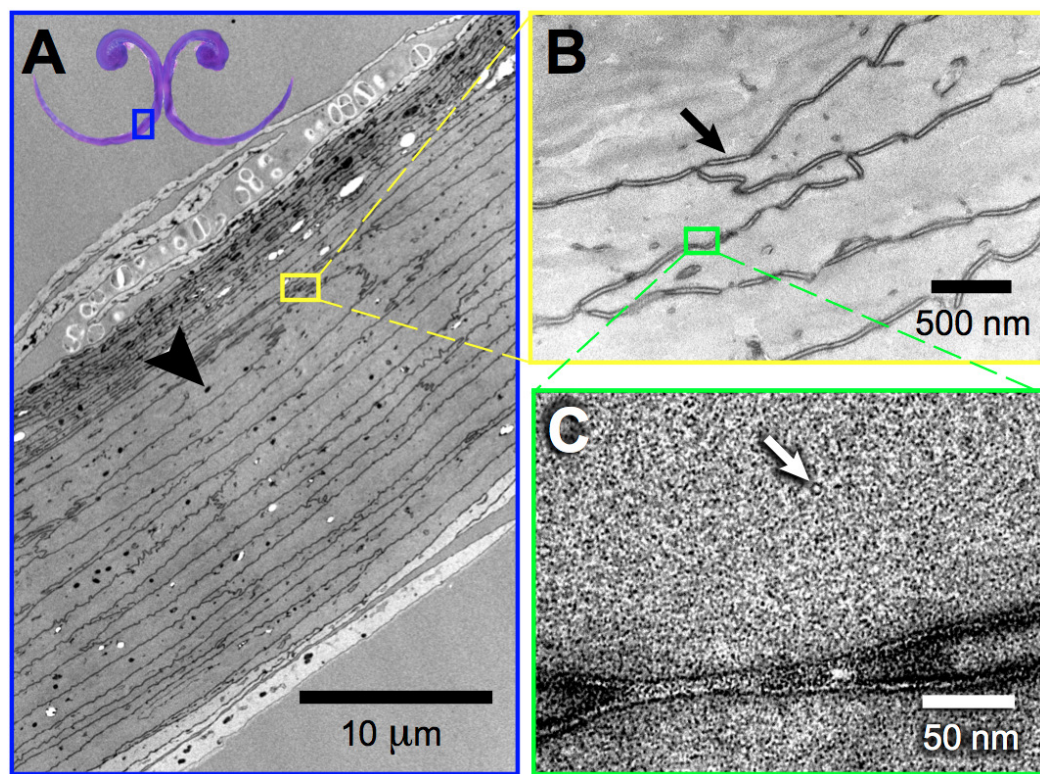


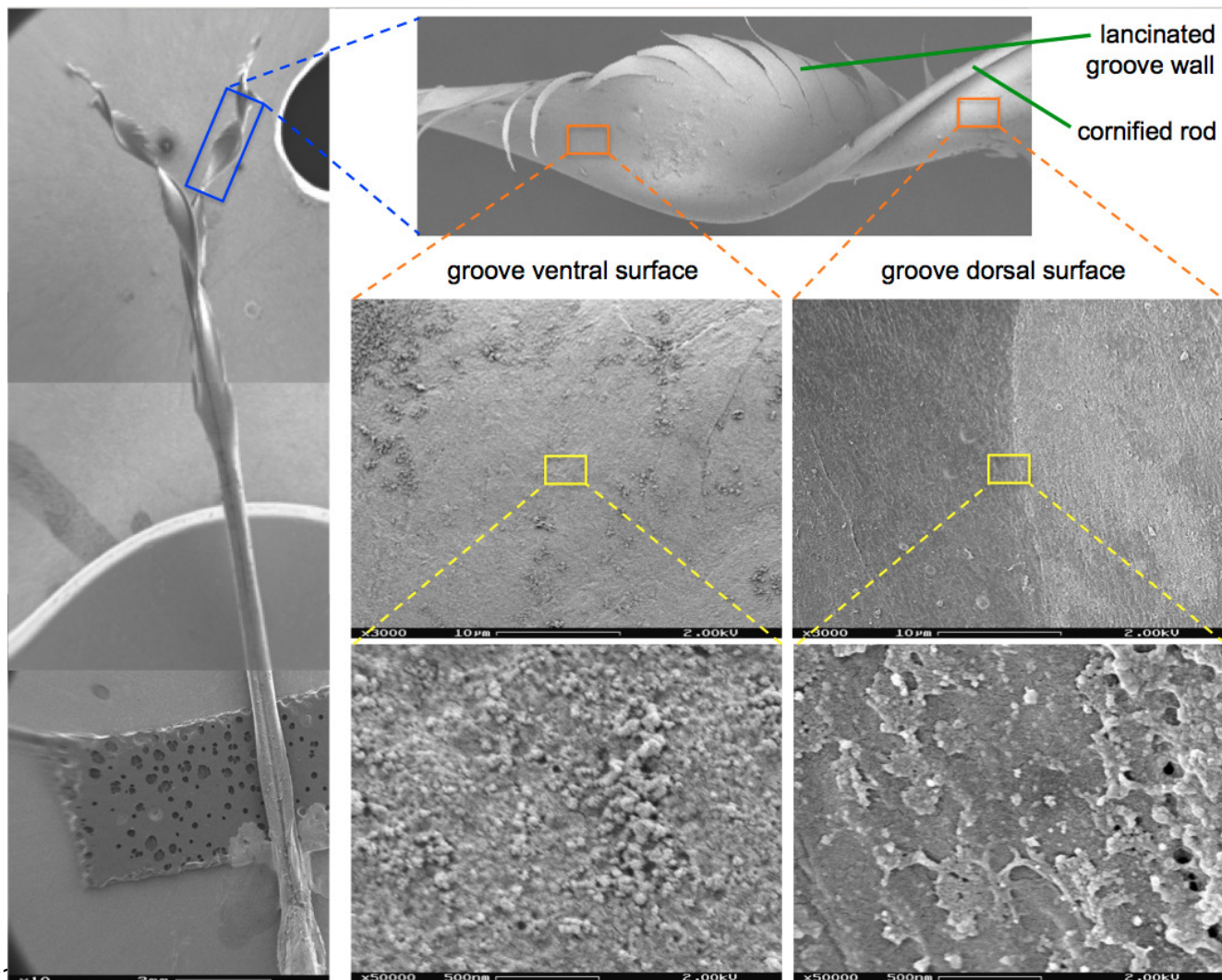
Figure 4. High-magnification morphology of a cross section at the rostral half (grooves) of a Ruby-throated Hummingbird tongue. (A) Transmission electron micrograph showing the difference in layer composition (more densely packed near the dorsal surface), and potential melanin (black arrow head) granules. Vapor-stained with RuO_4 . (B) The cellular outlines are connected corneo-desmosomes (black arrow). Stained with uranyl acetate (UA), lead citrate (LC), and RuO_4 (vapors). (C) Keratinous matrix showing the microfibrils (white arrow). Stained with UA, LC, and RuO_4 .

In the grooved (rostral) half of the tongue, two layers of the *stratum corneum* can be distinguished: a thicker one underlying the ventral (convex) surface of the grooves, which I refer to as ‘cornified layer’, and a thinner one underlying the dorsal (concave) surface of the grooves (Fig. 3B). The cornified layer is made of larger cells, it is less densely packed, and it contains less granules than the layer closer to the dorsal surface (Fig. 4A). This latter layer may contain some flattened granular-cornified cells but I do not refer to it as *stratum granulosum* since that name is mostly applied in mammal tissues (Baumel *et al.* 1993). It is plausible that some of the germinative layers of this keratinized stratified squamous epithelium could be found at the basal portions of the dorsal rods (Fig. 3B), but most of it is restricted to the caudal half of the tongue (Weymouth *et al.* 1964).

Probably related to the abovementioned differences in underlying tissue, I found qualitative differences between the dorsal and ventral surfaces of the tongue grooves (Fig. 5). These surfaces were cleaned in the same manner (see Methods: SEM), therefore differential accumulation of nectar or dirt residue does not appear to be a confounding factor. At the 10- μ m scale the ventral tongue groove surface seems to have more desquamated regions in comparison with dorsal side, which appears smoother. Furthermore, at the 500-nm scale the ventral surface presented a rougher aspect than the dorsal surface (Fig. 5, *bottom right*). Given that the accelerating voltage can alter the level of surface detail visualized I kept constant 2 kV for all the comparisons. To conclude that there are significant differences between dorsal and ventral surfaces of the hummingbird tongue, it would be necessary to quantify differences in roughness; the best way to do this is by using Atomic Force Microscopy (*e.g.* Ghosh *et al.* 2013). Alternative techniques (*e.g.* Nanda *et al.* 1998; Fujii 2011; Kremer *et al.* 2015) include

the use of optical interferometry (*e.g.* white light scanner), and 3-D reconstructions of tilted SEM micrographs (stereomicroscopy).

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382

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Figure 5. Scanning electron microscopy of a Rufous Hummingbird tongue. On the left, an overview of the entire tongue, although my observations focused on the rostral half (grooves). On the top right, a close up of a longitudinally twisted section of a tongue groove, indicating the cornified rod of the lingual tip and the lacerations of the groove wall. On the middle and bottom right, micrographs of the ventral and dorsal surfaces of the tongue grooves (as indicated by the zooming squares), showing qualitative differences in rugosity.

389

391

392 Ex-vivo *experiments*

393 I recorded expansive filling (*sensu* Rico-Guevara *et al.* 2015) in the *post-mortem*
 394 experiments (Fig. S2, Video S2). This observation indicates that physical (structural) rather than
 395 muscular forces are responsible for the expansion and filling of the tongue. I flattened the
 396 grooves by closing the bill tips and leaving only a small aperture to extrude the tongue through
 397 (see methods), reproducing our previous observations in free-living birds (Rico-Guevara and
 398 Rubega 2011; Rico-Guevara *et al.* 2015), and registered that the flattened grooves expanded
 399 spontaneously upon contact with nectar in tongues of deceased specimens (Video S3).
 400 Additionally, I observed that the separation of the tips and the relaxation of the fringed regions
 401 occurred in *post-mortem* experiments (Video S4). Consequently, nectar trapping (*sensu* Rico-
 402 Guevara and Rubega 2011) would be the first step of the fluid collecting system and is
 403 immediately followed by expansive filling. I hypothesize that the main force driving the
 404 expansive process and therefore the filling of the tongue with nectar is the elastic energy that can
 405 be stored in the cornified groove walls.

406

407 I explain the hypothesis as follows: 1) The process starts when the tongue is dorso-
 408 ventrally compressed upon protrusion; when the tongue is extruded, only a thin layer of nectar
 409 remains inside the grooves. Such thin layer acts as an adhesive (Stephan adhesion) maintaining
 410 the dorsoventrally flattened (elliptical) configuration of the grooves even after they pass the
 411 extrusion point (bill tip). The attractive forces between the nectar and the tongue (adhesion,
 412 cohesion and surface tension) are able to resist the elastic energy stored in the grooves' walls
 413 (cornified layers), and thus keep the grooves flattened. This stable flattened configuration is

414 conserved during the trip of the tongue across the air space between the bill tip to the nectar pool.
 415 In the dorsal portion of the tongue, where the groove's inside upper edge meets the rod, the free
 416 (outer) edge of the groove is prevented from rolling outward by a narrow sheet of nectar joining
 417 it to the rod. The surface tension at this exposed nectar sheet keeps the grooves "zipped up" by
 418 preventing air from entering the groove itself. Surface tension at the tip of the tongue also keeps
 419 the grooves stuck to each other, forming a unitary structure. 2) Once the tongue passes the
 420 compression point at the bill tips, there is a slight expansion in the tongue grooves (because of
 421 the cessation of compressive forces). The expansion of the grooves is arrested at the point in
 422 which the attractive forces between the tongue walls and the nectar balance out the elastic forces
 423 of the grooves walls. This creates an initial transient equilibrium that maintains the flattened
 424 configuration (*cf.* Rico-Guevara *et al.* 2015). 3) Once the tongue tip contacts the nectar surface,
 425 the free supply of fluid eliminates the surface tension that was holding the grooves together,
 426 allowing the area of the grooves that is inside the nectar to open (*cf.* Rico-Guevara and Rubega
 427 2011). This opening of the ends of the grooves allows the nectar molecules from the nectar pool
 428 to start interacting with the nectar molecules inside the grooves (*i.e.* elasticity-induced flow, Fig.
 429 6). On the dorsal surface of the length of the grooves still outside the nectar pool (more proximal
 430 to the bird's mouth), the surface tension of the fluid sheet between the rods and the groove walls
 431 holds the grooves in the rolled, flattened position. 4) Molecules of liquid entering the tongue
 432 grooves at the boundary where the tongue enters the nectar pool start moving proximally through
 433 the grooves, creating a jet of fluid that fills the grooves following their expansion (*cf.* Rico-
 434 Guevara *et al.* 2015). This continued destabilization of the initial transient equilibrium causes the
 435 area of the grooves outside the nectar to expand which in turn causes them to fill, creating a
 436 positive feedback that forces the grooves open along their entire length. This creates a filling

front wave, because the expansive process happens from the point of contact with the nectar backwards (Fig. 6). 5) The expansion stops when most of the potential elastic energy is released (and the grooves are fully reshaped into their cylindrical configuration) and when the remaining elastic energy is counteracted by the surface tension at the zipped dorsal slit (*cf.* Rico-Guevara and Rubega 2011). At this point the grooves have achieved their maximum capacity, and they are completely filled with nectar.

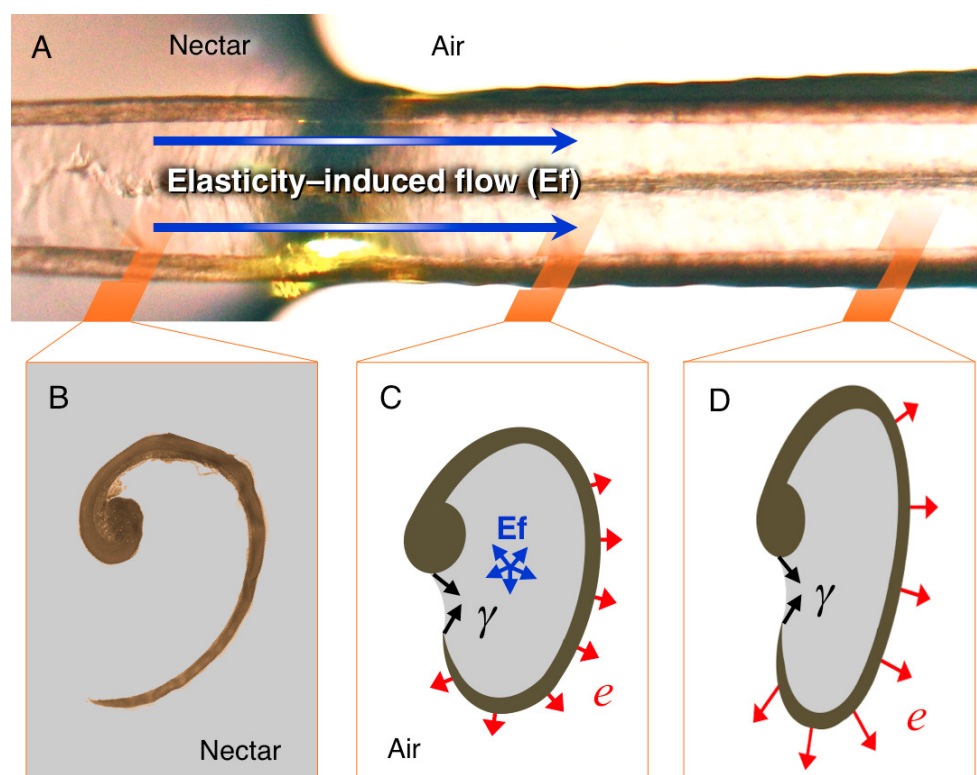


Figure 6. Elasticity-induced flow hypothesis. (A) Dorsal photograph of a hummingbird tongue tip just after contacting the nectar surface. Given the flattened configuration of the grooves on the right, there would be elastic energy stored which induces inward flow. (B) Cross section (light microscope photograph) of a hummingbird tongue in its “relaxed” configuration inside the nectar. (C) Hypothetical cross section showing the elasticity-induced flow (E_f in blue), the surface tension (γ in black), and the elastic potential energy (e in red). (D) Hypothetical cross section for a portion of the tongue not yet affected by the expansive flow. Strong nectar-wall adhesion keeps the groove in a flattened configuration, and surface tension along the groove slit prevents bubble infiltration. Elastic potential energy is larger when the bending of the wall is more pronounced; yielding a pressure differential that pumps the nectar into each groove.

455

456 Discussion

457 *Gross morphology of hummingbird tongues*

458 Hummingbird tongues entirely lack papillae, a rare condition in vertebrate tongues
 459 (Schwenk 2000; Iwasaki 2002) and even among birds (review in Erdoğan and Iwasaki 2014).
 460 Avian lingual papillae are involved in manipulation of solid food (*e.g.* prey apprehension,
 461 holding, cutting, filtering, shelling, Iwasaki *et al.* 1997; Kobayashi *et al.* 1998; Jackowiak *et al.*
 462 2010; 2011; Guimarães *et al.* 2104; Skieresz-Szewczyk and Jackowiak 2014) and caudal
 463 intraoral transport of solid items (review in Parchami *et al.* 2010). Hummingbirds have
 464 remarkable feeding modes; first, about half of their diet (*cf.* Stiles 1995) is composed of floral
 465 nectar that is collected inside the tongue grooves; this process does not involve adhesion of the
 466 liquid to intra-papillar spaces, as in the case of bats (Birt *et al.* 1997; Harper *et al.* 2013) or
 467 lorikeets (Homberger 1980, p. 41). Second, the other half of their diet (*cf.* Stiles 1995) consists of
 468 arthropods, which most hummingbirds capture by flycatching (Stiles 1995; Rico-Guevara 2008).
 469 Yanega and Rubega (2004) showed that the flycatching mechanism in hummingbirds involves an
 470 expansion of the gape (see also Smith *et al.* 2011) and most of the aerial prey are captured at the
 471 base rather than at the tip of the bill; therefore, little or no intraoral lingual transport is necessary.
 472 Other hummingbirds, especially from subfamily Phaethornithinae ('hermits'), consume mostly
 473 substrate-captured prey (*e.g.* spiders, Stiles 1995). This is also the case of reproductive females
 474 of many species across the entire family, which have higher protein requirements (Rico-Guevara
 475 2008; Hardesty 2009). In the process of consuming substrate prey or prey that are generally
 476 captured near the bill tip, hummingbirds, as other birds, can use inertial transport (*cf.* Mobbs
 477 1979; catch and throw, Zweers *et al.* 1997; or cranioinertial feeding, Tomlinson 2000; Gussekloo

and Bout 2005; also called ballistic transport, Baussart *et al.* 2009; Baussart and Bels 2011; Harte *et al.* 2012) while flying, or lingual transport (Yanega 2007). Hummingbirds have evolved the ability to protract their tongues past the bill tips to feed on nectar, but the purpose of the extreme protrusion that they can achieve (*e.g.* Fig. 7A) is still a mystery. Thus, hummingbirds can reach the rostral portions of their bills with the tongue base (to perform lingual transport for instance), without dragging their tracheae rostrally, because of the development of an accordion-like tube (*tuba elastica*, Zusi 2013) between the epiglottis and the tongue base which can contain a large part of the hyobranchial apparatus during tongue protrusion (*cf.* Weymouth *et al.* 1964; Fig. 7B). This *tuba elastica* appears to be a modification of the fibrous attachment between the rostral process of the cricoid cartilage and the rostral process of the *basihyale* (Soley *et al.* 2015). Hummingbirds' lack of lingual papillae may be explained by their arthropod hunting and consumption strategies, and their liquid food collecting method: Grooves with smooth surfaces are easier to extrude nectar from.

Besides lacking papillae, hummingbird tongues are also unique because of their *alae linguae*, which are flattened projections at the base of the tongue Fig. 7C). These two flaps are located and oriented at the same place and in the same general direction as the papillary crest in other birds. However, these structures do not present caudally directed conical papillae, as it is usual in avian tongues (*e.g.* Erdoğan and Alan 2012; Erdoğan *et al.* 2012b). In comparison to the width of the tongue, these flaps are greatly elongated laterally in Sicklebill hummingbirds (*Eutoxeres*, Fig. 7C), which have strongly decurved bills. These flaps are thin and flexible at touch, as well as positioned dorso-laterally forming a V-shaped structure. These flaps in hummingbirds have no parallel among nectar-feeding birds (Lucas 1894; Scharnke 1932, 1933;

501 Rand 1961, 1967; Bock 1972; Morioka 1992; Pratt 1992; Downs 2004; Chang *et al.* 2013), or
 502 birds in general (*e.g.* Erdoğan and Alan 2012; Erdoğan *et al.* 2012a, b; Erdoğan and Iwasaki
 503 2014; Erdoğan and Pérez 2015). I hypothesize that the *alae linguae* could aid to move the nectar
 504 backwards during its intraoral transport (Rico-Guevara 2014) and to drag proximally arthropod
 505 prey that are caught at different places along the bill length (*cf.* Yanega 2007). In terms of
 506 general shape, hummingbird tongues are not triangular and dorsoventrally flattened as in most
 507 birds (review in Erdoğan and Pérez 2015), instead, as it is the case of other nectarivorous birds,
 508 these tongues are cylindrically shaped (*e.g.* Bock 1972; Downs 2004; Chang *et al.* 2013). Lastly,
 509 I found that hummingbird tongues near the tip also lacked taste buds and salivary glands (found
 510 in other birds, review in Erdoğan *et al.* 2012a), in agreement with previous work by Weymouth
 511 and collaborators (1964).

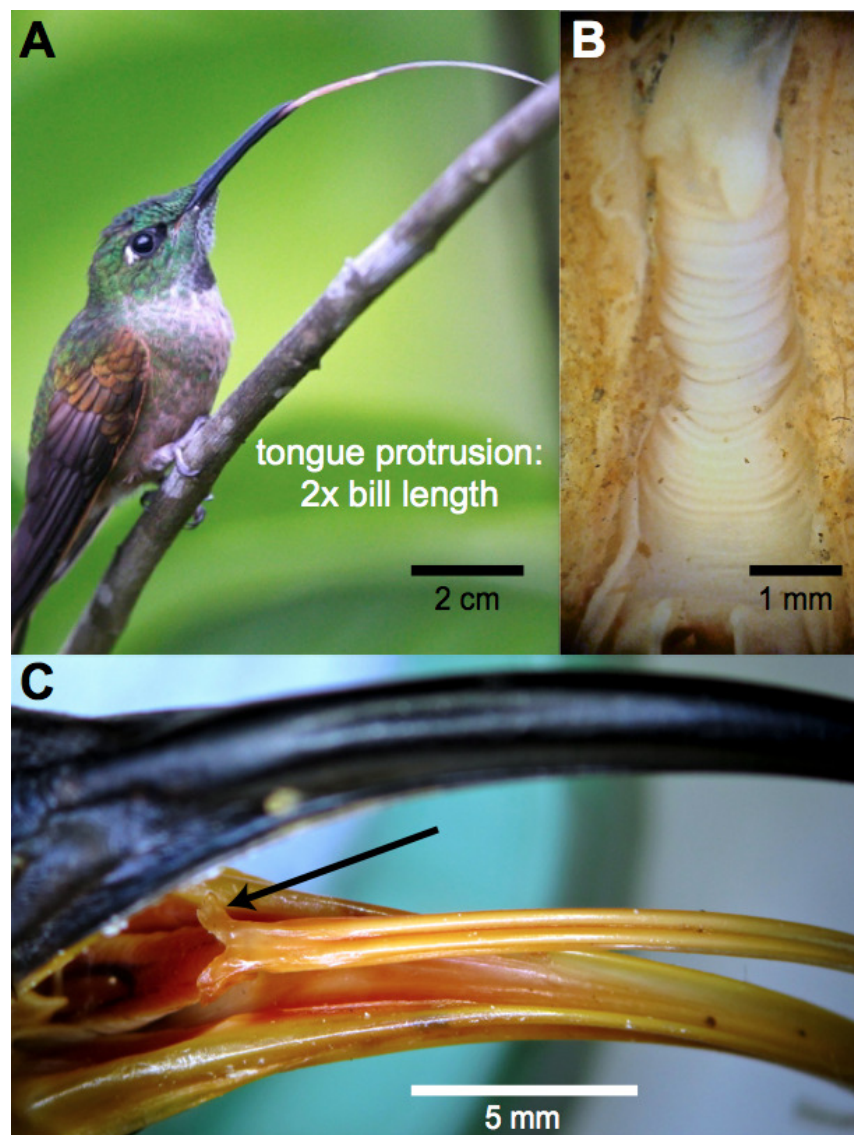


Figure 7. Gross morphology of hummingbird tongues. (A) Photograph of a Fawn-breasted Brilliant (*Heliodoxa rubinoides*) stretching its tongue apparatus (courtesy of Jim DeWitt –Frozen Feather Images). (B) Dissecting microscope photograph of the throat region in a dissected specimen. Featuring a White-necked Jacobin (*Florisuga mellivora*) showing the accordion-like structure or *tuba elastica* in its retrieved position. The *tuba elastica* can contain the basihyal and ceratobranchial bones allowing them to move independently from the rest of the surrounding tissue and permitting the extreme protraction of the tongue. (C) Macro photograph of the bill and tongue-base of a White-tipped Sicklebill (*Eutoxeres aquila*). Note the *alae linguae* at the base of the tongue (black arrow), which are enlarged in comparison to other hummingbirds.

525

526 *Ultrastructural characteristics of hummingbird tongues*

527 The rostral portions of the hummingbird tongue, the ones that collect the food, are mostly
 528 transparent and their tissues are extremely thin (Figs. 2, 8AC), a rare condition in vertebrates. In
 529 most avian tongues the *stratum corneum* at the ventral surface comprises less than 10% of the
 530 lingual tissue in a cross section (Erdoğan *et al.* 2012a; Erdoğan and Iwasaki 2014). Different
 531 from most birds, the cornified ventral layer in hummingbirds accounts for between 50%, near the
 532 cornified rod and near the groove base, and 100%, at the edge of the groove wall and at the
 533 tongue tip, of the tissue in cross sections (Figs. 2, 3A, 8BD, S1). I suggest that most of the
 534 germinative layers of this keratinized stratified squamous epithelium, for which its superficial
 535 layer the *stratum corneum*, disappear before reaching the most rostral portions of the
 536 hummingbird tongue; similar to what would be expected in cross sections of human nail
 537 overhangs. Therefore, the caudal half of the hummingbird tongues is made of dead cornified
 538 tissue that is shaped by the interaction with the bill, and it is constantly replaced from the rostral
 539 half. A thick (cornified) layer of β -keratin can increase mechanical resistance on a surface that is
 540 compressed and scraped by the serrated edges of the bill tip ~ 14 times a second (Ewald and
 541 Williams 1982) and literally tens of thousands of times a day (Rico-Guevara 2014). Future
 542 experiments to test the hypothetical high percentage (50-100%) of β -keratin in the hummingbird
 543 tongue grooves could use *in situ* hybridization, immunolabeling for β -keratins (*e.g.* in Alibardi *et*
 544 *al.* 2009) or selective biodegradation of β -keratin (*e.g.* Lingham-Soliar *et al.* 2010; Lingham-
 545 Soliar and Murugan 2013).

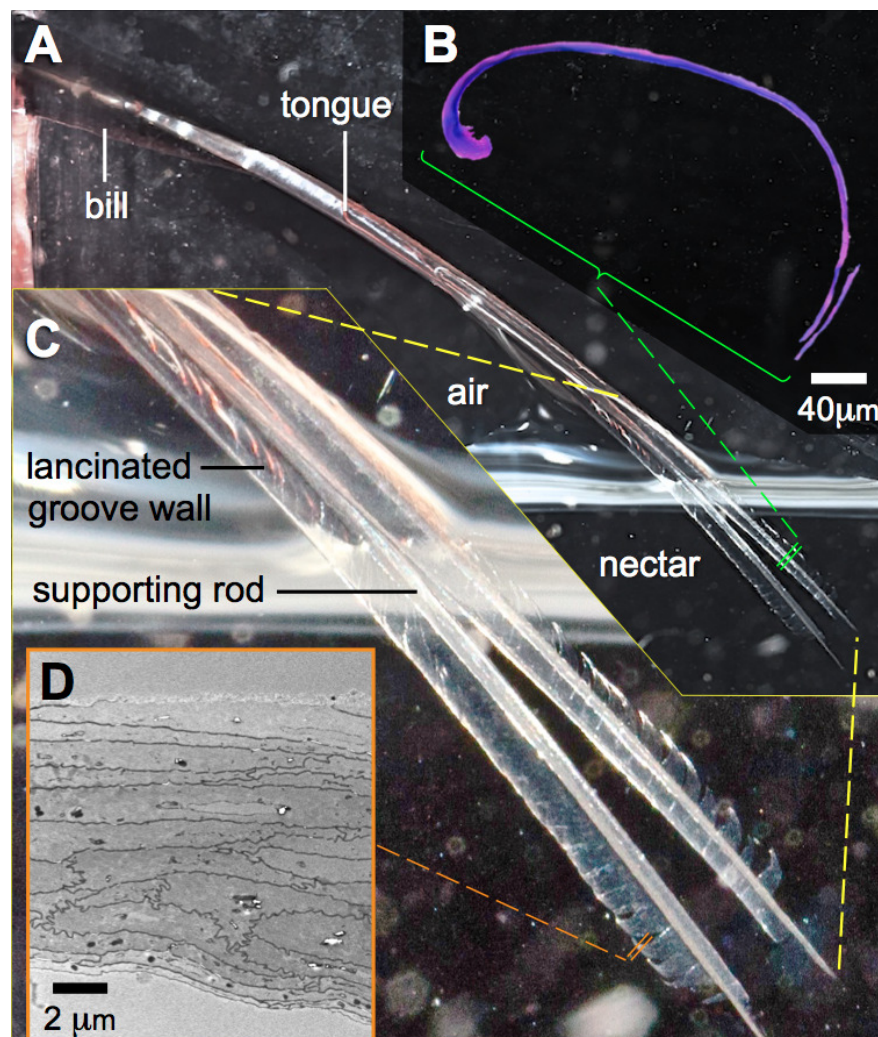


Figure 8. Tongue groove morphology at the most distal portions (near the tip) in a Ruby-throated Hummingbird. (A) Photograph showing the tongue protrusion, its bifurcation, and the relaxed morphology of the grooves inside the nectar (courtesy of Don Carroll). (B) Cross section (light microscope) showing the reduction in cornified rod diameter and the thinning in the *stratum corneum* composing the grooves (which at this point is composed only of the cornified layer). (C) Close up to the tongue tip showing the membranous appearance of the grooves and the presence of diagonal cuts in the tissue (lancinated groove walls). (D) Electron micrograph showing the structure of the cornified layer, note the reduction in the number of cell layers and the absence of delineated boundaries in the dorsal surface (on top).

I found differences between the layers of tissue underlying the dorsal and ventral surfaces of the tongue grooves (Fig. 3B). These differences may be explained by the organization of the tissues (Fig. 4A), but they may also be influenced by differential composition and organization between proteins (fibrous vs. matrix components) and/or the presence of β -keratin (reviewed by Alibardi *et al.* 2009), which has been found in the rostral ventral epithelium of other avian tongues (review in Carver *et al.* 1990). On the ventral surface of the tongue grooves I found thick *stratum corneum* (*cf.* Fig. 4 in Kadhim *et al.* 2013; Figs. 5, 6 in Jackowiak *et al.* 2015), but without the underlying *lamina propria* characteristic of heavily cornified areas in bird tongues (Farner 1960; Kadhim *et al.* 2013). This *stratum corneum* in the tongue surface is common in birds (Farner 1960; Erdoğan *et al.* 2012a; Erdoğan and Iwasaki 2014), however, as opposed to hummingbirds, in several bird species the *stratum corneum* is better developed on the dorsal lingual surface (Iwasaki 2002; Erdoğan *et al.* 2012a). I found more sloughing cell layers in the histology and TEM preparations in the dorsal compared to the ventral surface, which indicates that the ventral surface is underlain by harder keratin (*cf.* Lucas and Stettenheim 1972). Interestingly, my results are consistent with the idea that dorsal and ventral surfaces of hummingbird tongues have different rugosities (Figs. 5, 8D), which may have direct implications for their hydrophobicity, *i.e.* increased roughness may significantly increase contact angle of a water droplet and decrease contact angle hysteresis, which would augment its hydrophobicity (*e.g.* Michael and Bhushan 2007). Therefore, the dorsal tongue groove surface, which is less rugose, may be more hydrophilic than the ventral groove surface, and potentially facilitating the fluid trapping process described by Rico-Guevara and Rubega (2011).

582 *Microanatomy of hummingbird tongues*

583 Using the data from the micro-CT scans I digitally decoupled the feeding apparatus
584 components (segmenting in Avizo®) and constructed three-dimensional models to study the bill
585 and tongue match. Hummingbird tongues, as well as most avian tongues, correspond to the shape
586 of the interramal region (oropharyngeal cavity floor), although commonly not to its size (e.g.
587 Abou-Zaid and Al-Jaloud 2010; Tivane *et al.* 2011; review in Abumandour 2014). Nevertheless,
588 it is worth noting that avian tongues are not larger than the oropharyngeal cavity (as it is the case
589 in some nectarivorous bats, Muchhala 2006), instead, to reach far away from the tip of their bills,
590 some hyoid apparatus had become greatly elongated (e.g. Video S5). In hummingbirds, the
591 tongue grooves fit perfectly the rostral portion of the oropharyngeal cavity and match both lower
592 and upper bill internal walls (Fig. 1), which is of vital importance for the efficient offloading of
593 nectar (*cf.* Rico-Guevara and Rubega 2011) and intraoral transport (Rico-Guevara 2014). My
594 study presents the first high-resolution (5- μ m voxels) CT scan of a vertebrate tongue
595 satisfactorily stained to highlight soft tissue. A study on flamingos presented detailed CT scans
596 of the head (including the tongue) stained with a novel injection technique (Holliday *et al.* 2006),
597 but it focused on vascular anatomy at lower resolution than in the present study. Within the last
598 five years other studies have used a variety of techniques to enhance visualization of soft tissue
599 in vertebrates (reviews in Gignac and Kley 2014; Lautenschlager *et al.* 2014; Gignac *et al.*
600 2016), but they have not been focused on tongues, or worked at the micro scale of the present
601 study. This three-dimensional modeling of hummingbird tongues allows for the clarification of
602 some misconceptions; for instance, it has been suggested that the mathematical model derived
603 for capillary filling provides a rationale for the shape of hummingbird tongues (Kim *et al.* 2012).
604 Specifically, that the semi-cylindrical shape of the grooves (cylinders with a dorsal slit) can be

explained by an optimal opening angle of a cross section, which matches a peak of energy intake rates (Fig. 4 in Kim *et al.* 2012). I prefer a more parsimonious explanation: starting with a dorso-ventrally flattened tongue as an ancestral condition (*cf.* Emura *et al.* 2010; Shah and Aziz 2014), evolution would maximize the nectar-holding capacity by selecting for a cylindrical structure. In the same way in which a sphere is the shape with the lowest surface area to volume ratio, for an elongated structure (like a tongue), a cylindrical configuration achieves the greatest capacity for a given amount of tissue (in this case, the groove walls). It is worth noting that the tongue tip whilst outside the nectar ends in a conical shape (Fig. 1 in Rico-Guevara *et al.* 2015), due to a shortening of the cross-sectional groove wall length (Figs. 2, 3), which helps to trap and retain the nectar at high licking rates (Rico-Guevara and Rubega 2011). Rostrally, the groove wall membranes exhibit diagonal to perpendicular cuts in the tissue starting from their lateral edges (Fig. 8C), forming lancinated groove walls (Lucas 1891; also called lamellae, Rico-Guevara and Rubega 2011). Such cuts may originate by wear during the extruding action of the serrated bill tips on the rostral tongue portions (Lucas 1891, Rico-Guevara 2014), and may facilitate the bending of the tongue tip and trapping of fluid drops while mopping the inside of nectar chambers. Wearing at the tongue tip seems to counteract the continuous elongation of the tongue by the growing tissue at the base of the grooves (*cf.* Fig. 2), and unpublished descriptions of hummingbirds with ‘dislocated’ tongues (feeding from artificial feeders with the tongue always hanging to one side from the bill base) report that their tongues are unusually long and/or that become longer with time.

Additionally, microCT data could inform the mathematical models necessary to make predictions about feeding efficiency across the varying morphology of hummingbird species. For

instance, by calculating the total and partial groove capacities depending on immersion lengths (conditioned by the nectar pools on the flowers they visit) the expected amount of liquid extracted can be obtained, and then compared to performance measurements in the wild. Further calculations of the intraoral flow on nectar (based on the bill-tongue internal coupling) taking into account a range of liquid properties that vary in nature (*e.g.* composition, viscosity, temperature, etc.) will provide information on the limiting step of the fluid collection and transport system. Such approach would generate falsifiable quantitative predictions about the action of the feeding apparatus, and the volumes of nectar that can be collected and the speed at which they can be transported, for nectars of different concentrations and at different temperatures (hummingbirds feed from flowers at elevations as high as 5000 m, Carpenter 1976). ~~Which~~ will shed new light on the long-standing debate about the reason of the mismatch between hummingbird nectar concentration preferences (Hainsworth 1976; Roberts 1996; Morgan *et al.* 2016) and the concentration of the nectar of the flowers they pollinate (review in Nicolson *et al.* 2007). The predictions from these mathematical models available only with the MicroCT reconstruction data, could be tested with additional experiments under controlled conditions using *post mortem* tongues (building on the *ex-vivo* experiments presented here), and by measuring nectar extraction rates (fluid volume uptake [$\mu\text{l/s}$]) in free-living nectarivores living under extreme environmental conditions.

Biophysics of nectar collection

The *post-mortem* observations (*e.g.* Videos S3, S4) are consistent with the idea that expansive filling and nectar trapping are processes that do not incur in any extra energy than that necessary to squeeze the nectar out of the tongue and inside the bill, making this elastic

651 micropump a highly efficient device (Rico-Guevara 2014). This is because at the surface of the
 652 nectar pool, the attractive forces (adhesion and cohesion) holding the groove walls together get
 653 weaker because more molecules of fluid are available to fill the internal groove space. This
 654 creates an imbalance, with elastic forces dominating, that results in reshaping of the groove walls
 655 away from the flattened configuration. Molecules of nectar are pulled inside the grooves through
 656 the release of the elastic energy stored on the reshaping tongue groove walls (Fig. 6). Since the
 657 grooves are sealed on top (by surface tension in the zipped dorsal slit), the release of the elastic
 658 energy (reshaping of the grooves) pulls more and more nectar molecules inside the grooves until
 659 they reach a stable cylindrical configuration. The net result of this process is that the portions of
 660 the tongue that remain outside the liquid expand and are filled quickly with nectar, thereby
 661 improving fluid collection efficiency. Thus, the tongue filling is achieved through the transition
 662 from a high potential energy state (flattened grooves) to a low potential energy state (filled
 663 grooves). In summary, the elastic properties of the cornified layer make plausible our elasticity-
 664 induced flow hypothesis. This is ecologically relevant because when the bill tip is almost in
 665 contact with the nectar surface (most likely scenario in the wild given hummingbird flowers'
 666 internal morphology), the process described above is sufficient to fully load the fringed distal
 667 portion of the tongue. Nevertheless, when the bill tip is not in contact with the surface of the
 668 nectar (e.g. hummingbirds visiting flowers with corollas longer than their bills), but instead there
 669 is a space between the bill tip and the nectar pool, the portion of the tongue that remains outside
 670 the liquid would be filled with fluid by the interaction of the aforementioned physical forces in a
 671 process I hypothesize as follows: As the tongue is protruded the grooves are dorso-ventrally
 672 flattened by the bill tips, and once the tongue tip contacts the nectar surface the fluid starts to
 673 penetrate the flattened grooves (because of cohesion of water molecules in the nectar pool and

water molecules in the nectar remaining trapped inside the tongue). When the grooves expand, their walls start releasing the potential energy stored by the bending (flattening by the bill tips). At this point, the excess Laplace pressure due to the nectar flowing inside the grooves plus the releasing of the potential energy whilst the grooves' walls are recovering their semi-cylindrical shape, create a positive feedback between the groove's internal space expansion and the nectar flow. The net result of this process is that the portion of the tongue that remains outside the nectar is also loaded with nectar (Fig. 6). Additionally, if there are empty portions of the tongue located more proximally, which are not being squeezed (therefore flattened) by the bill tips, the nectar filling the grooves (by adhesive and cohesive forces) could close them while moving proximad thereby allowing complete loading of the grooves (including the portion "hidden" inside the bill). Alternatively, the complete filling of the tongue may be achieved by the bill-tongue interaction, involving mechanisms like suction, surface tension transport, hydrostatic pressure motion, etc. However, this would be dependent on, and pertains to, the intra-oral transport of the nectar, which remains understudied.

Conclusions

A variety of anatomical structures allow hummingbirds to protrude their tongues and drag food backwards. Hummingbird tongue shape matches the shape of the internal bill walls, which is important to understand and model the squeezing of the tongue and movement of the nectar to the throat. The rostral portions of the tongue are mostly made of a cornified layer (β -keratin) that is replaced from the tongue basal portions, and worn at the tip by the interaction with the bill tips upon nectar extrusion. Interestingly, if the dorsal and ventral surfaces have different rugosities

that may have direct implications to their hydrophobicity, *i.e.* increased roughness may significantly increase contact angle (of a water droplet) and decrease contact angle hysteresis (*e.g.* Michael and Bhushan 2007). Therefore, the inner tongue groove surface may be more hydrophilic than the outer groove surface, potentially helping the fluid trapping process (Rico-Guevara and Rubega 2011) and maintaining the surface tension zip at the dorsal slit along the grooves (Fig. 9).

Hummingbird tongues are thinner than other bird tongues (references above), and that the groove walls are between ~10 and 30 μm thick, which makes them highly pliable. In addition, the tissue architecture of the cornified layer resembling a brick-wall configuration, along with its keratinous composition, grants non-stretchable properties to the groove walls. Hence, hummingbird tongues are easily bent to extrude the nectar inside the bill (Rico-Guevara and Rubega 2011), yielding to storage of elastic potential energy in the groove walls, which is then released when the tongue is reinserted in the nectar (Rico-Guevara 2014), thereby improving liquid uptake efficiency. The proper functioning of hummingbird tongue grooves as dynamic structures depends on the balance between pliability and elasticity; in particular, the latter has to be strong enough to help the pumping process to extract nectar but weak enough to keep the grooves flattened until they contact the nectar surface (Rico-Guevara *et al.* 2015). Several scaling models and applications have been developed on the basis of recent discoveries of biological phenomena and underlying physical explanations (see Vogel 2011), which opens the way for deeper studies of the influence of the surface characteristics (*e.g.* differential hydrophilicity) and the tissue composition of the groove walls on the elastic properties of hummingbird tongues.

720

721 The present work raises anew the question: How do hummingbirds feed? Much work
 722 remains before we can explain the whole nectar feeding process in hummingbirds and other
 723 nectarivores. Achieving a fuller understanding of the mechanics of the nectar-feeding process
 724 may help eliminate the disparity between the theoretical predictions of how birds should act, and
 725 empirical observations of what they actually do. A detailed three-dimensional morphological
 726 description that allows for detail mathematical modeling will aid in understanding different
 727 aspects of their food collection efficiency limits and deviations of predicted *vs.* realized
 728 performance, which are the building blocks of foraging and coevolution principles (review in
 729 Pyke 2016). This paper sets the bases for morpho-functional comparisons between
 730 hummingbirds and other nectar feeding organisms, as an example of convergent and alternative
 731 ways to maximize food collection efficiency in nature.

732

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746

747 **Competing Interests**

748 The author declares that he has no competing interests.

749

750 **Animal Ethics**

751 This article does not contain any studies with live animals performed by the author.

References

- Abou-Zaid DF, Al-Jaloud NA (2010) The structural adaptations of the lingual apparatus of the grey heron, *Ardea cinerea*. Egypt J Exp Biol 6:307–317
- Abumandour MM (2014) Gross anatomical studies of the oropharyngeal cavity in Eurasian Hobby (Falconinae: *Falco Subbuteo*, Linnaeus 1758). IJLSR 1:80–92
- Alibardi L, Dalla Valle L, Nardi A, Toni M (2009) Evolution of hard proteins in the sauropsid integument in relation to the cornification of skin derivatives in amniotes. J Anat 214:560–586
- Baldwin MW, Toda Y, Nakagita T, O'Connell MJ, Klasing KC, Misaka T, Edwards SV, Liberles SD (2014) Evolution of sweet taste perception in hummingbirds by transformation of the ancestral umami receptor. Science 345:929–933
- Baumel JJ, King AS, Breazile JE, Evans HE, Vanden Berge JC (1993) Handbook of avian anatomy: nomina anatomica avium, 2nd edn R.A. Paynter Jr. ed. Nuttall Ornithological Club, Cambridge
- Baussart S, Korsoun L, Libourel PA, Bels V (2009) Ballistic food transport in toucans. J Exp Zool 311:465–474
- Baussart S, Bels V (2011) Tropical hornbills (*Aceros cassidix*, *Aceros undulatus*, and *Buceros hydrocorax*) use ballistic transport to feed with their large beaks. J Exp Zool 315:72–83
- Birt P, Hall LS, Smith GC (1997) Ecomorphology of the tongues of Australian megachiroptera (Chiroptera: Pteropodidae). Aust J Zool 45:369–384
- Bock WJ (1972) Morphology of the tongue apparatus of *Ciridops anna* (Drepanididae). Ibis 114:61–78
- Bock WJ (1999) Functional and evolutionary morphology of woodpeckers. Ostrich 70:23–31
- Bozzola JJ, Russell LD (1999) Specimen preparation for transmission electron microscopy. In: Bozzola JJ, Russell LD (eds) Electron microscopy: Principles and techniques for biologists. Jones and Bartlett, Sudbury, MA, pp 21–31
- Carpenter FL (1976) Ecology and evolution of an Andean hummingbird (*Oreotrochilus estella*). Univ Calif Publ Zool 106:1–74
- Carver, WE, Knapp, LW, Sawyer, RH (1990). β -keratin expression in avian tongue cell aggregates. J Exp Zool A Ecol Genet Physiol 256:333–338

- Chang YM, Lin HY, Hatch KA, Yao CT, Shiu HJ (2013) Brush-tipped tongue structure of the Taiwan Yuhina (*Yuhina brunneiceps*) and White-eared Sibia (*Heterophasia auricularis*). Wilson J Ornithol 125:204–208
- Darwin C (1841) The Zoology of the voyage of H.M.S. Beagle: Part III, Birds. Smith, Elder and Co, London, 156 p
- Downs CT (2004) Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. Ostrich 75:169-175
- Dummett CO, Barens G (1974) Avian oral pigmentation. J Periodontol 45:426–433
- Emura S, Okumura T, Chen H (2010) Scanning electron microscopic study of the tongue in the jungle nightjar (*Caprimulgus indicus*). Okajimas Folia Anat Jpn 86:117–120
- Erdoğan S, Alan A (2012) Gross anatomical and scanning electron microscopic studies of the oropharyngeal cavity in the European magpie (*Pica pica*) and the common raven (*Corvus corax*). Microscopy research and technique 75:379–387
- Erdoğan S, Sağsöz H, Akbalik ME (2012a) Anatomical and Histological Structure of the Tongue and Histochemical Characteristics of the Lingual Salivary Glands in the Chukar Partridge (*Alectoris chukar*, Gray 1830). British Poultry Science 53:307–315
- Erdoğan S, Pérez W, Alan A (2012b) Anatomical and scanning electron microscopic investigations of the tongue and laryngeal entrance in the long-legged buzzard (*Buteo rufinus*, crettschmar, 1829). Microscopy research and technique 75:1245–1252
- Erdoğan S, Iwasaki SI (2014) Function-related morphological characteristics and specialized structures of the avian tongue. Ann Anat 196:75–87
- Erdoğan S, Pérez W (2015) Anatomical and scanning electron microscopic characteristics of the oropharyngeal cavity (tongue, palate and laryngeal entrance) in the southern lapwing (Charadriidae: *Vanellus chilensis*, Molina 1782). Acta Zool 96:264–272
- Ewald PW, Williams WA (1982) Function of the bill and tongue in nectar uptake by hummingbirds. Auk 99:573–576
- Farner DS (1960) Chapter XI. Digestion and the Digestive System. In: Marshall AJ (ed) Biology and Comparative Physiology of Birds, Vol. I. Academic Press, New York, pp 411–467
- Filshie BK, Rogers GE (1962) An electron microscope study of the fine structure of feather keratin. J Cell Biol 13:1–12
- Fleming TH, Muchhala N (2008) Nectar-feeding bird and bat niches in two worlds: Pantropical comparisons of vertebrate pollination systems. J Biogeogr 35:764–780

- Fujii Y (2011) Comparison of Surface Roughness Estimations by X-ray Reflectivity Measurements and TEM observations. IOP Conf Ser: Mater Sci Eng 24:012008
- Ghosh S, Bowen J, Jiang K, Espino DM, Shepherd DE (2013) Investigation of techniques for the measurement of articular cartilage surface roughness. Micron 44:179–184
- Gignac PM, Kley NJ (2014) Iodine-enhanced micro-CT imaging: Methodological refinements for the study of the soft-tissue anatomy of post-embryonic vertebrates. J Exp Zool 322:166–176
- Gignac PM, Kley NJ, Clarke JA et al (2016) Diffusible iodine-based contrast-enhanced computed tomography (diceCT): an emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. J Anat. doi: 10.1111/joa.12449
- Guimarães JP, de Britto MR, Le Bas A, Miglino MA (2014) Ultrastructural aspects of the tongue in Magellanic Penguins *Spheniscus magellanicus* (Forster, 1781). Acta Sci Biol Sci 36:491–497
- Gussekloo SW, Bout RG (2005) The kinematics of feeding and drinking in palaeognathous birds in relation to cranial morphology. J Exp Biol 208:3395–3407
- Hainsworth FR (1973) On the tongue of a hummingbird: Its role in the rate and energetics of feeding. Comp Biochem Physiol 46:64–78
- Hainsworth FR, Wolf LL (1976) Nectar characteristics and food selection by hummingbirds. Oecologia 25:101–113
- Handschuh S, Baeumler N, Schwaha T, Ruthensteiner B (2013) A correlative approach for combining microCT, light and transmission electron microscopy in a single 3D scenario. Front Zool 10:44–59
- Hardesty J (2009) Using nitrogen-15 to examine protein sources in hummingbird diets. Ornith Colomb 8:19–28
- Harper CJ, Swartz SM, Brainerd EL (2013) Specialized bat tongue is a hemodynamic nectar mop. Proc Natl Acad Sci USA 110:8852–8857
- Harte M, Legreneur P, Pelle E, Placide MA, Bels V (2012) Ballistic food transport in birds: the example of *Casuaris casuaris*. Comput Methods Biomech Biomed Engin 15:137–139
- Hayat MA (2000) Principles and techniques of electron microscopy: Biological applications. 4th Edition. Academic Press, London, 564 p
- Holliday CM, Ridgely RC, Balanoff AM, Witmer LM (2006) Cephalic vascular anatomy in flamingos (*Phoenicopterus ruber*) based on novel vascular injection and computed tomographic imaging analyses. Anat Rec 288:1031–1041

- Homberger DG (1980) Funktionell-morphologische untersuchungen zur radiation der ernährungs-und trinkmethoden der papageien (Psittaci). Dissertation. Universität Zürich
- Homberger DG, Meyers RA (1989) Morphology of the lingual apparatus of the domestic chicken, *Gallus gallus*, with special attention to the structure of the fasciae. *Am J Anat* 186:217–257
- Iwasaki S-I, Asami T, Chiba A (1997) Ultrastructural study of the keratinization of the dorsal epithelium of the tongue of Middendorff's Bean Goose, *Anser fabalis middendorffii* (Anseres, Anatidae). *Anat Rec* 247:149–163
- Iwasaki S-I (2002) Evolution of the structure and function of the vertebrate tongue. *J Anat* 201:1–13
- Jackowiak H, Andrzejewski W, Godynicki S (2006) Light and scanning electron microscopic study of the tongue in the cormorant *Phalacrocorax carbo* (Phalacrocoracidae, Aves). *Zool Sci* 23:161–167
- Jackowiak H, Skieresz-Szewczyk K, Kwiecinski Z, Trzcielinska-Lorych J, Godynicki S (2010) Functional morphology of the tongue in the Nutcracker (*Nucifraga caryocatactes*). *Zool Sci* 27:589–594
- Jackowiak H, Skieresz-Szewczyk K, Godynicki S, Iwasaki S-I, Meyer W (2011) Functional morphology of the tongue in the Domestic Goose (*Anser anser f. domestica*). *Anat Rec* 294:1574–1584
- Jackowiak H, Skieresz-Szewczyk K, Kwieciński Z, Godynicki S, Jackowiak K, Leszczyszyn A (2015) Light microscopy and scanning electron microscopy studies on the reduction of the tongue microstructures in the White Stork (*Ciconia ciconia*, Aves). *Acta Zool* 96:436–441
- Johnson DJ, Sikorski J (1965) Alpha-keratin: Molecular and fine structure of α -keratin (IV). *Nature* 205:266–268
- Jung, J. Y., Naleway, S. E., Yaraghi, N. A., Herrera, S., Sherman, V. R., Bushong, E. A., ... & McKittrick, J. (2016). Structural analysis of the tongue and hyoid apparatus in a woodpecker. *Acta biomaterialia*, 37, 1-13.
- Kadhim KK, Zuki ABZ, Babjee SMA, Noordin MM, Zamri-Saad M (2013) Morphological and histochemical observations of the red jungle fowl tongue *Gallus gallus*. *Afr J Biotechnol* 10:9969–9977
- Kim W, Peaudecerf F, Baldwin MW, Bush JW (2012) The hummingbird's tongue: A self-assembling capillary syphon. *Proc Biol Sci* 279:4990–4996

- Kobayashi K, Kumakura M, Yoshimura K, Inatomi M, Asami T (1998) Fine structure of the tongue and lingual papillae of the penguin. *Arch Histol Cytol* 61:37–46
- Kremer A, Lippens S, Bartunkova S, Asselbergh B, Blanpain C, Fendrych M, Guérin CJ (2015) Developing 3D SEM in a broad biological context. *J Microsci* 259:80–96
- Lautenschlager S, Bright JA, Rayfield EJ (2014) Digital dissection—using contrast-enhanced computed tomography scanning to elucidate hard-and soft-tissue anatomy in the Common Buzzard *Buteo buteo*. *J Anat* 224:412–431
- Lingham-Soliar T, Bonser RH, Wesley-Smith J (2010) Selective biodegradation of keratin matrix in feather rachis reveals classic bioengineering. *Proc Biol Sci* 277:1161–1168
- Lingham-Soliar T, Murugan N (2013) A new helical crossed-fibre structure of β -keratin in flight feathers and its biomechanical implications. *PloS One* 8:e65849
- Lucas FA (1891) On the structure of the tongue in humming birds. *Proc US Nat Mus* 14:169–172
- Lucas FA (1894) The Tongue of the Cape May Warbler. *Auk* 11:141–144
- Lucas AM, Stettenheim PB (1972). *Avian anatomy. Integument. Agriculture Handbook 362*, U.S. Department of Agriculture, Washington, D.C.
- Martin WCL (1833) *The naturalist's library: A general history of humming-birds or the Trochilidae*. H.G. Bohn, London, 276 p
- Michael N, Bhushan B (2007) Hierarchical roughness makes superhydrophobic states stable. *Microelectron Eng* 84:382–386
- Metscher BD (2009) MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiol* 9:11
- Mobbs AJ (1979) Methods used by the Trochilidae hummingbirds when capturing insects. *Avic Mag* 851:26–30
- Morgan KV, Hurly TA, Martin L, Healy SD (2016) Presentation order affects decisions made by foraging hummingbirds. *Behav Ecol Sociobiol* 70: 21–26
- Morioka H (1992). Tongue of two species of *Prionochilus* from the Philippines, with notes on feeding habits of flowerpeckers (Dicaeidae). *Jap J Ornithol* 40:85–91
- Muchhala N (2006) Nectar bat stows huge tongue in its rib cage. *Nature* 444:701–702
- Nanda KK, Sarangi SN, Sahu SN (1998) Measurement of surface roughness by atomic force microscopy and Rutherford backscattering spectrometry of CdS nanocrystalline films. *Appl Surf Sci* 133:293–297

- Newton A, Gadow H, Lydekker R, Roy CS, Shufeldt RW (1896) A dictionary of birds. A & C Black, London, 1066 p
- Nicolson SW, Nepi M, Pacini E (2007) Nectaries and nectar. Springer Netherlands, Dordrecht, 396 p
- Ortiz-Crespo, F. (2003). Los colibríes: historia natural de unas aves casi sobrenaturales. FUNDACYT, Quito
- Parakkal PF, Alexander NJ (1972) Keratinization: a survey of vertebrate epithelia. Academic Press, New York, 59 p
- Parchami A, Dehkordi RAF, Bahadoran S (2010) Scanning electron microscopy of the tongue in the golden eagle *Aquila chrysaetos* (Aves: Falconiformes: Accipitridae). WJZ 5:257–263
- Paton DC, Collins BG (1989) Bills and tongues of nectar-feeding birds: A review of morphology, function and performance, with intercontinental comparisons. Austral Ecol 14:473–506
- Pratt HD (1992) Is the Poo-uli a Hawaiian honeycreeper (Drepanidinae)? Condor 94:172–180
- Pyke GH (2016) Plant–pollinator co-evolution: It's time to reconnect with Optimal Foraging Theory and Evolutionarily Stable Strategies. Perspect Plant Ecol Evol Syst 19:70–76
- Rand AL (1961) The Tongue and Nest of Certain Flowerpeckers, Aves: Dicaeidae. Natural History Museum, Chicago
- Rand AL (1967) The flower-adapted tongue of a Timaliinae bird and its implications. Field Museum of Natural History
- Rico-Guevara A (2008) Morfología y forrajeo para buscar artrópodos por colibríes altoandinos. Ornitol Colomb 7:43–58
- Rico-Guevara A (2014) Morphology and function of the drinking apparatus in hummingbirds. Dissertation. University of Connecticut
- Rico-Guevara A, Rubega MA (2011) The hummingbird tongue is a fluid trap, not a capillary tube. Proc Natl Acad Sci USA 108:9356–9360
- Rico-Guevara A, Fan T-H, Rubega MA (2015) Hummingbird tongues are elastic micropumps. Proc Biol Sci 282:20151014
- Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212

- 1026 Roberts WM (1996) Hummingbirds' nectar concentration preferences at low volume: The
1027 importance of time scale. *Anim Behav* 52:361–370.
1028
- 1029 Rubega MA (2000) Feeding in birds: Approaches and opportunities. In: Schwenk K (ed)
1030 Feeding: Form, function and evolution in tetrapod vertebrates. Academic Press, San Diego,
1031 pp 395–408
1032
- 1033 Schwenk K (2000) An introduction to tetrapod feeding. In: Schwenk K (ed) Feeding: Form,
1034 function and evolution in tetrapod vertebrates. Academic Press, San Diego, pp 21–61
1035
- 1036 Schwenk K (2001) Extrinsic versus intrinsic lingual muscles: a false dichotomy. *Bull Mus Comp*
1037 *Zool* 156:219–235
1038
- 1039 Scharnke H (1931) Beiträge zur morphologie und entwicklungsgeschichte der zunge der
1040 Trochilidae, Meliphagidae und Picidae. *J Ornithol* 79:425–491
1041
- 1042 Scharnke H (1932) Ueber den Bau der Zunge der Nectariniidae, Promeropidae und Drepanididae
1043 nebst Bemerkungen zur Systematik der blütenbesuchenden Passeres. *J Ornithol* 80: 114–123
1044
- 1045 Scharnke H (1933) Ueber eine rückgebildete Honigfresser-Zunge. *J Ornithol* 81:355–359
1046
- 1047 Shah SW, Aziz NA (2014) Morphology of the lingual apparatus of the Swiftlet, *Aerodramus*
1048 *fuciphagus* (Aves, Apodiformes, Apodidae). *JMAU* 2:100–103
1049
- 1050 Shufeldt RW (1900) On the osteology of the woodpeckers. *Proc Am Philos Soc* 39:578–622
1051
- 1052 Skieresz-Szewczyk K, Jackowiak H (2014) Scanning electron microscopy investigation of the
1053 filter-feeding apparatus in the domestic goose (*Anser anser* f. domestica) and the domestic
1054 duck (*Anas platyrhynchos* f. domestica). In: Méndez-Vilas A (ed) Microscopy: Advances in
1055 scientific research and education. Formatex Research Center, Badajoz, Spain, p 84–88
1056
- 1057 Smith ML, Yanega GM, Ruina A (2011) Elastic instability model of rapid beak closure in
1058 hummingbirds. *J Theor Biol* 282:41–51
1059
- 1060 Soley JT, Tivane C, Crole MR (2015) Gross morphology and topographical relationships of the
1061 hyobranchial apparatus and laryngeal cartilages in the ostrich (*Struthio camelus*). *Acta Zool*
1062 96:442–451
1063
- 1064 Stiles FG (1981) Geographical aspects of bird-flower coevolution, with particular reference to
1065 Central America. *Ann Mo Bot Gard* 68:323–351
1066
- 1067 Stiles FG (1995) Behavioral, ecological and morphological correlates of foraging for arthropods
1068 by the hummingbirds of a tropical wet forest. *Condor* 97:853–878
1069
- 1070 Tivane C, Rodrigues MN, Soley JT, Groenwald HB. (2011) Gross anatomical features of the
1071 oropharyngeal cavity of the ostrich (*Struthio camelus*). *Pesq Vet Bras* 31:543–550

- Tomlinson CAB (2000) Feeding in paleognathous birds. In: Schwenk K (ed) Feeding: Form, function and evolution in tetrapod vertebrates. Academic Press, San Diego, pp 359–394
- Villard P, Cuisin J (2004) How do woodpeckers extract grubs with their tongues? A study of the Guadeloupe Woodpecker (*Melanerpes herminieri*) in the French West Indies. Auk 121:509–514
- Vogel S (2011) Surface tension helps a tongue grab liquid. Proc Natl Acad Sci USA 108:9321–9322
- Weymouth RD, Lasiewski RC, Berger AJ (1964) The tongue apparatus in hummingbirds. Cells Tissues Organs 58:252–270
- Xue T, Trent JS, Osseo-Asare K (1989) Characterization of Nafion® membranes by transmission electron microscopy. J Memb Sci 45:261–271
- Yanega GM, Rubega MA (2004) Feeding mechanisms: Hummingbird jaw bends to aid insect capture. Nature 428:615
- Yanega GM (2007) A comparative study of the functional morphology and ecology of insectivory in hummingbirds. Dissertation. University of Connecticut
- Zusi RL (2013) Introduction to the skeleton of hummingbirds (Aves: Apodiformes, Trochilidae) in functional and phylogenetic contexts. Ornithol Monogr 77:1–94
- Zweers, G., de Jong, F., Berkhoudt, H. & Vanden Berge, J.C. (1995) Filter feeding in flamingos (*Phoenicopterus ruber*). Condor, 97, 297–324
- Zweers GA, Berge JV, Berkhoudt H (1997) Evolutionary patterns of avian trophic diversification. Zoology 100:25–57