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



First submission

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Virginia Abdala / 29 Apr 2017

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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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## 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 function~~ing~~ 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 *~~P~~araglossum* (*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 tool~~s~~ 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 tongues have been studied for ~~around~~ two centuries (Martin 1833; Darwin 1841; Lucas 1891; Scharnke 1931; Weymouth *et al.* 1964; Hainsworth 1973), many aspects of their morphology and function still remain to be understood. The tongues of hummingbirds are forked at their tips (Martin 1833; Darwin 1841; Scharnke 1931; Hainsworth 1973), ending in two tube-like grooves with fringed edges (Lucas 1891). These grooves are exclusively rostral structures and the interior of the tongue base is not hollow (Scharnke 1931; Weymouth *et al.* 1964). There is only one study focusing on the morphology of the entire length of the tongue grooves (Hainsworth 1973), which unfortunately is lacking histological details. The most rostral cross section micrograph near the base of the tongue grooves (Weymouth *et al.* 1964)~~,~~ shows at least two distinct layers of tissue composing the dorsal and ventral surfaces of the grooves, which are not further described. Studies on nectar feeding in living birds suggest that the functional traits enabling hummingbird to extract liquid are related to the structural configuration of the tongue tip (Rico-Guevara and Rubega 2011; Rico-Guevara *et al.* 2015), rather than to active movements of their parts through muscle action. A deeper study of the entire length of hummingbird tongues is essential to understand the underlying architectural properties enabling the observed nectar extraction mechanisms. Because previous studies (*e.g.* Weymouth *et al.* 1964; Zusi 2013) have described in detail the hyobranchial apparatus, ~~and~~ the structure of the root, and body of the tongue (up to the bifurcation point) in hummingbirds, the present study presents only descriptions of the structures of the rostral portion of the tongue grooves, and in addition a description of the coupling between the bill and tongue. Understanding the morphology of the rostral portion of the grooves and the bill-tongue fit is crucial to understand the nectar-feeding mechanics in hummingbirds (*e.g.* Rico-Guevara 2014). Furthermore, ~~since~~ the proposed mechanism of nectar collection involves 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 (Handschuh *et al.* 2013; Jung *et al.* 2016).

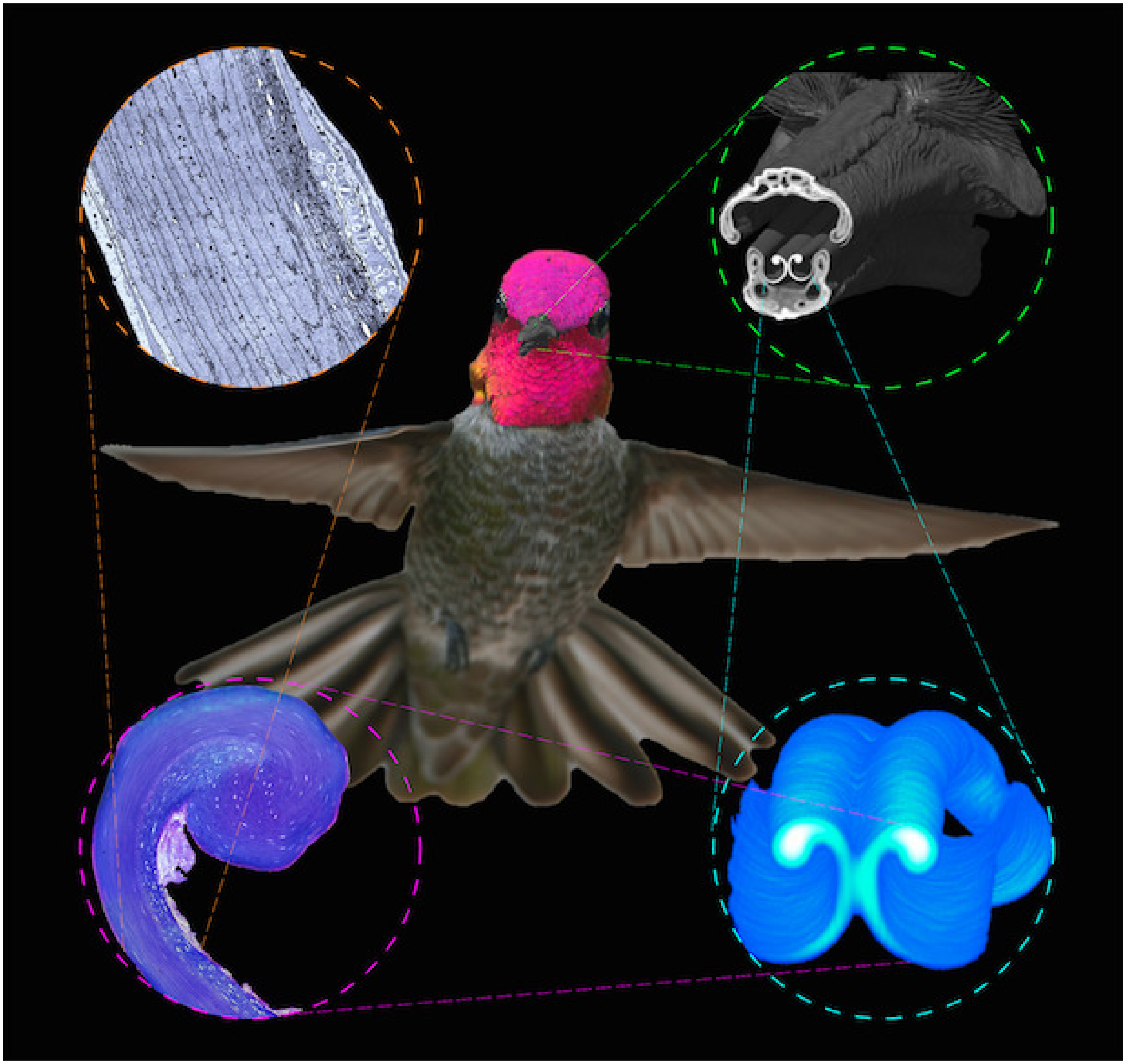

**Figure 1. Depiction of the techniques used to study the hummingbird feeding apparatus.** In the center, a photograph of an Anna's Hummingbird hovering (courtesy of Robert McQuade). Inside the upper right circle (green), a microCT scan coronal cutaway section portraying both the bill and tongue. Inside 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 i~~nside 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)~~. F~~or 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 t~~o obtain detailed morphological data at the micrometric scale and visualize the tongue soft tissues, I employed a staining protocol with osmium tetroxide ($OsO_4$, *cf.* Metscher 2009) with the difference that I did not embed~~ded~~ 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) $OsO_4$ 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 $SiO_2$, 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 fuchsine 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 $RuO_4$ vapors, or $RuO_4$ 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) $OsO_4$ (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 connect~~s~~, when moving rostrally through cross sections (Fig. 2, XS 2-5), effectively dividing the tongue body (*cf.* XS 13 in Weymouth *et al.* 1964) and giving rise to a bifid tongue. At XS 3 the semi-cylindrical configuration characteristic of the tongue grooves is already conspicuous (*cf.* XS 14 in Weymouth *et al.* 1964).

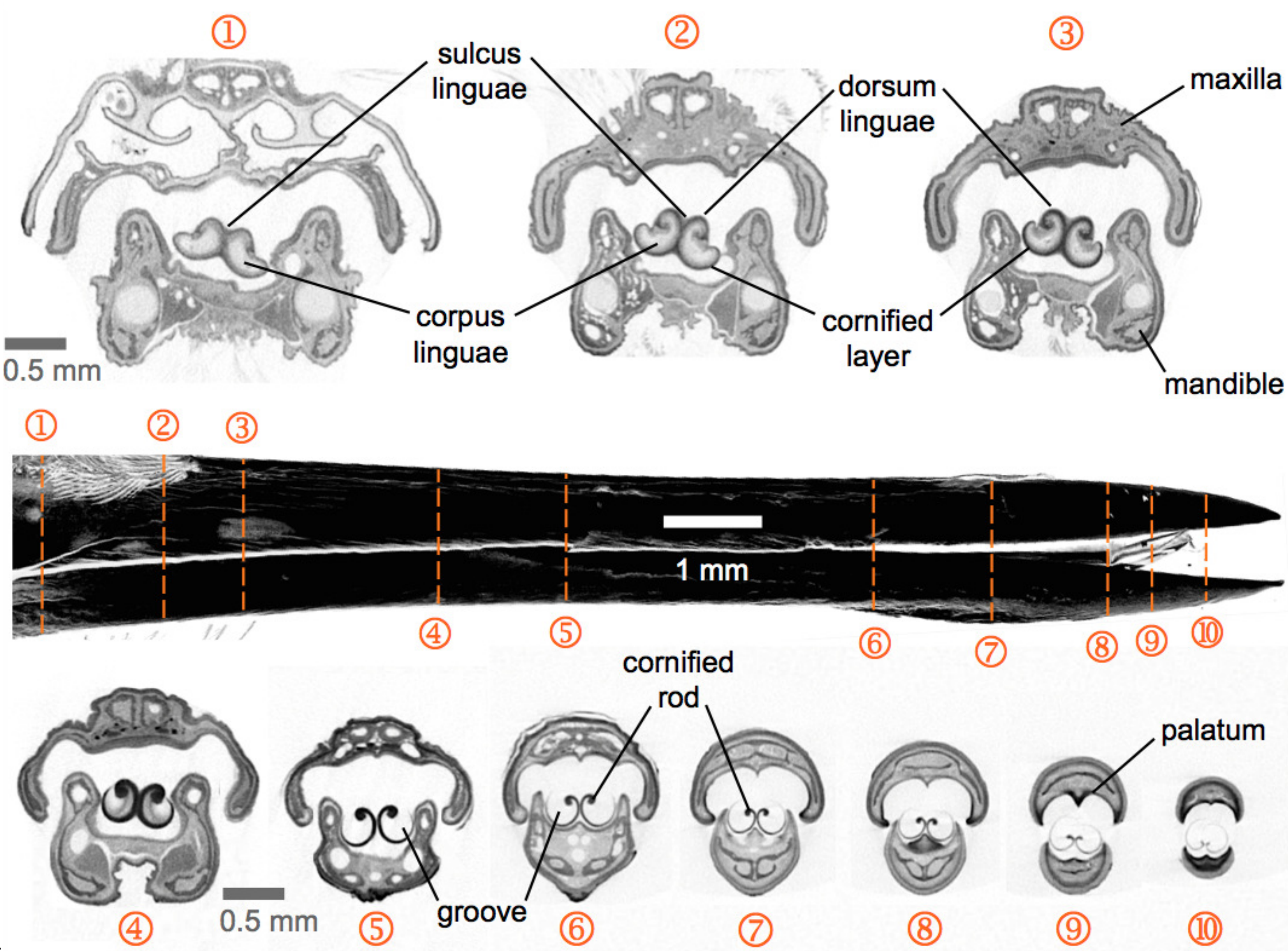


**Figure 2. Selected feeding apparatus cross sections (1-10) from a microCT scan of an Anna's Hummingbird**. Black structure in the middle of the figure is a lateral view of the bill from the reconstructed scan, and the dashed orange lines crossing it correspond to the numbered cross sections. Upper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

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

*Histology and Electron Microscopy*

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

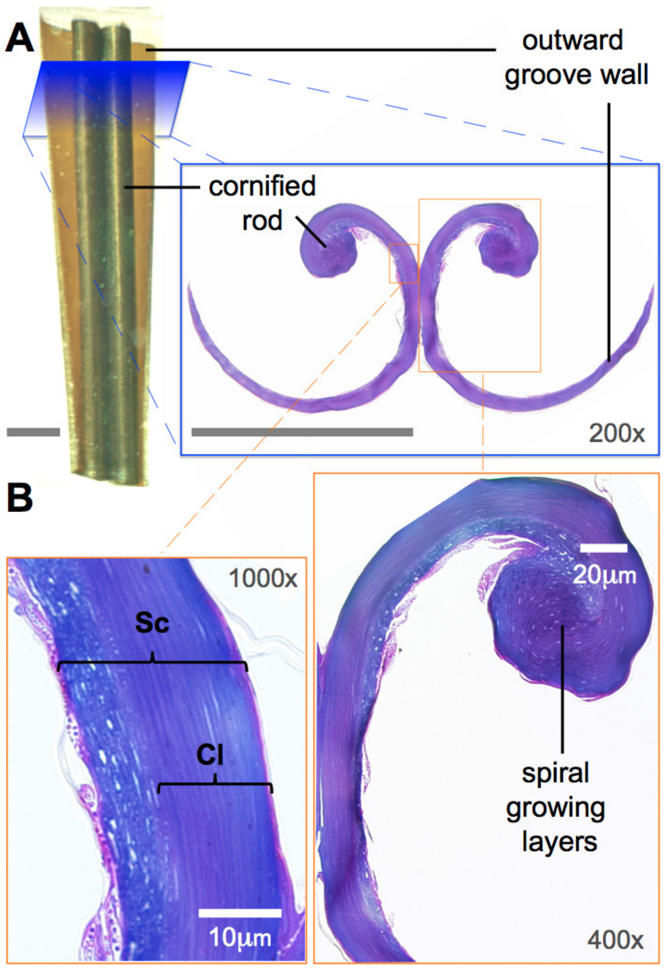


**Figure 3. Low-magnification morphology of the rostral half (grooves) of a Ruby-throated Hummingbird tongue**. (A) On the left, a section of the tongue embedded in resin; dorsal view oriented with the caudal end of the section at the top. On the right, a corresponding cross section (light microscope) 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 Barens 1974). The cell boundaries are continuous lines of corneo-desmosomes (*e.g.* black arrow, Fig. 4B). I found structures of ~35 Å 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).

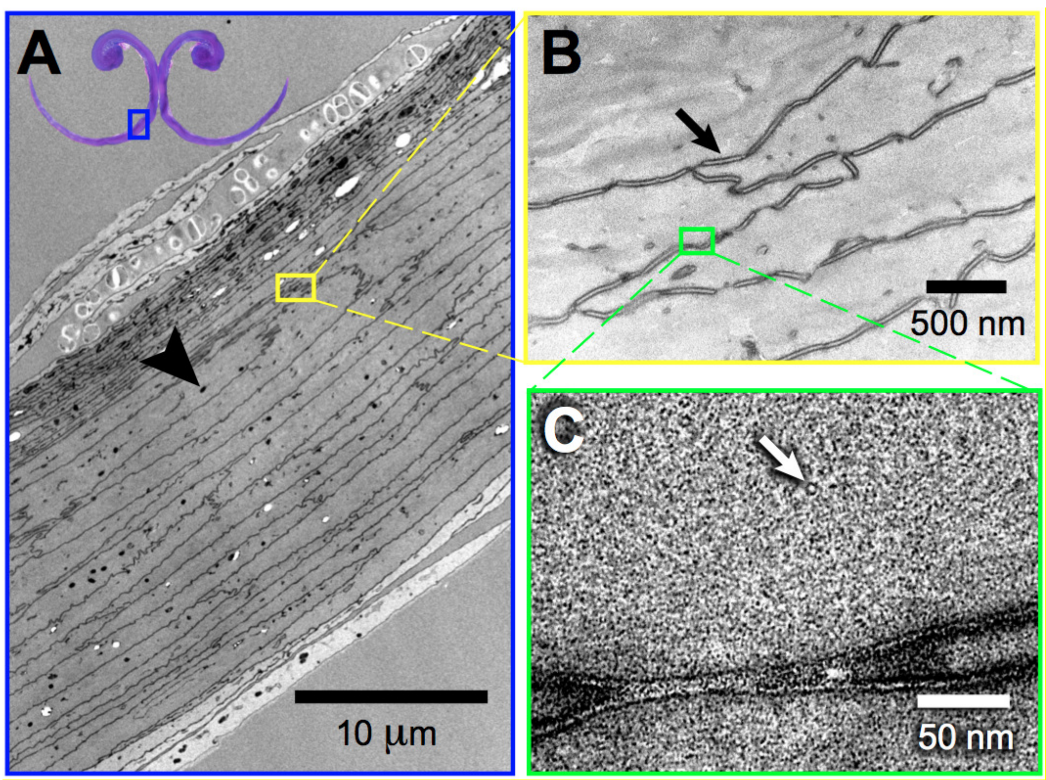


**Figure 4. High-magnification morphology of a cross section at the rostral half (grooves) of a Ruby-throated Hummingbird tongue.** (A) Transmission electron micrograph showing the difference in layer composition (more densely packed near the dorsal surface), and potential melanin (black arrow head) 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-µm 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).

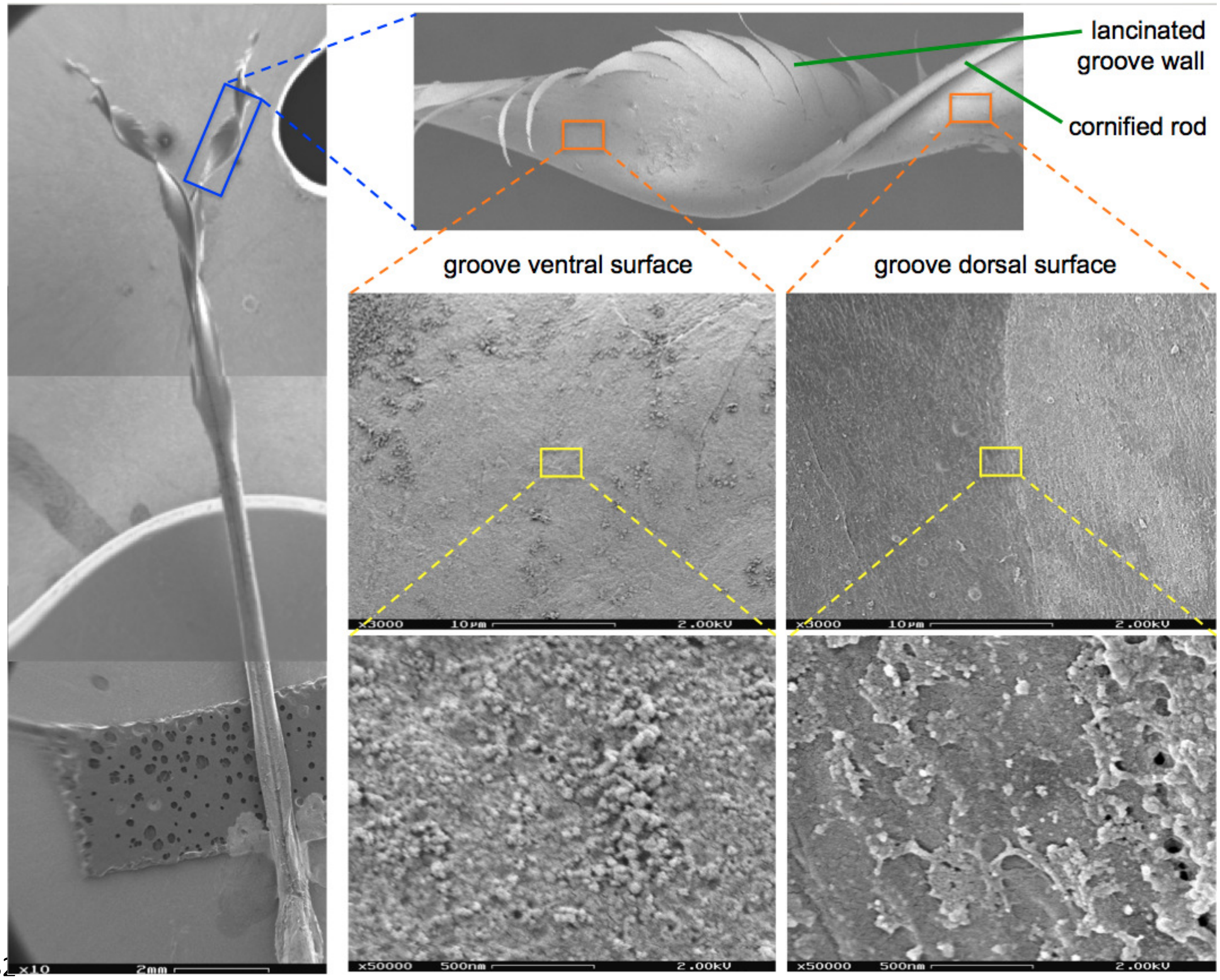


**Figure 5. Scanning electron microscopy of a Rufous Hummingbird tongue.** On the left, an overview of the entire tongue, although my observations focused on the rostral half (grooves). On the top right, a close up of a longitudinally twisted section of a tongue groove, indicating the cornified rod of 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.

Ex-vivo *experiments*

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

I explain the hypothesis as follows: 1) The process starts when the tongue is dorso-ventrally compressed upon protrusion; when the tongue is extruded, only a thin layer of nectar remains inside the grooves. Such thin layer acts as an adhesive (Stephan adhesion) maintaining the dorsoventrally flattened (elliptical) configuration of the grooves even after they pass the extrusion point (bill tip). The attractive forces between the nectar and the tongue (adhesion, cohesion and surface tension) are able to resist the elastic energy stored in the grooves' walls (cornified layers), and thus keep the grooves flattened. This stable flattened configuration is conserved during the trip of the tongue across the air space between the bill tip to the nectar pool. In the dorsal portion of the tongue, where the groove's inside upper edge meets the rod, the free (outer) edge of the groove is prevented from rolling outward by a narrow sheet of nectar joining it to the rod. The surface tension at this exposed nectar sheet keeps the grooves "zipped up" by preventing air from entering the groove itself. Surface tension at the tip of the tongue also keeps the grooves stuck to each other, forming a unitary structure. 2) Once the tongue passes the compression point at the bill tips, there is a slight expansion in the tongue grooves (because of the cessation of compressive forces). The expansion of the grooves is arrested at the point in which the attractive forces between the tongue walls and the nectar balance out the elastic forces of the grooves walls. This creates an initial transient equilibrium that maintains the flattened configuration (*cf.* Rico-Guevara *et al.* 2015). 3) Once the tongue tip contacts the nectar surface, the free supply of fluid eliminates the surface tension that was holding the grooves together, allowing the area of the grooves that is inside the nectar to open (*cf.* Rico-Guevara and Rubega 2011). This opening of the ends of the grooves allows the nectar molecules from the nectar pool to start interacting with the nectar molecules inside the grooves (*i.e.* elasticity-induced flow, Fig. 6). On the dorsal surface of the length of the grooves still outside the nectar pool (more proximal to the bird's mouth), the surface tension of the fluid sheet between the rods and the groove walls holds the grooves in the rolled, flattened position. 4) Molecules of liquid entering the tongue grooves at the boundary where the tongue enters the nectar pool start moving proximally through the grooves, creating a jet of fluid that fills the grooves following their expansion (*cf.* Rico-Guevara *et al.* 2015). This continued destabilization of the initial transient equilibrium causes the area of the grooves outside the nectar to expand which in turn causes them to fill, creating a 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.

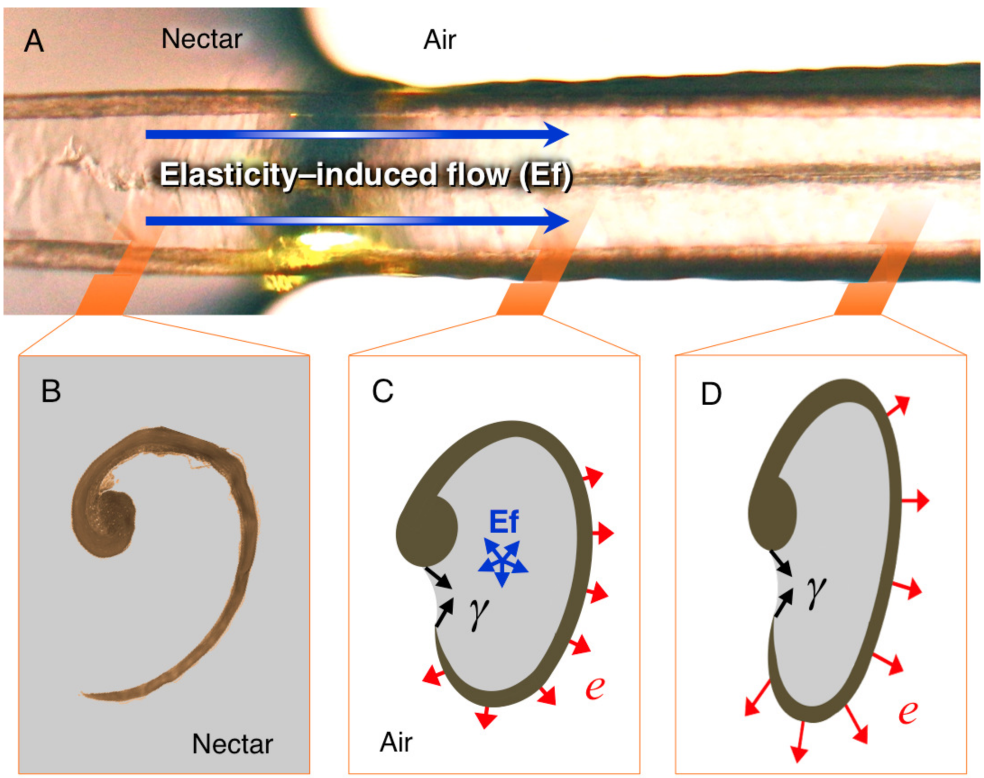


**Figure 6. Elasticity-induced flow hypothesis.** (A) Dorsal photograph of a hummingbird tongue tip just after contacting the nectar surface. Given the flattened configuration of the grooves on the right, there would be elastic energy stored which induces inward flow. (B) Cross section (light microscope photograph) of a hummingbird tongue in its "relaxed" configuration inside the nectar. (C) Hypothetical cross section showing the elasticity-induced flow (Ef in *blue*), the surface tension ($\gamma$ 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.

**Discussion**

*Gross morphology of hummingbird tongues*

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

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

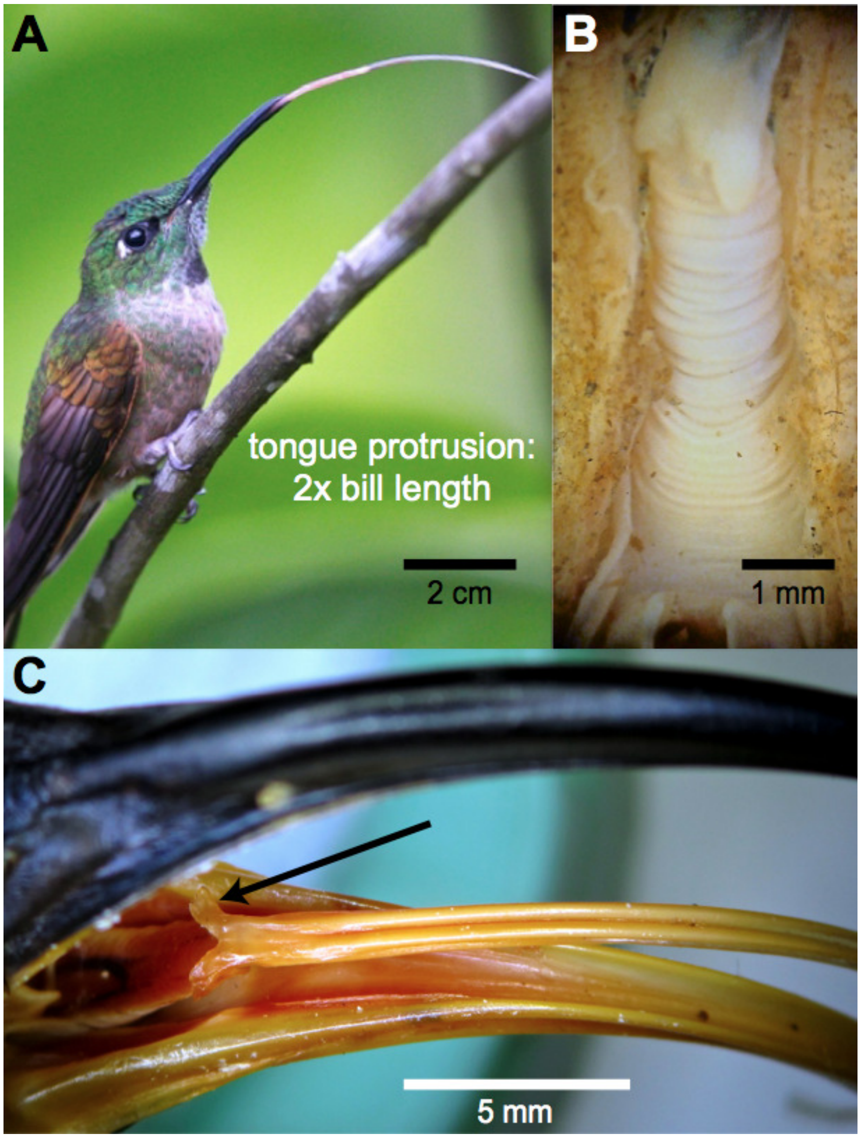


**Figure 7. Gross morphology of hummingbird tongues. (**A) Photograph of a Fawn-breasted Brilliant (*Heliodoxa rubinoides*) stretching its tongue apparatus (courtesy of Jim DeWitt –Frozen Feather Images). (B) Dissecting microscope photograph of the throat region in a dissected specimen~~. Featuring~~ 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.

*Ultrastructural characteristics of hummingbird tongues*

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

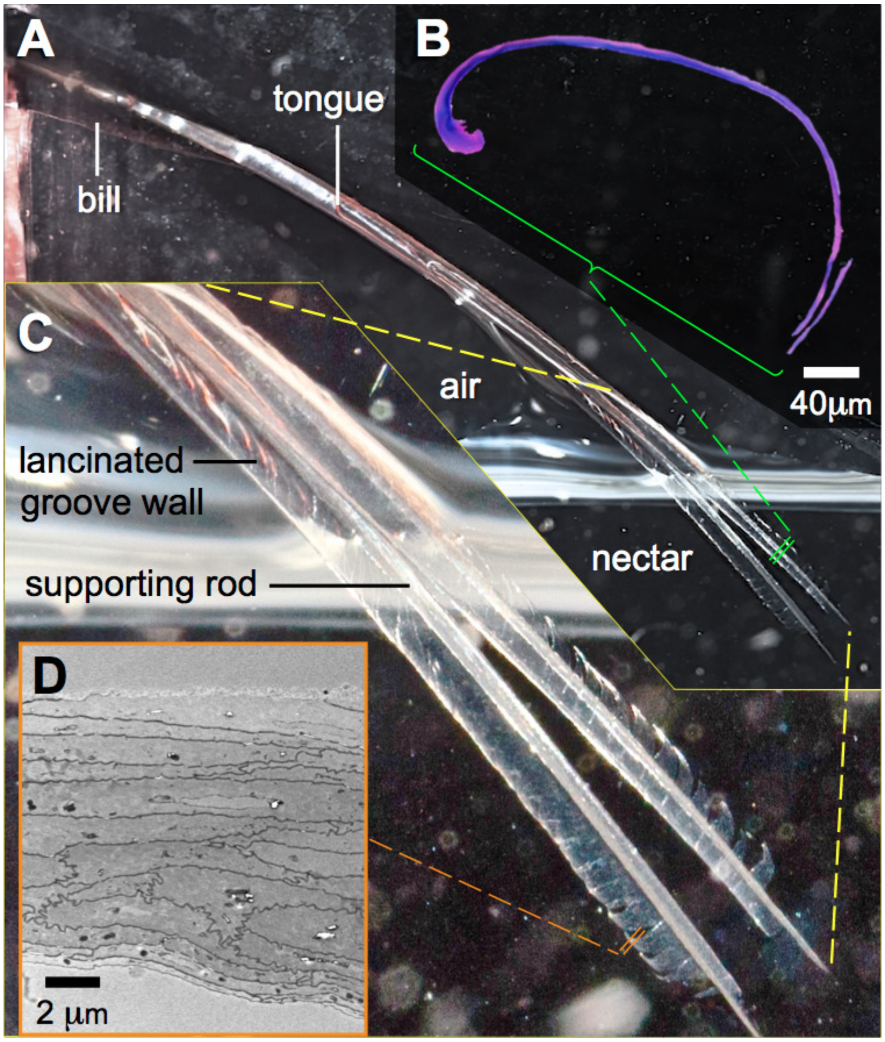


**Figure 8. Tongue groove morphology at the most distal portions (near the tip) in a Ruby-throated Hummingbird.** (A) Photograph showing the tongue protrusion, its bifurcation, and the relaxed morphology of the grooves inside the nectar (courtesy of Don Carroll). (B) Cross section (light microscope) 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 grove surface, and potentially facilitating the fluid trapping process described by Rico-Guevara and Rubega (2011).

*Microanatomy of hummingbird tongues*

Using the data from the microCT scans I digitally decoupled the feeding apparatus components (segmenting in Avizo©) and constructed three-dimensional models to study the bill and tongue match. Hummingbird tongues, as well as most avian tongues, correspond to the shape of the interramal region (oropharyngeal cavity floor), although commonly not to its size (*e.g.* Abou-Zaid and Al-Jalaud 2010; Tivane *et al.* 2011; review in Abumandour 2014). Nevertheless, it is worth noting that avian tongues are not larger than the oropharyngeal cavity (as it is the case in some nectarivorous bats, Muchhala 2006), instead, to reach far away from the tip of their bills, some hyoid apparatus ~~had~~ become greatly elongated (*e.g.* Video S5). In hummingbirds, the tongue grooves fit perfectly the rostral portion of the oropharyngeal cavity and match both lower and upper bill internal walls (Fig. 1), which is of vital importance for the efficient offloading of nectar (*cf.* Rico-Guevara and Rubega 2011) and intraoral transport (Rico-Guevara 2014). My study presents the first high-resolution (5-µm voxels) CT scan of a vertebrate tongue satisfactorily stained to highlight soft tissue. A study on flamingos presented detailed CT scans of the head (including the tongue) stained with a novel injection technique (Holliday *et al.* 2006), but it focused on vascular anatomy at lower resolution than in the present study. Within the last five years other studies have used a variety of techniques to enhance visualization of soft tissue in vertebrates (reviews in Gignac and Kley 2014; Lautenschlager *et al.* 2014; Gignac *et al.* 2016), but they have not been focused on tongues~~,~~ or worked at the micro scale of the present study. This three-dimensional modeling of hummingbird tongues allows for the clarification of some misconceptions; for instance, it has been suggested that the mathematical model derived for capillary filling provides a rationale for the shape of hummingbird tongues (Kim *et al.* 2012). 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 [$\mu$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 micropump a highly efficient device (Rico-Guevara 2014). This is because at the surface of the nectar pool, the attractive forces (adhesion and cohesion) holding the groove walls together get weaker because more molecules of fluid are available to fill the internal groove space. This creates an imbalance, with elastic forces dominating, ~~that~~ results in reshaping of the groove walls away from the flattened configuration. Molecules of nectar are pulled inside the grooves through the release of the elastic energy stored on the reshaping tongue groove walls (Fig. 6). ~~Since~~ the grooves are sealed on top (by surface tension in the zipped dorsal slit), the release of the elastic energy (reshaping of the grooves) pulls more and more nectar molecules inside the grooves until they reach a stable cylindrical configuration. The net result of this process is that the portions of the tongue that remain outside the liquid expand and are filled quickly with nectar, thereby improving fluid collection efficiency. Thus, the tongue filling is achieved through the transition from a high potential energy state (flattened grooves) to a low potential energy state (filled grooves). In summary, the elastic properties of the cornified layer make plausible our elasticity-induced flow hypothesis. This is ecologically relevant because when the bill tip is almost in contact with the nectar surface (most likely scenario in the wild given hummingbird flowers' internal morphology), the process described above is sufficient to fully load the fringed distal portion of the tongue. Nevertheless, when the bill tip is not in contact with the surface of the nectar (e.g. hummingbirds visiting flowers with corollas longer than their bills), but instead there is a space between the bill tip and the nectar pool, the portion of the tongue that remains outside the liquid would be filled with fluid by the interaction of the aforementioned physical forces in a process I hypothesize as follows: As the tongue is protruded the grooves are dorso-ventrally flattened by the bill tips, and once the tongue tip contacts the nectar surface the fluid starts to 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 grove 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.

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

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**Competing Interests**

The author declares that he has no competing interests.

**Animal Ethics**

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

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