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# Morphology and Function of the Drinking Apparatus in Hummingbirds

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# *Morphology and Function of the Drinking Apparatus in Hummingbirds*

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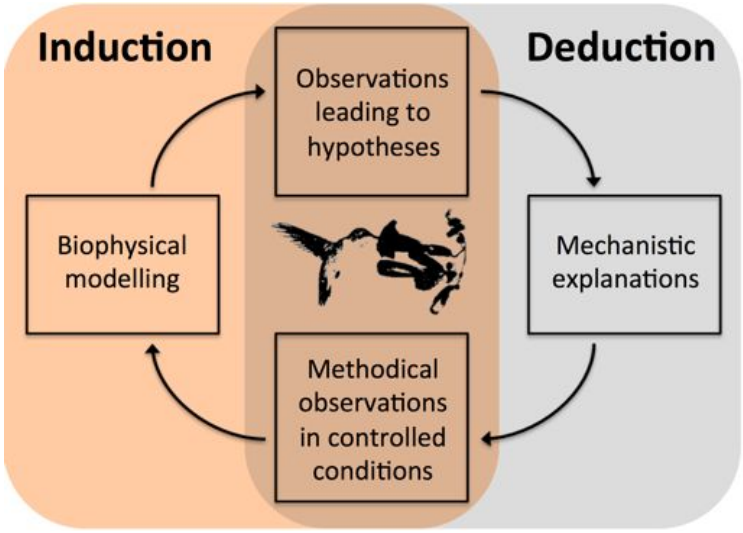
University of Connecticut, 2014

My research aims to answer the questions: How do hummingbirds feed? And, how do the mechanics of feeding define the limits and adaptive values of feeding behaviors? I meticulously study every step of nectar capture and ingestion. My dissertation chapters are organized following a morpho-functional and feeding sequence approach: 1) Feeding Apparatus Morphology; with emphasis on the understudied morphology of the tongue grooves and bill tongue coupling. 2) Tongue Tip Dynamics; how hummingbird tongues entrap nectar. 3) Tongue Grooves Functioning: how the tongue acts as an elastic micropump while collecting nectar. 4) Bill Tip Mechanics; internal bill structures that aid in offloading nectar from the tongue. 5) Intraoral Transport: how the nectar flows inside the bill to the throat where it can finally be swallowed.

My results demonstrate that capillarity equations are unsuitable to calculate energy intake rate, which is the building unit of foraging theories; therefore a development of a new theoretical framework to study hummingbird energetics and foraging ecology is needed. I describe previously unknown methods of tongue-based nectar collection, report undocumented tongue and bill structures, and offer the first test of intraoral transport hypotheses.

Alejandro Rico-Guevara – University of Connecticut, 2014

I followed the scientific method cycle of deduction and induction. To understand the determinants of hummingbird feeding mechanics in an ecological context, I tested biophysical model predictions using data from wild birds.




Elucidating the drinking mechanism of hummingbirds will facilitate downstream calculations of the rates at which birds can obtain nectar along several environmental axes (*e.g.* altitudinal and latitudinal ranges, migrations, corolla morphology, *etc.*). This will in turn inform how and where the limits of nectar uptake have shaped the distribution, ecology and evolution of hummingbirds.

*With their enchanting appeal and unique physical capabilities, hummingbirds captivate people of all ages. As such, they serve as ambassadors to the natural world, fostering public appreciation for scientific and conservation efforts aimed at preserving these fascinating birds, and the biodiversity upon which they depend.*

# Morphology and Function of the Drinking Apparatus in Hummingbirds

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# Chapter 1

## Ultrastructure and three-dimensional microanatomy of hummingbird tongues


### Abstract

A central challenge of biological studies is to describe the link between the underlying mechanisms (*e.g*. organismal morphology) and the emergent phenomena (*e.g.* performance, ecological, and evolutionary implications) seen in live organisms. To meet this challenge, it is necessary to identify and quantify the causal link between variation in traits and performance capabilities of their possessors. A complete understanding of the feeding structures is fundamental when the goal is to study how animals survive by obtaining energy in the most efficient manner. For hummingbirds, the tongue is the organ that enables exploiting the nectarivorous niche, and therefore it is of vital importance to study its anatomy and variation in the family. I used complementary techniques to study hummingbird tongues; histology, transmission and scanning electron microscopy, and micro-computed tomography to describe larger anatomical features and the three-dimensional arrangement of the tongue inside the bill. We assessed the variation in the structure of hummingbird tongues, by surveying 18 species covering the range of variation in length and curvature of hummingbird bills. We found that hummingbird tongues are unique among vertebrates in that they are composed mainly of cornified epithelium (beta-keratin), lack papillae, and fill entirely the distal portion of the mouth cavity. This puzzle-piece match between bill and tongue will be determinant for the study of intraoral transport of nectar in hummingbirds. Likewise, the structural composition and tissue architecture of the tongue groove walls provide the tongue with elastic properties that will be central to the study of tongue-nectar interactions during the feeding process.

## Introduction

When vertebrates started to colonize terrestrial environments (~ 370 mya, Ward *et al.* 2006) they faced a variety of challenges from locomotion to feeding (see recent review in Ashley-Ross *et al.* 2013). Around 300 mya tetrapods achieved novel feeding mechanisms (*cf.* Anderson *et al.* 2013); the tongue and hyobranchial apparatus took over the manipulation and transport of food through modulation of water flow in earlier aquatic vertebrates (Schwenk and Rubega 2005). The tongue is a key evolutionary novelty for terrestrial feeding (Schwenk 2000a), largely overlooked in earlier studies of feeding mechanics (Schwenk and Rubega 2005). Birds evolved a reduced tongue with reduced lingual musculature and tongue "joints" compensating for diminished manipulative capabilities (Tomlinson 2000), but then subsequently evolved the most morphologically diverse array of feeding structures among tetrapods (Rubega 2000). Hence, some birds evolved complex tongues, some of which are elongated and protractible beyond their bill tips to access their food (*e.g.* woodpeckers, Villard and Cuisin 2004; nectar-feeding birds, Paton and Collins 1989). Hummingbirds are arguably the most specialized nectar-feeding vertebrates (Stiles 1981, Fleming and Muchhala 2008), and have evolved to feed on flowers well enough to make their living out of spatially scattered, small volumes of nectar.

The first studies about hummingbird feeding inferred that the birds visited flowers searching for arthropods inside of them instead of nectar (Gould 1861, p. 15). In fact, it was believed that the bifid tongues were organs for prehension, extruding beyond their bills to access crevices or to delve in flower corollas in order to entrap insects (Audubon and MacGillivray 1856, Lucas 1891).

Now we know that hummingbirds indeed visit flowers to extract nectar, and that they are the group of vertebrates that have thrived in the nectarivore niche for the longest time (> 30 mya, Mayr 2004, Louchart *et al.* 2008, McGuire *et al.* 2014). Since hummingbirds collect the floral nectar using their tongues, we would expect that this organ would reflect extreme specialization. Hummingbird tongues have been studied for around two centuries, and 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), ending in two tube-like grooves with fringed edges (Lucas 1891). These grooves are exclusively distal structures and the interior of the tongue base is not hollow (Scharnke 1931, Weymouth *et al.* 1964). Weymouth *et al.* (1964) published the only histological micrographs available of a hummingbird tongue; other histologists only reported drawings (*e.g.* Scharnke 1931, Hainsworth 1973). There is only one study focusing on the morphology of tongue grooves (Hainsworth 1973), unfortunately lacking histological details. The most distal cross section micrograph presented by Weymouth *et al.* (1964) shows at least two distinct layers of tissue comprising the inner and outer surfaces of the tongue grooves, but the authors do not offer descriptions of them. Our studies on nectar feeding in living birds suggest that the functional traits making hummingbird tongues highly efficient at extracting liquid are related to the structural configuration of the tongue tip, rather than to active movements of their parts through muscle action (Rico-Guevara and Rubega 2011, Rico-Guevara 2014 Chapt. 3). A deeper study of the distal portion of hummingbird tongues is essential to understand the underlying architectural properties enabling the observed nectar extraction mechanisms.

The aim of this paper was to use cutting-edge techniques to study hummingbird tongue morphology; specifically, we used histology (image processing in Auto-Montage), transmission and scanning electron microscopy, focusing on the distal portion of the tongue or lingual grooves (understudied by Weymouth *et al.* 1964), and high-resolution X-ray computed tomography (microCT) to describe larger anatomical features and the three-dimensional arrangement of the tongue inside the bill. To our knowledge, there has only been one other study merging microCT, light, and electron microscopy in order to examine morphological features by linking them across disparate spatial scales; Handschuh *et al.* (2013) demonstrated the overimposition of these techniques on a juvenile bivalve. Lastly, in order to assess the variation in the structure of the lingual apparati in hummingbirds, we surveyed the gross morphology of the tongue of several species. We aimed to encompass the range of variation in length and curvature of hummingbird bills, under the assumption that these correspond to variation in lingual traits (*cf.* Zusi 2013).

## Methods

### *Morphological survey of museum specimens*

We examined the tongues of hummingbird specimens in the following museums: Instituto de Ciencias Naturales, Universidad Nacional de Colombia (ICN); National Museum of Natural History, Smithsonian Institution, Washington D.C. (USNM); the American Museum of Natural History, New York, NY (AMNH); and the Vertebrate Research Collection, University of Connecticut, Storrs, CT. We measured the lingual apparati at magnifications of 10-50x using Wild-Heerbrugge dissecting microscopes or a Leica GZ6 stereomicroscope.

Samples comprised 3 adult specimens per species of 18 hummingbird species; we included representatives of 8 out of the 9 currently recognized clades for Trochilidae (*cf.* McGuire *et al.* 2014), encompassing the full range of bill lengths and including the species with the most extreme bill curvature (90°), the White-tipped Sicklebill (*Eutoxeres aquila*). We measured exposed culmen (feathers to bill tip), total tongue length (tongue base to groove tips), groove length (taken for consistency in the right groove only, but we did not notice asymmetry), length of the fringed region (presence of lamellae on the right groove), bifurcation length (from the split point of the grooves to the tip of the right groove), and tongue thickness (right groove diameter at its base).

*High-resolution X-ray computed tomography (microCT)*

We dissected three salvaged specimens, a Ruby-throated Hummingbird (*Archilochus colubris*), an Anna's Hummingbird (*Calypte anna*), and a Short-tailed Woodstar (*Myrmia micrura*) to scan their heads. In order to obtain detailed morphological data at the micrometric scale and visualize the tongue soft tissues, we developed a staining protocol by modifying (via trial and error) a common technique for transmission electron microscopy using osmium tetroxide ($OsO_4$), but without embedding in resin (*cf.* Metscher 2009). Recently, a variety of alternative techniques have been used to enhance visualization of soft tissue during microCT imaging, especially by using iodine compounds (reviewed by Gignac and Kley 2014). We opted for osmium instead of iodine because, although they both seem to bind to lipids (Bozzola and Russell 1999, Gignac and Kley 2014), the former stabilizes tissue proteins and they do not coagulate during dehydration with alcohol (see below, 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 (30 minutes apart) of ethanol solutions (50, 70, 95, and 100% [vol/vol] ethanol–thrice–). The specimens were stored in 100% ethanol at 4°C and shipped with surrounding ice packs to be scanned at The University of Texas High-Resolution X-ray Computed Tomography Facility, using a custom built high-resolution X-ray computed tomographic scanner. Scans were performed at 70 kV and 10W, with Xradia 0.5 and 4X objectives, 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. We obtained 16bit TIFF images that were reconstructed by Xradia Reconstructor, and the total of slices per specimen was between 2223 and 2854, with scan times between 4 and 7 hours.

*Histological preparations*

We dissected one salvaged Ruby-throated Hummingbird (*Archilochus colubris*) to extract its tongue, which was cut into ~3-mm long sections and fixed (modified Karnovsky's fixative) 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 (50, 70, and 100% [vol/vol] ethanol), and embedded in epoxy resin (a mixture of Embed812, Araldite 502 and DDSA, blocks polymerized at 60°C for 48 hours). We obtained semi-thin cross sections (1 μm) with a glass knife using a Leica Ultracut Ultramicrotome, which were mounted on glass slides, and 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). We used Auto-Montage software (Syncroscopy Inc.) to compile images of multiple optical planes obtaining pseudo-planar fields of view with improved visualization of the tissue structures.

*Transmission electron microscopy (TEM)*

Using the fixed and embedded sections (epoxy resin processed in a Microwave Tissue Processor, Pelco Biowave Pro) of the tongue from the histological preparations, we 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), 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)*

We dissected two salvaged specimens, one Rufous Hummingbird (*Selasphorus rufus*) and one Ruby-throated Hummingbird (*Archilochus colubris*) to extract their tongues. The tongues were flattened with microslides (*e.g.* Fig. S1), 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 washed three times in the cacodylate buffer and then dehydrated in a graded series of ethanol solutions (50, 70, and 100% [vol/vol] ethanol). The first tongue was dried with a critical point dryer (Polaron E3000) for 2 h. During critical point drying (CPD) procedure, the sample (in this case the tongue) is put into liquid $CO_2$ under pressure, and the temperature is raised until the critical point is reached where the gaseous and liquid $CO_2$ have the same density and are miscible. Unfortunately, CPD caused the outer edges of the tongue in the distal region (forming the grooves) to spiral inward while drying, and only a small proportion of the inner surface of the tongue was visible after CPD.

Given that one of the objectives of our ultrastructural study was to characterize and contrast the inner and outer surfaces of the tongue grooves, we considered several potential solutions to overcome this obstacle in our second tongue:

1) To generate a surface replica using a variety of materials (*cf.* Goldman *et al.* 1969); since we were working on a very thin structure (~ 20 μm thick) that is curled to start with, we decided against this.

2) Use hexamethyldisilazane (HMDS) and other chemical drying methods (revision in Ting-Beall *et al.* 1995) in place of CPD. Given that chemical drying also produces shrinkage and distortion, we also decided against this.

3) Environmental SEM or cryo-SEM. Special facilities, which we did not have access to, are required, so this solution was not available.

4) Pinning or wiring the tongue in the desired position. Because of the small absolute size of the tongue grooves, a large percentage of tissue would have been damaged by either the pins or wires, and by the attaching surface, and thus we rejected this alternative.

5) Making partial horizontal cuts perpendicular to the long axis to relieve stress during drying, and realigning just after CPD when the tissue is still elastic. The problem with this approach is that in the distal portion of the grooves there are already natural incisions of the tissue (forming the lamellae) which are oriented approximately at a 45 degree angle to the long axis of the tongue, thus perpendicular cuts would result in the loss of tissue.

6) Inserting minute pins through the tissue along the long axis of the tongue. At tongue tissue thicknesses of ~ 20 μm (at the margins of the lamellae) this would be extremely challenging, and we did not attempt it.

7) Cutting the tongue into sections, and reassembling them on the stub after drying. We were doubtful that we could successfully reconstruct the overall appearance of the tongue, and concerned about damage at edges of the cuts, both important in mapping tissue types on the photographed regions afterwards.

8) Processing in polycarbonate envelopes or biopsy bags stapled or sewn flat. We believed stapling and/or sewing would likely produce damage similar to the wiring alternative.

9) Finally, we opted for using nylon mesh biopsy capsules and tissue cassettes to keep the tissue from spiraling inward. We inserted the tissue between layers of filter paper (chemically stable and allows adequate fluid exchange) to prevent mechanical damage from the mesh. The end result was fortuitous, in that although the tongue surface did not remain flat on the second sample (it twisted longitudinally), we could visualize and photograph the regions of interest, including equal access to both inner and outer surfaces, using SEM.

After CPD, we sputter coated (Polaron E5100) the tongues with gold and palladium, and attached them to aluminum SEM stubs using double-sided carbon tape and coating the anterior ends of the tongues with silver paint, connecting them to the aluminum stubs in order to reduce charging effects. We 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. The tongues were stored in a vacuum desiccator at the UConn EM Laboratory during imaging intervals.

## Results

*Morphological survey of museum specimens*

We found that bill and tongue lengths do not scale isometrically among hummingbird species (squares in Fig. 1). In short-billed species the tongue is disproportionately longer than the bill (exposed culmen) relative to long-billed species; for instance, the shortest tongue in our survey belonged to a Purple-Backed Thornbill (*Ramphomicron microrhynchum*) and was 11.9 mm, about twice as long as the bird's bill (6.1 mm), while the longest tongue belonged to a Sword-billed Hummingbird (*Ensifera ensifera*) and was 119 mm, just slightly longer (relatively) than its bill (103 mm). In fact, almost all the species sampled had tongues longer than their bills; the only exception was the White-tipped Sicklebill (*Eutoxeres aquila*) in which the exposed culmen was slightly longer than its tongue (top left, Fig. 1). In a similar way, tongue thickness does not increase proportionally to tongue length across species (filled circles in Fig. 1); although there is a trend of increasing thickness from short to long-tongued species, no species has an average tongue thickness above 0.73 mm (Table S1). On the contrary, groove length scales isometrically with tongue length (Fig. 1, Table S3); the grooves always comprise (slightly more than) the distal half of the tongue across all the hummingbird species sampled. Excluding the Sword-billed Hummingbird from the dataset, bifurcation length seems to increase diffusely with tongue length across species with a similar slope to the groove-total tongue length relationship, and fringed region length appears to also increase diffusely but with a much lower slope. Interestingly, in the Sword-billed Hummingbird both bifurcation and fringed region lengths are shorter than the expected for its tongue length (bottom right, Fig. 1).

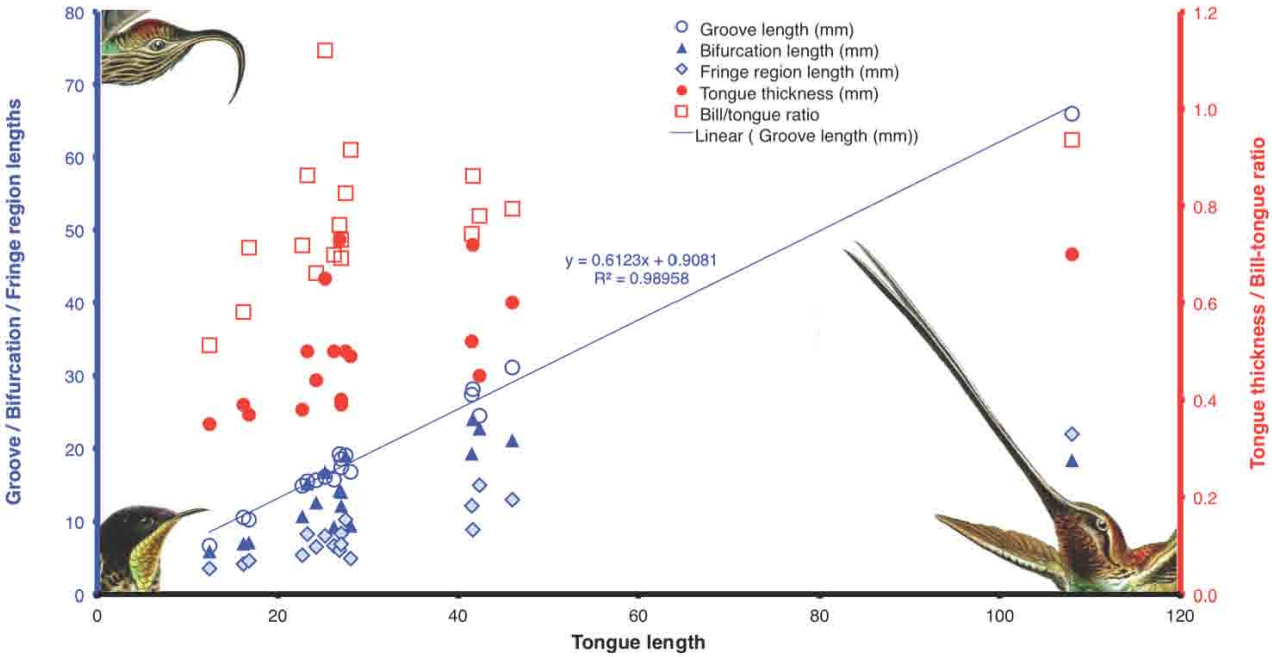


**Figure 1. Tongue and bill gross morphology comparisons among hummingbird species.** On the *X-axis* the total tongue length (see Table S3 for sampling details) is plotted against *left Y-axis*, which includes groove (blue empty circles), bifurcation (blue triangles) and fringed region (blue diamonds) lengths -all in mm-, and against the *right Y-axis* that includes tongue thickness (red filled circles) -in mm- and the dimensionless ratio between bill and tongue lengths (red squares). Note the unusual bill/tongue ratio above 1 in the Sicklebill (top left), the low values for all the parameters in the Thornbill (bottom left), and the gap between tongue lengths of all of the species and the Swordbill (right). Drawings by Ernst Haeckel (public domain) do not cover any data points.

*High-resolution X-ray computed tomography (microCT)*

We successfully used a staining protocol with osmium tetroxide (see methods) to allow visualization of soft tissue at the micro-scale. We achieved superior imaging of low-density (electron-lucent) materials, for example the cornified epithelium the tongue is made of; other computer tomography procedures do not provide enough resolution beyond dense tissues like bones (Figure 2). We used an Xradia MicroCT scanner to recreate virtual models of the internal three-dimensional arrangement of the lingual structures when the bill is closed (*e.g.* Movie S1).

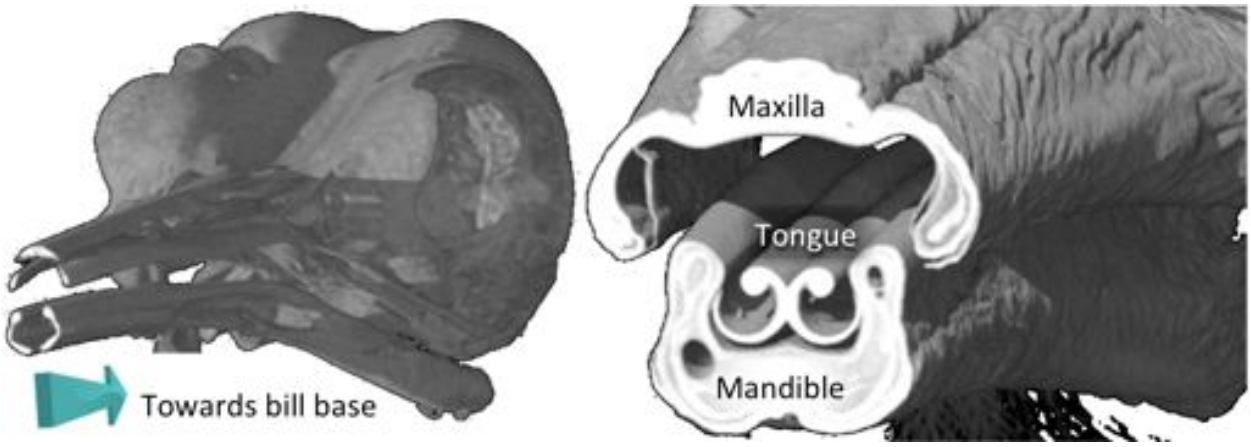


**Figure 2. Comparison of the image resolution between reconstructions.** Contrast the Micro-CT scan without staining soft tissues (skull on the left) and a Micro-XCT scan using $OsO_4$ staining (zoom to bill and tongue on the right). Note how with this enhanced visualization technique, the fit between tongue and bill becomes observable.

We present the first complete cross-section series of a hummingbird feeding apparatus, which is vital for understanding the potential functional interactions between bill and tongue. We started with the most proximal section selected arbitrarily at the nasal operculum (Fig. 3, cross section [XS] 1), however we include a complete head scan in which the entire hyobranchial apparatus can be visualized (Movie S2). In Fig. 3 - XS1, it is noticeable that the tongue is dorso-ventrally flattened, and the tongue body has started to divide into two chambers due to an ingrowth of the dorsal and ventral epithelia (*cf.* XS 11 in Weymouth *et al.* 1964). At XS 1 we observe a dark layer of tissue almost completely surrounding the lingual body; this layer becomes thicker at the ingrowth region and eventually connects (when moving distally through cross sections), separating the two chambers of tissue (Fig. 3, XS 2; *cf.* XS 13 in Weymouth *et al.* 1964). At XS 3 the dark layer, presumably keratinized tissue, becomes even darker; and the thin layer of tissue external to the dark layer, on the dorsal and ventral ingrowth regions, starts to disappear. In this section the semi-cylindrical configuration characteristic of the tongue grooves (*cf.* Fig. 2) is already conspicuous (*cf.* XS 14 in Weymouth *et al.* 1964).

At XS 4 is apparent that the tissue inside the former chambers of the lingual body is thinner, leaving an empty space dorso-laterally (*cf.* XS 15-17 in Weymouth *et al.* 1964), and the dorsal supporting rods (Fig. 4A) become thicker and more robust, probably because they are the sole structural support of the distal half of the tongue. By XS 5 there is no tissue inside the keratinized semi-cylindrical grooves, and the two sides of the lingual body are completely separated (*i.e.* bifurcated tongue).

It is worth noting that there is almost no change between the tongue appearance and size between XS 5 and 6, which is about 3 mm and corresponds 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 distal portion of the tongue (Fig. 3, XS 9-10) is characterized by a reduction of the rods and a thinning in the keratinized tissue comprising the grooves (*cf.* Fig. S7).

This staining technique allowed us to assess the internal spaces of the bill throughout its entire length, but an in-depth description of the bill structures will be given in Chapter 4. It will suffice to say that from XS 1 to 4 it is evident how the tongue fills the internal spaces (when the bill is shut), leaving only a small space dorso-laterally. Such space matches the position of the two flaps present at the tongue base in hummingbirds also called “tongue wings” (Scharnke 1931, XS 2 in Weymouth *et al.* 1964). From XS 5 to 10, a reduction in the internal space and a tighter coupling between bill and tongue shape is evident.

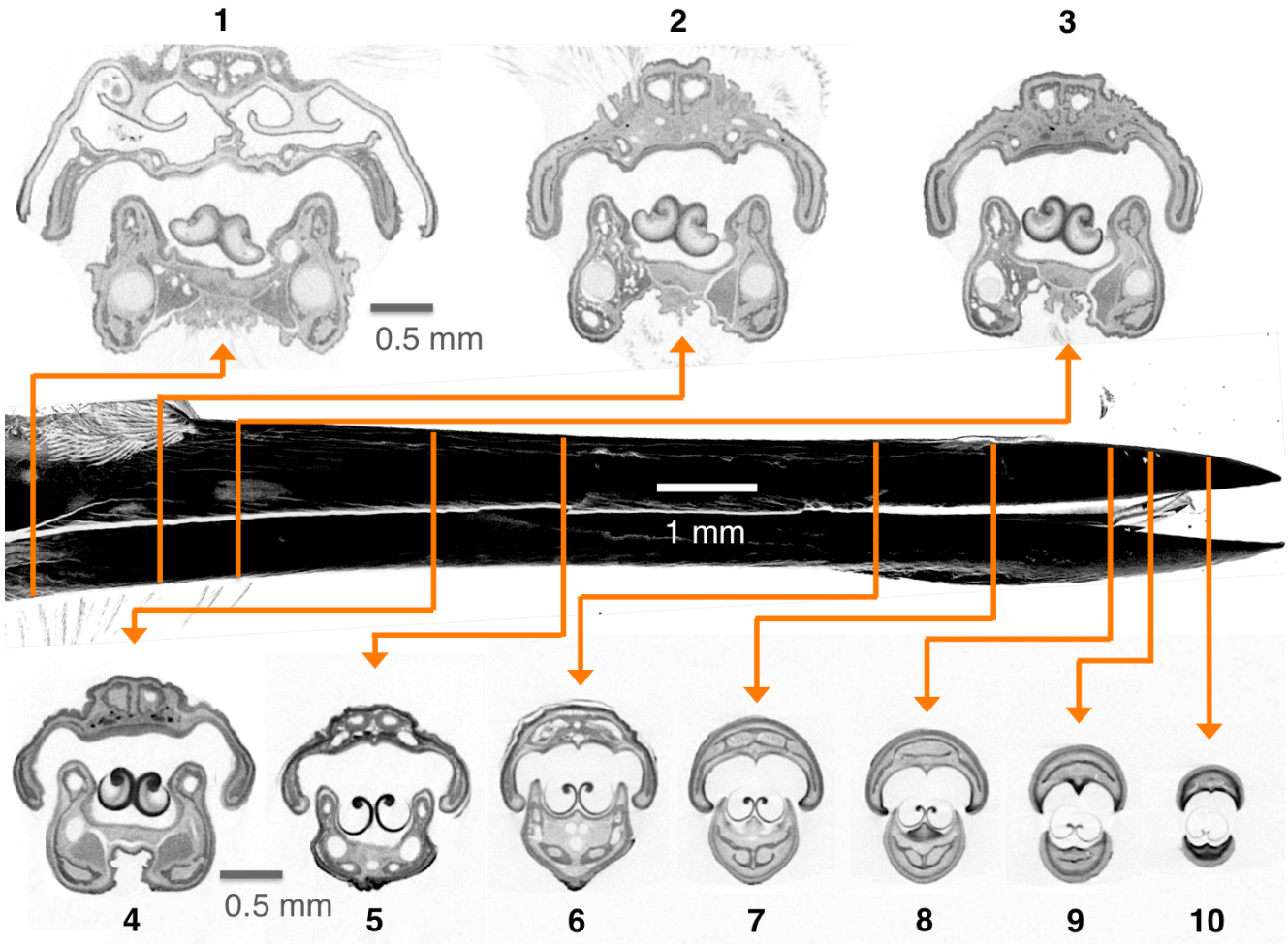


**Figure 3. Selected cross sections (1-10) from a microCT scan of a hummingbird bill and tongue.** Black structure in the middle of the figure is a lateral view of the bill from the reconstructed scan, and the orange lines crossing it correspond to the cross sections pointed at by the arrows.

*Histological preparations*

We successfully reconstructed pseudo-planar fields (using Auto-Montage) in which we could study the different tissues in the cross sections at magnifications up to 1,000x (Fig. 4).

We focused our observations in the distal half of the tongue where its edges roll inwards forming semi-cylindrical grooves (Fig. 4A), to complement the work of Weymouth *et al.* (1964) focused on the proximal half. On the inner surfaces of the grooves, which developmentally seem to correspond to the dorsal surface of the tongue (discussion on *microCT* results), we found keratinized stratified squamous epithelium (with some nuclei still visible, Fig. 4B). This stratified epithelium included stratum granulosum, spinosum and (mostly) corneum in a gradient with higher proportions at and near the supporting rod (Fig. 4B 1000x, Fig. S3) decreasing towards the tongue edges (outward groove walls, Fig. 4B 400x). On the outer surfaces of the grooves, which developmentally seem to correspond to the ventral surface of the tongue, we found a relatively thick keratinized band when compared to the stratum corneum in the inner surface (Fig. 1B 1000x). The outermost half of the groove wall in cross section (*cf.* Fig. 4A), is entirely composed of the keratinized band due to the disappearance of the dorsal stratified squamous epithelium (Fig. 4B, Fig. S4).

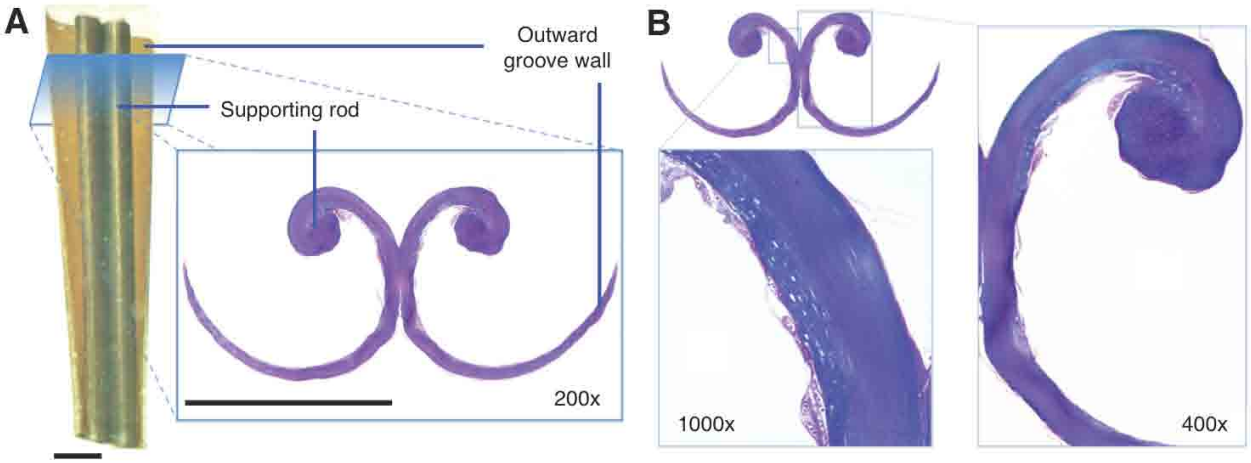


**Figure 4. Low magnification morphology of the distal half (grooves) of a hummingbird tongue. A)** On the left, a section of the tongue embedded in resin. On the right, a cross section (light microscope) showing the semi-cylindrical configuration of the grooves. Unlabeled scale bars = 250 μm. **B)** Histological details of the supporting rod and groove walls, showing the different epithelial strata and keratinized band.

*Transmission electron microscopy (TEM)*

We were able to clearly visualize cell boundaries, dark corpuscles, and even bacteria in the inner surface of the hummingbird tongue grooves (Fig. 5A). We found that the layers of tissue were thinner (more closely compacted together) near the inner surface in comparison to the outer surface (Fig. S5).

We also observed irregular elliptical dark spots in the stratified epithelium near the inner surface of the grooves (black arrow head, Fig. 5A; Fig. S6), which likely are keratohyalin granules. We also found smaller, elliptical-to-circular dark spots distributed more evenly throughout the tongue tissue (white arrow head, Fig. 5A; Figs. S4, S5, S7), possibly melanin granules.

We noticed that the cell boundaries were continuous lines of corneo-desmosomes (*e.g.* black arrow, Fig. 5B) binding the keratin filaments across cells. Zooming in, the diameter of the microfibrils was ~35 Å (*e.g.* white arrow, Fig. 5C) similar to what it has been found in feathers (*cf.* Filshie and Rogers 1962).

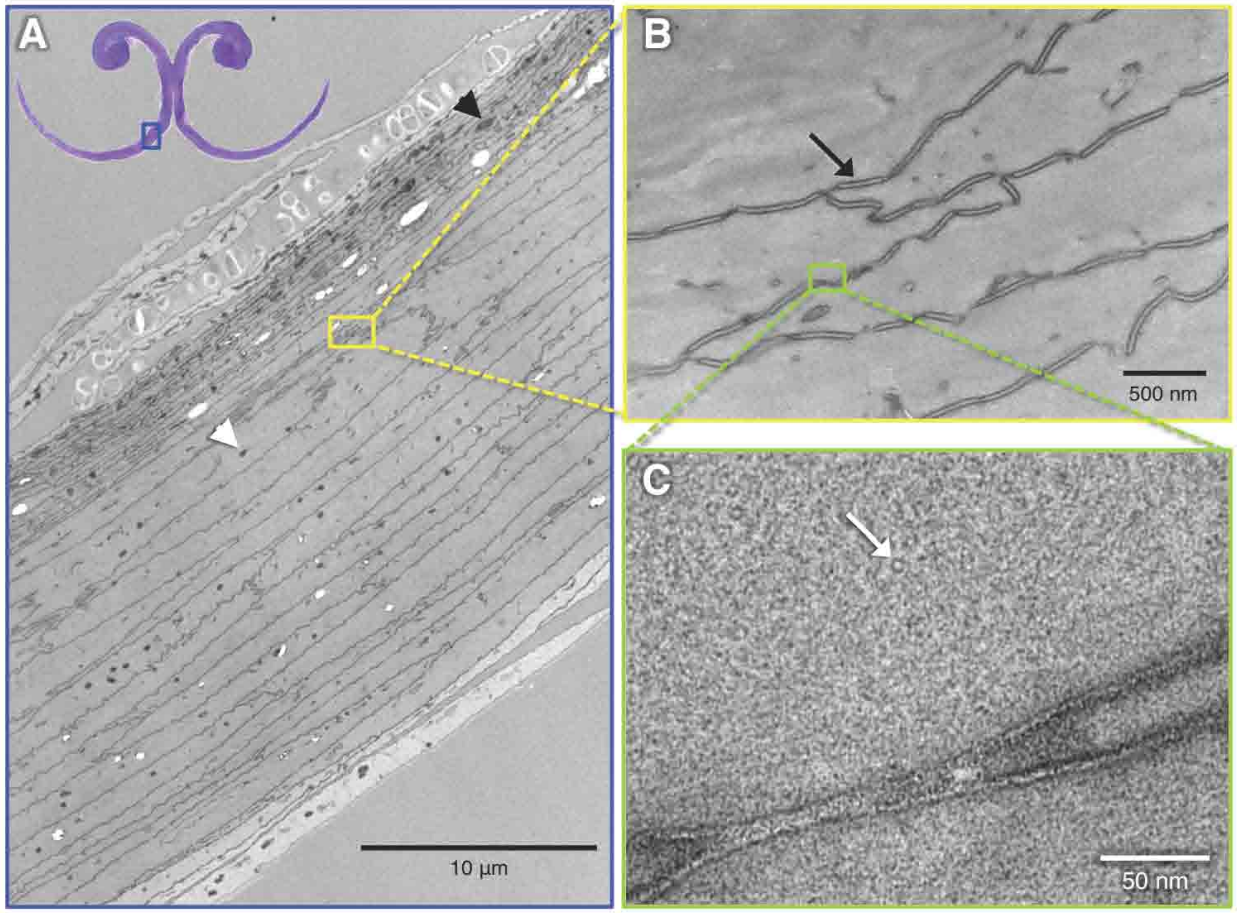


**Figure 5. High magnification morphology of a cross section at the distal half (grooves) of a hummingbird tongue. A)** Transmission electron micrograph showing the difference in layer composition (more densely packed near the inner surface), keratohyalin (black arrow head) and melanin (white arrow head) 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 $RuO_4$.

We found bacteria growing on the inner surface of the hummingbird tongue. Bacterial growth was restricted to the inner (dorsal) most external tissue layers in the region near the groove rod (Fig. 6A). We did not find bacteria in the most distal portions of the tongue, where the rod has almost disappeared (Fig. 4B, S4). We identified coccus-shaped (*e.g. Staphylococcus sp.* Onyango *et al.* 2012) and bacillus-shaped bacteria (Fig. 6B) similar to the ones found in rat tongues (Picoli *et al.* 2006), our only reference because lingual microbial fauna is understudied in birds (*e.g.* Carvalho *et al.* 2003).

One possibility is that the bacteria we found are keratinolytic (*e.g. Bacillus sp.* Williams *et al.* 1990, Pandian *et al.* 2012), and they were degrading the tongue surface; another possibility is that the microorganisms we found are actually remnants of the microbial communities found in floral nectar (bacteria, Álvarez-Pérez *et al.* 2012, Vannette *et al.* 2013; yeast, Herrera *et al.* 2008, 2009).

The occurrence of layers of tissue interspersed with the microbes is puzzling; if they are living in keratinized pouches in the tongue surface they could be commensals and/or be using the birds to colonize recently opened flowers (*e.g.* Aizenberg-Gershtein *et al.* 2013).

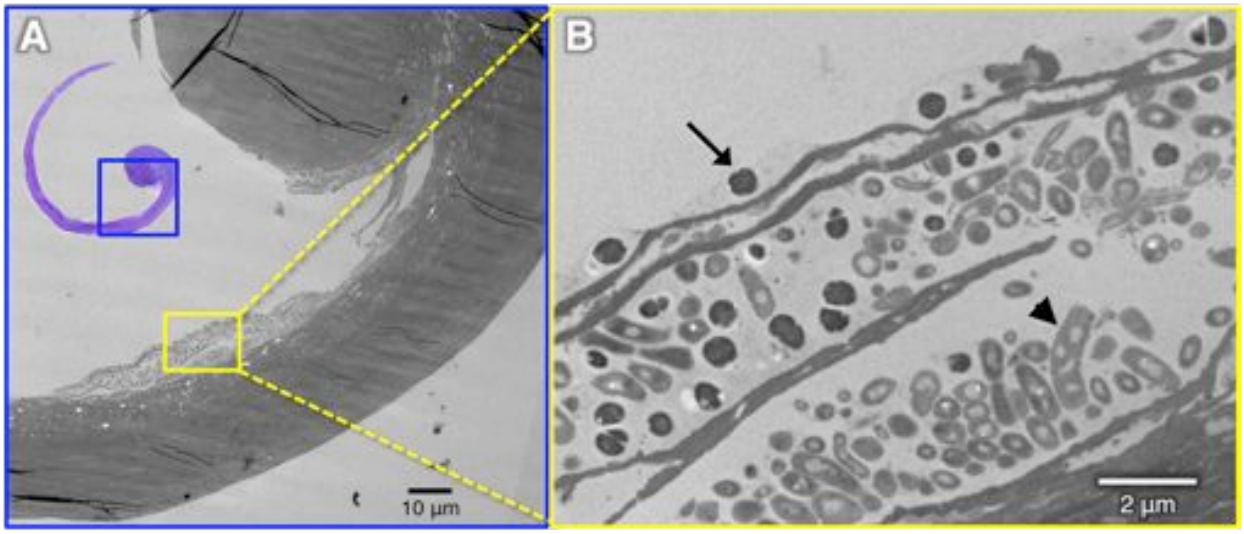


**Figure 6. Bacteria in a hummingbird tongue. A)** Transmission electron micrograph showing bacterial foci along the inner surface of the tongue groove. On Formvar, stained with uranyl acetate (UA) and lead citrate (LC). **B)** Coccus-shaped (black arrow) and bacillus-shaped (black arrow head) bacteria intercalated with keratin layers. On Formvar, stained with UA and LC.

*Scanning electron microscopy (SEM)*

We found qualitative differences between the inner and outer surfaces of the tongue grooves (Fig. 7). At the 10-μm scale the outer tongue groove surface exhibited granulated regions with a seemingly random distribution. In contrast, at the same scale, the inner groove surface lacked dense granular regions, *i.e.* it was smoother.

We zoomed in a non-granular region of the outer surface, and at the 500-nm scale it presented a rougher aspect than the inner surface (Fig. 7, *bottom right*). Given that the accelerating voltage can alter the level of surface detail visualized we were careful to maintain constant 2 kV for our comparisons (Fig. 7).

It would be desirable to quantify differences in roughness between the inner and the outer surfaces of the tongue; 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) include the use of optical interferometry (*e.g* white light scanner), and 3-D reconstructions of tilted SEM micrographs (stereomicroscopy) using commercial (*e.g.* MeX by Alicona) or open source (*e.g.* Gwyddion) software, however the use of these methods was outside the scope of this study.

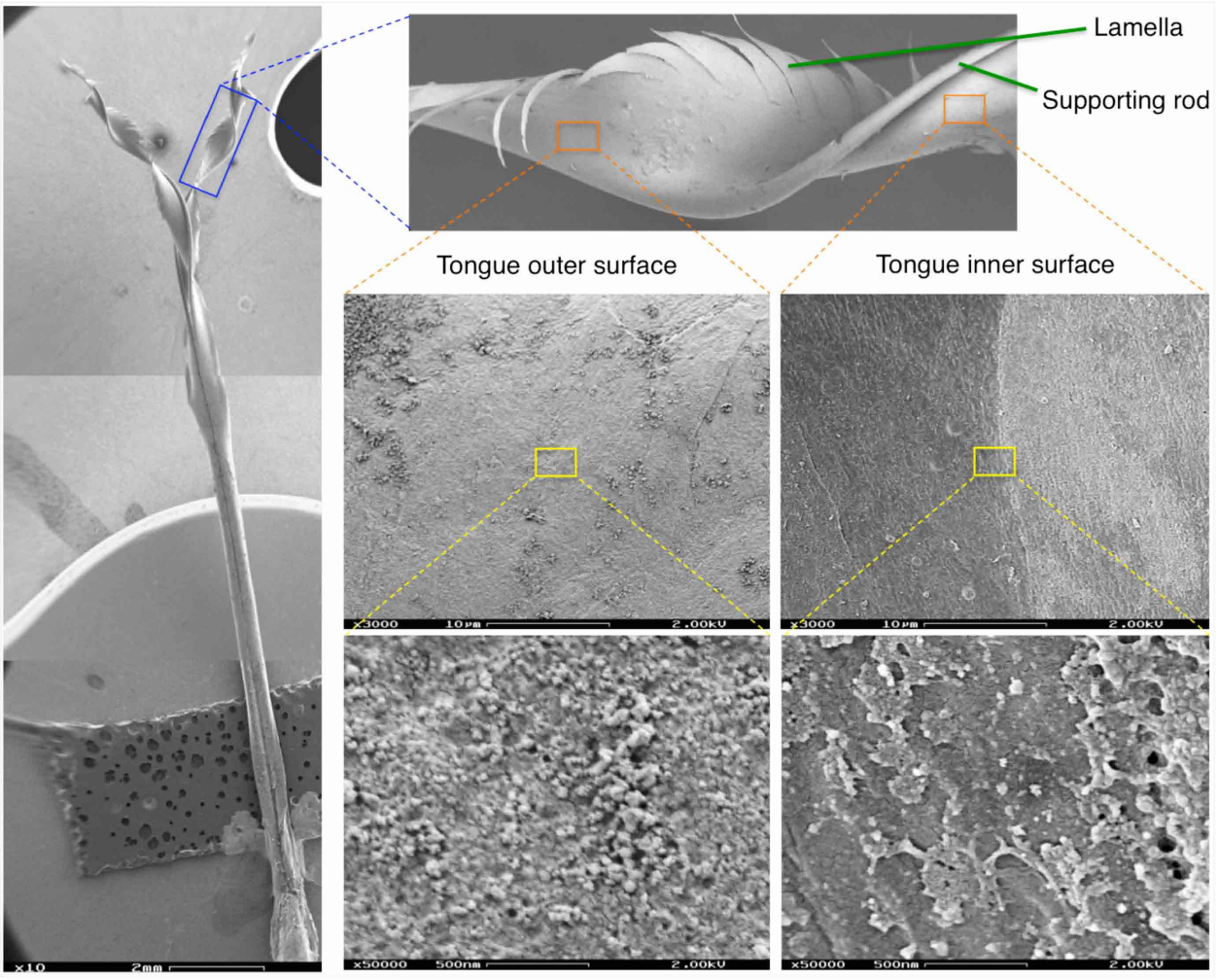


**Figure 7. Scanning electron microscopy on a hummingbird tongue.** On the left, an overview of the entire tongue, our observations focused on the distal half (grooves). On the top right a close up of a longitudinally twisted section of a tongue groove, indicating the supporting rod and lamellae. On the middle and bottom right, micrographs of the inner and outer surfaces of the tongue grooves, showing qualitative differences in rugosity.

We found structures on the inner (dorsal) surface of the hummingbird tongue (*e.g.* Fig. 8) that do not seem to correspond to commonly found structures in other bird tongues (*e.g.* taste buds of salivary glands, *cf.* Kudo *et al.* 2008, Guimarães *et al.* 2009, Erdoğan *et al.* 2012a). Additionally, no taste buds were found in the distal half of the tongue of several birds studied by Moore and Elliott (1946), Nalavade and Varute (1977) and Kudo *et al.* (2008). If the unidentified structure (Fig. 8) accomplishes any sensorial function (*e.g.* Leitner and Roumy 1974a, b; Berkhoudt 1979; Jackowiak *et al.* 2011) it must be innervated.

However, the few studies that have investigated neuro-histology in bird tongues have not reported nerves in the distal portions of the tongue (Scharnke 1931, Weymouth *et al.* 1964, Purwar 1975, Baecker *et al.* 1983, Wild 1990).

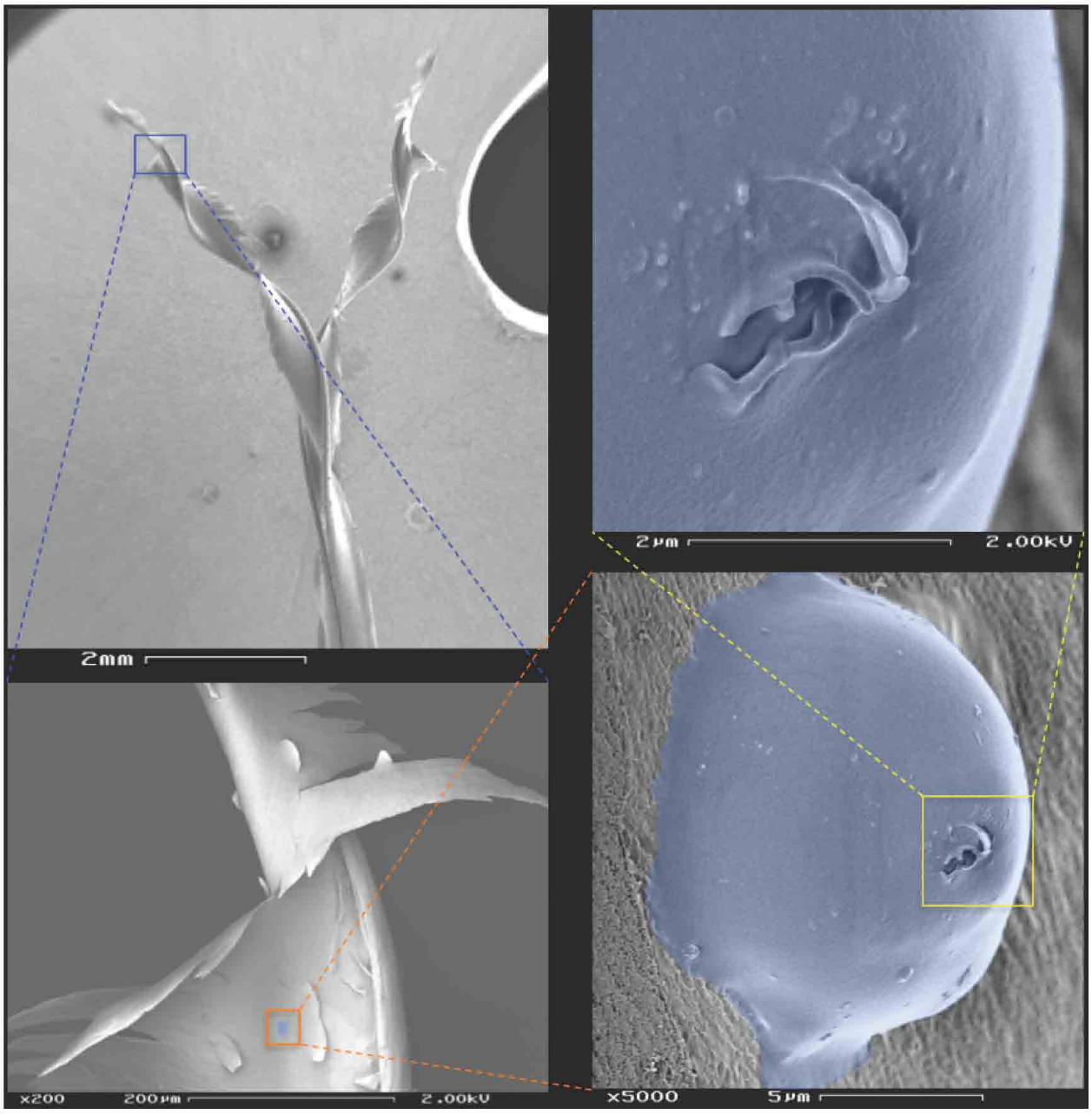


**Figure 8. Scanning electron micrographs of unknown structures in a hummingbird tongue.** Top left, an overview of the distal half of the tongue (grooves). Bottom right, close up of a rip in the tongue tissue caused by our attempts to flattening it during critical point drying. Bottom left, a zoomed micrograph on the inner surface of the tongue groove in which a peculiar protuberance can be observed (highlighted blue). Top right, close up to the top of the protuberance showing the details of the structures found.

## Discussion

Although bird tongues were important in early ornithological studies because of their originally conceived taxonomic value (Lucas 1896, Gardner 1925), they were left in oblivion with only a few thorough works on functional morphology of bird feeding (*e.g.* Homberger 1980). Recently however, an upsurge in morphological work has been published after the advent of electron microscopy (review in Erdoğan and Iwasaki, 2013). Here we discuss our morphological findings in the context of what has been found in other birds, discussing the functional implications of the structural composition and architectural organization of hummingbird tongues.

### *Gross morphology of hummingbird tongues in the context of vertebrate lingual apparati*

Most bird tongues differ from mammal tongues in the absence of elaborated musculature (with parrots as the only exception, *cf.* Schwenk 2000b). Birds control the movement of their tongues by muscles attached to the hyobranchial apparatus, the "intrinsic hylolingual muscles" (Tomlinson 2000, but see Schwenk 2001) which find their most anterior attachments in the *Os entoglossum* (Newton *et al.* 1896, *cf. Paraglossals* Weymouth *et al.* 1964, Fig. S10). Some birds have to protrude their tongues to procure their food, *e.g.* woodpeckers (Shufeldt 1900), and nectar-feeding birds (Paton and Collins 1989); woodpeckers however, have the ability to actively change the direction of the tongue tip (*cf.* Bock 1999), a capacity that is lacking in hummingbirds (Zusi 2013). In most birds, the distal third of the tongue is entirely free of musculature (review in Erdoğan and Iwasaki, 2013) but in hummingbirds from half (Scharnke 1931, Weymouth *et al.* 1964) to three fourths of the tongue lacks muscles, bone and/or cartilage support.

Two longitudinal rods provide longitudinal rigidity to the distal membranous tube-like grooves in hummingbird tongues (Fig. 1 in Rico-Guevara and Rubega 2011, Figs. 3, 4), thinning gradually until disappearing at the tips (Fig. 3). Therefore, hummingbirds rely solely on tongue-fluid interactions to collect their main source of energy, the floral nectar. Evolution has shaped their tongues to take advantage of the nectar properties (*e.g*. surface tension) and the elastic properties of the tongue walls to create highly efficient micropumps (Chapter 3).

We found that hummingbird tongues lack papillae, a rare condition in vertebrate tongues (Schwenk 2000b, Iwasaki 2002) and even in birds (review in Erdoğan and Iwasaki, 2013). Avian lingual papillae are involved in manipulation of solid food (*e.g.* holding, cutting, filtering, shelling, Iwasaki *et al.* 1997, Kobayashi *et al.* 1998, Jackowiak *et al.* 2010, 2011) and proximal intraoral transport of solid items (reviews in Parchami *et al.* 2010a, Elsheikh and Al-Zahaby, 2014); however, hummingbirds have singular feeding modes. First, about half of their diet (*cf.* Stiles 1995) is composed of floral nectar that is collected inside the tongue grooves, a process which 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 in the subfamily Trochilinae are mostly captured by flycatching (Stiles 1995, Rico-Guevara 2005). 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; in this case little or no lingual transport is necessary.

Other hummingbirds, especially from the other hummingbird subfamily (Phaethornithinae), consume more substrate-captured prey (*e.g.* spiders, Stiles 1995), than do reproductive females of many species across the entire family (Rico-Guevara 2008, Hardesty 2009). In the process of consuming substrate prey, or prey that are generally captured near the bill tip, hummingbirds can use inertial transport (*cf.* Mobbs 1979, catch and throw, Zweers *et al.* 1997, or cranioinertial feeding, Tomlinson 2000, Gussekloo and Bout 2005; ballistic transport, Baussart *et al.* 2009, Baussart and Bels 2011, Harte *et al.* 2012) while flying, or lingual transport (Yanega 2007). Hummingbirds' lack of lingual papillae may be explained by their liquid food collecting method (grooves with smooth surfaces are easier to extrude nectar from) and their arthropod hunting and consumption strategies. When using lingual transport, hummingbirds use the base of their tongues (Yanega 2007) where they present two backward projections corresponding to the papillary crest edges in other birds (*i.e.* tongue wings, Scharnke 1931, Weymouth *et al.* 1964, Figs. S11 and S12). Hummingbirds can reach the distal portions of their bills with their tongue wings, 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 containing part of the hyobranchial apparatus (*i.e.* the basihyal, Weymouth 1964, Fig. S11).

The association between hummingbirds' ability to protrude their tongues beyond their bills tips and their nectarivore lifestyle, specifically to probe deep inside the flowers, was recognized early on (*cf.* Martin 1833, Darwin 1841, Lucas 1891). Yet, the selective forces and limits to the extent of tongue protrusion are still poorly understood.

In some hummingbird species extensible tongues may provide access to a wider spectrum of resources (*e.g.* by allowing them to visit flowers with corollas longer than their bills, usually pollinated by other hummingbird species). However, at the end of all floral resource spectra there is one species which would not need to reach further to cover all possible conspecific niches: the Sword-billed Hummingbird (*Ensifera ensifera*).

A proxy for maximum tongue protrusion is the length of the hyobranchial apparatus. The basihyals and ceratobranchials are relatively constant in length among hummingbird species, but the length of the epibranchials (which wrap around the skull, Fig. S13) is highly variable (Table 2 in Zusi 2013, but see Table S2). In woodpeckers, which also exhibit long tongue protrusion, the epibranchials surround the skull and end either inside one of the nostrils, or encircling the orbits (Shufeldt 1900). In some hummingbird species the tips of extremely elongated epibranchials end in one of the nasal cavities as well (*e.g.* species of the genus *Heliodoxa*, Fig. 29 in Zusi 2013).

We found that the free ends of the epibranchials of *E. ensifera* insert through the maxillary rhamphotheca following the maxillary dorsal bar for about 25 mm (Fig. S14); hummingbirds from the genera *Heliodoxa* and *Ensifera* present extended basal feathering at the bill base (*cf.* Hilty and Brown 1986) possibly related to extra space beneath loose skin covered with feathers for the insertion of the epibranchials. In the Long-billed Hermit (*Phaethornis longirostris*) the movement of the epibranchials in and out of the nasal region is visible beneath the thin and flexible maxillary rhamphotheca while the bird is drinking nectar (pers. obs.).

In the hummingbird species with the shortest tongue ~ 12.4 mm (Table S1), the Purple-Backed Thornbill (*Ramphomicron microrhynchum*), the hyobranchial apparatus is ~ 20 mm long (Fig. S14), while in the species with the longest tongue ~ 108 mm (Table S1), the Sword-billed Hummingbird (*E. ensifera*), the hyobranchial apparatus is ~ 67 mm long (Fig. S15).

In 6 species sampled, the hyobranchial apparatus is 2 to 3 times as long as the tongue grooves, but in the Sword-billed Hummingbird this ratio is 1:1 (Table S2). It is possible that most hummingbird species (other than *E. ensifera*) may be able to protrude their tongues as far as their tongue length permits (epibranchials are as long as their tongues, Table S2), but it seems that *E. ensifera* only needs to protrude its tongue as far as necessary to withdraw nectar from the tongue grooves by squeezing them with its bill tips. This hypothesis is consistent with our tongue measurement results; both bifurcation and fringed region lengths in *E. ensifera* are substantially shorter than expected for its tongue length (Fig. 1).

Bifurcation and the appearance of lamellae (the fringed region) seem to be related to channeling and extruding the tongue through a wringer device at the tongue tips with central dividers (prongs) and serrated, flexible tomia (unpub. data). Studying the drinking mechanics of morphological extremes like *E. ensifera* or the White-tipped Sicklebill (*Eutoxeres aquila*), with its relatively short tongue (Fig. 1), large tongue wings (Fig S12), and strongly decurved bill and tongue (Fig. S16), would shed light on the constraints of nectar-feeding related traits in hummingbirds.

*Ultrastructural uniqueness of hummingbird tongues*

A striking result from our SEM micrographs is that both dorsal and ventral surfaces of the distal portion of the hummingbird tongue (Fig. 7) are smoother compared to the tongue surfaces of other birds (*e.g*. myna and wagtail, Dubale and Thomas 1978; bean goose, Iwasaki *et al.* 1997; budgerigar, Martinez *et al.* 2003; sea eagle, Jackowiak and Godynicki 2005; cormorant, Jackowiak *et al.* 2006; peregrine falcon and kestrel, Emura *et al.* 2008; ostrich, Jackowiak and Ludwig 2008; owl, Emura and Chen 2008, Emura *et al.* 2009a; woodpecker, Emura *et al.* 2009b; eagle, Parchami *et al.* 2010a; nutcracker, Jackowiak *et al.* 2010; finch, Dehkordi *et al.* 2010; emu, Crole and Soley 2010; quail, eagle, Parchami *et al.* 2010b; rhea, Santos *et al.* 2011; hoopoe, El-Bakary 2011; domestic goose, Jackowiak *et al.* 2011; raven, Erdoğan and Alan 2012; seagull, Onuk *et al.* 2013; kingfisher, El-Bakary 2012, El-Beltagy 2013; crow, Elsheikh and Al-Zahaby, 2014; SEM micrographs in these papers were taken at magnifications comparable to those in our Fig. 7). The tongue apex in all of these birds is covered by desquamate non-keratinized stratified epithelium; this is probably the result of constant abrasion and the need to replace the keratinized protective layer (but see Skieresz-Szewczyk *et al.* 2012).

In hummingbirds, however, contact with solid items is not expected to influence the tongue surface morphology, despite the original notion that hummingbird tongues have evolved to trap minute insects as well as nectar inside the flowers (Audubon and MacGillivray 1856, Gould 1861, Lucas 1891).

In contrast, most of the wear of hummingbird tongues seems to be happening at the edges of the groove walls, where the keratinized tissue is lacerated during the extrusion (*cf.* Ewald and Williams 1982) process (scraping against the bill edges) and thereby producing the lamellae (*cf.* Lucas 1891). We infer that there should be continuous production of keratin at the base of the grooves counteracting the wear at the tongue tips.

Few studies have employed SEM in other nectarivorous bird tongues, but in the reported micrographs the tongue surface of other nectar-feeding birds seems smooth as well (*e.g.* Pauw 1998, an SEM image labeled as a dissecting microscope picture; Downs 2004; Emura *et al.* 2010). Interestingly, it seems that dorsal and ventral surfaces have different rugosities, 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 inner tongue groove surface (less rugose) may be more hydrophilic than the outer grove surface, potentially facilitating the fluid trapping process described by Rico-Guevara and Rubega (2011).

A notable difference between the lingual morphology of most of birds (citations above) and the hummingbird tongues that we studied is the extreme difference in thickness; only the tongues of some nectar-feeding birds (*e.g.* excluding lorikeets) thin distally and remain between thicknesses of less than half a millimeter, thereby conferring a membranous appearance. Unfortunately there are few studies that show micrographs of nectar-feeding bird tongues (*e.g.* Weymouth *et al.* 1964, Moreau *et al.* 1969, Chang *et al.* 2013), but almost all of them (except lorikeets and other parrots) seem to have thin membranous structures in the distal tongue regions.

Dorsal and ventral surfaces of avian tongues are covered by stratified squamous epithelium; in most birds the dorsal epithelium is keratinized, and in rare cases the ventral epithelium is non-keratinized (review in Erdoğan and Iwasaki, 2013). Hence, in some birds, the ventral lingual surface is keratinized, while the dorsal surface is non-keratinized (reviews in Igwebuike and Anagor 2013a, b). Such parakeratinization (*cf.* Kadhim *et al.* 2013a) seems to match the diet and feeding mechanism of each species.

Usually, the dorsal epithelium is thicker than the ventral one (Erdoğan *et al.* 2012b, Igwebuike *et al.* 2013, Kadhim *et al.* 2013b); despite being thinner, in some cases (for species see Moore and Elliott 1946, Erdoğan and Iwasaki, 2013) the ventral epithelium becomes extremely keratinized at the distal region (tongue apex) and stiffens, forming a "lingual nail" (Susi 1969, Homberger 1986; or *cuticula cornea lingualis,* McLelland 1979; or *nagel*, Homberger 1980). One shared characteristic of dorsal and ventral epithelia is that they are nurtured by a well-developed layer of vascularized tissue, even at the tongue apex (Igwebuike and Eze 2010, Igwebuike *et al.* 2013, Elsheikh and Al-Zahaby 2014). Our light microscopy cross sections of the tongue apex (Figs. 1, S3, S4, S7) show tissue types similar to the ones found by Kadhim *et al.* (2013b), however hummingbirds do not present layers of vascularized tissue in the distal half of the tongue. On the dorsal surface of the tongue (inner surface of the groove) we found keratinized epithelium, mostly stratum corneum, but without many desquamating cells (*cf.* Fig. 3 in Kadhim *et al.* 2013b). On the ventral (outer) surface of the tongue grooves we found a relatively thick keratinized band, without underlying lamina propria (*cf.* Fig. 4 in Kadhim *et al.* 2013b). In most avian tongues the apical keratinized layer at the ventral surface comprises less than 10% of the lingual tissue in a cross section (Erdoğan and Iwasaki, 2013).

In hummingbirds we found that the keratinized ventral layer accounts for between 50% (near the supporting 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. 4, S4, S7). There is slightly more stratification (including stratum granulosum and spinosum) at the groove supporting rod, which suggests that the growing layers that generate and nurture the keratinocytes disappear at the base of the grooves (*cf.* Fig. 10 in Weymouth *et al.* 1969). Therefore we hypothesize that the grooves are continuously replaced from their base (like nails in humans), in contrast to the replacement of the dorsal and ventral apical surfaces in most birds that occurs from the inside out (*cf.* Erdoğan and Iwasaki, 2013). We found an interesting cellular arrangement in the keratinized band; the cells appeared greatly elongated with the cytoplasm surrounded by an almost continuous line of corneo-desmosomes (Fig. 5, *cf.* Fig. 23 in Iwasaki *et al.* 1997), and overlapping organized into "brickwall" arrays.

We also found circular to elliptical dark spots throughout the tissue of the distal region in hummingbird tongues (Figs. 5, S5, S6). One possibility is that those spots correspond to keratohyalin granules, found in avian epidermal cells (Alexander 2012), and characteristic of lingual tissue keratinization in mammals (*cf.* Iwasaki and Miyata 1990), squamates (*e.g.* Iwasaki and Yoshihara 2003), and testudines (*e.g.* Iwasaki *et al.* 1996). However, keratohyalin granules have not been found in avian tongues (review in Iwasaki *et al.* 1997) and the spots found in the hummingbird tongue are comparatively small (~ 0.1 – 1 μm).

A second possibility is that the dark spots are coagulated free ribosomes (*cf.* Fig. 27 in Iwasaki *et al.* 1997), but they are usually found at differential densities across the layers of tissue in cross sections of the tongue (*e.g.* Iwasaki *et al.* 1997), and we did not find any consistent pattern of differential allocation of the dark spots in our cross sections (Figs. 5, S5). A third possibility is that they are multigranular bodies (*cf.* Alexander 2012, p. 27), but we did not find evidence of lamellated granules inside of them. The last possibility is that the dark spots correspond to melanin granules (*e.g.* Dummet and Barens 1974).

We found differences between the layers of tissue underlying the dorsal (inner) and ventral (outer) surfaces of the tongue grooves (Figs. 4, 5). These differences may be explained by the cellular organization (stratum corneum in the inner vs. keratinized band in the outer), but they may also be influenced by differential composition and organization between proteins (fibrous *vs.* matrix components) and/or the presence of a peculiar beta-folded peptide sequence known only in sauropsids, known as β-keratin (reviewed by Alibardi *et al.* 2009). We found that the microarchitecture of the outer (ventral) layers of cornified tissue is more similar to the one found in feathers (presumably β-keratin) than to that of tissues with α-keratin (*cf.* Filshie and Rogers 1962). Specifically, the diameter of the microfibrils in the hummingbird tongue tissue is ~35 Å (Fig. 5) similar to other β-keratin tissue microarchitectures (Alexander 2012, p. 33), and almost a third of the diameter of α-keratin microfibrils (Filshie and Rogers 1962, Johnson and Sikorski 1965). Differences in electron scattering produced by varying proportions of α- and β-keratin could explain our qualitative results about the outer layer of the tongue grooves being more electron-lucent (high % of β-keratin, Fig. S5) and the inner layers being more electron-dense (high % of α-keratin, see review in Alibardi and Sawyer 2002).

Both kinds of keratin have been reported in bird tongues; α-keratin is usually present in soft tissues while β-keratin prevails in hardened ones (specifically in the lingual anterior ventral region, Carver and Sawyer 1989; or lingual nail, Homberger and Brush 1986). Additionally, transition from α-keratin in the basal, spinosum and transitional layers to β-keratin in the corneous layer has been reported for scutate scales (Alibardi 2004), and masking (transition in %) of α- by β-keratin has been observed in the lingual nail of chicken (review in Alibardi and Sawyer 2002). Differences in α- and β-keratin composition may provide differential elasticity to the inner and outer surfaces, making a spring recovery process of the walls of the tongue grooves more efficient (*cf.* Rico-Guevara 2014 Chapt. 3). Interestingly, β-keratin is considered to hinder elasticity, and impede pliability, while increasing mechanical resistance (Alibardi *et al.* 2009), yet we have observed the tongue grooves to be very dynamic structures (Rico-Guevara and Rubega 2011, Rico-Guevara 2014 Chapt. 3) contrary to the conventional view of them as static tubes (*cf.* capillarity hypothesis, Martin 1833, Paton and Collins 1989, Kim *et al.* 2011). The appropriate 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 micropump but weak enough to keep the grooves flattened until they contact the nectar surface (Rico-Guevara 2014 Chapts. 3 and 4).

However, we believe that a thick outer layer (keratinized band) of β-keratin is a necessity to 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 *et al.* in prep.).

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 (review in Alibardi *et al.* 2009) or selective biodegradation of β-keratin (*e.g*. Lingham-Soliar *et al.* 2010, Lingham-Soliar and Murugan 2013).

*Three-dimensional microanatomy of hummingbird tongues*

Using the data from the microCT scans we digitally decoupled bill and tongue (segmenting, *e.g.* Fig. S8) and constructed three-dimensional models of the tongue-nectar interaction (Fig. S9) emulating the fluid trapping (Rico-Guevara and Rubega 2011) and the expansive filling processes (Rico-Guevara 2014 Chapt. 3). In this way, microCT data could inform mathematical models (*e.g.* calculating total and partial groove capacities depending on immersion lengths) that are the building blocks of foraging theories (Rico-Guevara 2014 Chapt. 3). Our study presents the first high-resolution (5-μm voxels) CT scan of a vertebrate tongue adequately 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 (review in Gignac and Kley 2014), but they have not been focused on tongues.

Our three-dimensional study of hummingbird tongues allows us to clarify 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, matching a peak on energy intake rates (Fig. 4 in Kim *et al.* 2012). We prefer a more parsimonious explanation: starting with a dorso-ventrally flattened tongue as an ancestral condition, 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 highest capacity for a given amount of tissue (groove walls).

An interesting axis of variation across species is the presence of melanin in the keratin of the groove walls; we have encountered tongues ranging from almost transparent to entirely black in different species (*cf.* morphological survey, Rico-Guevara and Rubega 2011). We expect that the percentage of melanin in the groove walls would influence their elasticity and subsequently the strength of the micropump, hence further comparative and scaling experiments are warranted. Several scaling models and applications have been developed on the basis of recent discoveries of biological phenomena and underlying physical explanations (see Vogel 2011), opening the way for a deeper study about the influence of the surface characteristics and the tissue composition of the groove walls on the elastic properties of the tongue (Rico-Guevara 2014 Chapt. 3).

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**Literature cited**

Aizenberg-Gershtein, Y., Izhaki, I., & Halpern, M. (2013). Do honeybees shape the bacterial community composition in floral nectar?. PloS One, 8(7), e67556.

Alexander, N. (2012). Keratinization: a survey of vertebrate epithelia. Elsevier.

Alibardi, L., & Sawyer, R. H. (2002). Immunocytochemical analysis of beta (β) keratins in the epidermis of chelonians, lepidosaurians, and archosaurians. Journal of Experimental Zoology, 293(1), 27-38.

Alibardi, L. (2004). Immunocytochemical and autoradiographic studies on the process of keratinization in avian epidermis suggests absence of keratohyalin. Journal of Morphology, 259(2), 238-253.

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. Journal of anatomy, 214(4), 560-586.

Álvarez-Pérez, S., Herrera, C. M., & Vega, C. (2012). Zooming in on floral nectar: a first exploration of nectar-associated bacteria in wild plant communities. FEMS microbiology ecology, 80(3), 591-602.

Anderson, P. S., Friedman, M., & Ruta, M. (2013). Late to the table: diversification of tetrapod mandibular biomechanics lagged behind the evolution of terrestriality. Integrative and comparative biology, 53(2), 197-208.

Ashley-Ross, M. A., Hsieh, S. T., Gibb, A. C., & Blob, R. W. (2013). Vertebrate Land Invasions–Past, Present, and Future: An Introduction to the Symposium. Integrative and comparative biology, 53(2), 192-196.

Audubon, J. J., & MacGillivray, W. (1856). The birds of America: From drawings made in the United States and their territories. New York: V.G. Audubon.

Baecker, B., Yanaihara, N., & Forssmann, W. G. (1983). VIP innervation of the tongue in vertebrates. Anatomy and embryology, 167(2), 173-189.

Baussart, S., Korsoun, L., Libourel, P. A., & Bels, V. (2009). Ballistic food transport in toucans. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 311(7), 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. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology, 315(2), 72-83.

Berkhoudt, H. (1979). The morphology and distribution of cutaneous mechanoreceptors (Herbst and Grandry corpuscles) in bill and tongue of the mallard (*Anas platyrhynchos* L.). Netherlands Journal of Zoology, 30(1), 1-34.

Birt, P., Hall, L. S., & Smith, G. C. (1997). Ecomorphology of the tongues of Australian megachiroptera (Chiroptera: Pteropodidae). Australian Journal of Zoology, 45(4), 369-384.

Bock, W. J. (1999). Functional and evolutionary morphology of woodpeckers. Ostrich, 70(1), 23-31.

Bozzola, J. J.; Russell, L. D. (1999). "Specimen Preparation for Transmission Electron Microscopy". Electron Microscopy : Principles and Techniques for Biologists. Sudbury, MA: Jones and Bartlett. pp. 21–31. ISBN 978-0-7637-0192-5.

Carlesso Santos, T., Yuri Fukuda, K., Plácido Guimarães, J., Franco Oliveira, M., Angelica Miglino, M., & Watanabe, L. S. (2011). Light and scanning electron microcopy study of the tongue in *Rhea americana*. Zoological science, 28(1), 41-46.

Carvalho, L. R. D., Farias, L. M., Nicoli, J. R., Silva, M. C. F., Corsino, A. T. S. M., Lima, L. A. D., ... & Pinto, M. E. B. M. (2003). Dominant culturable bacterial microbiota in the digestive tract of the American black vulture (*Coragyps atratus* Bechstein 1793) and search for antagonistic substances. Brazilian Journal of Microbiology, 34(3), 218-224.

Carver, W. E., & Sawyer, R. H. (1989). Immunocytochemical localization and biochemical analysis of α and β keratins in the avian lingual epithelium. American journal of anatomy, 184(1), 66-75.

Chang, Y. M., Lin, H. Y., Hatch, K. A., Yao, C. T., & Shiu, H. J. (2013). Brush-tipped tongue structure of the Taiwan Yuhina (*Yuhina brunneiceps*) and White-eared Sibia (*Heterophasia auricularis*). The Wilson Journal of Ornithology, 125(1), 204-208.

Crole, M. R., & Soley, J. T. (2010). Surface morphology of the emu (*Dromaius novaehollandiae*) tongue. Anatomia, histologia, embryologia, 39(4), 355-365.

Darwin, C. (1841). The Zoology of the voyage of H.M.S. Beagle: Part III, Birds. London: Smith, Elder and Co.

Dehkordi, R. A. F., Parchami, A., & Bahadoran, S. (2010). Light and scanning electron microscopic study of the tongue in the zebra finch *Carduelis carduelis* (Aves: Passeriformes: Fringillidae). Slovenian Veterinary Research, 47(4).

Downs, C. T. (2004). Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. Ostrich-Journal of African Ornithology, 75(3), 169-175.

Dubale, M. S., & Thomas, V. C. (1978). The Epidermal Structures of the Tongue and the Buccal Cavity of the Brahminy Myna (*Sturnus pagodarum* Gmelin) and the Wagtail (*Motacilla flava thunbergi* Billberg). Acta Zoologica, 59(3:4), 149-155.

Dummett, C. O., & Barens, G. (1974). Avian Oral Pigmentation. Journal of periodontology, 45(6), 426-433.

El-Bakary, N. E. (2011). Surface morphology of the tongue of the hoopoe (*Upupa epops*). Journal of American Science, 7(1).

El-Bakary, N. E. (2012). Scanning Electron Microscope Study of the Dorsal Lingual Surface of *Halcyon smyrnensis* (White Breasted Kingfisher). Global Veterinaria 9 (2): 192-195.

El-Beltagy, A. E. F. B. (2013) Comparative Studies on the Tongue of White-Throated Kingfisher (*Halcyon smyrnensis*) and Common Buzzard (*Buteo buteo*). Egypt. Acad. J. biolog. Sci., 4 (1): 1-14.

Elsheikh, E. H., & Al-Zahaby, S. H. (2014). Light and scanning electron microscopic study of the tongue in the hooded crow (Aves: *Corvus corone cornix*). The Journal of Basic & Applied Zoology.

Emura, S., & Chen, H. (2008). Scanning electron microscopic study of the tongue in the owl (*Strix uralensis*). Anatomia, histologia, embryologia, 37(6), 475-478.

Emura, S., Okumura, T., & Chen, H. (2008). Scanning electron microscopic study of the tongue in the peregrine falcon and common kestrel. Okajimas folia anatomica Japonica, 85(1), 11-15.

Emura, S., Okumura, T., & Chen, H. (2009a). Scanning electron microscopic study of the tongue in the Oriental scops owl (*Otus scops*). Okajimas folia anatomica Japonica, 86(1), 1.

Emura, S., Okumura, T., & Chen, H. (2009b). Scanning electron microscopic study of the tongue in the Japanese pygmy woodpecker (*Dendrocopos kizuki*). Okajimas folia anatomica Japonica, 86(1), 31.

Emura, S., Okumura, T., & Chen, H. (2010). Comparative studies of the dorsal surface of the tongue in three avian species by scanning electron microscopy. Okajimas folia anatomica Japonica, 86(4), 111-115.

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(3), 379-387.

Erdoğan, S., Pèrez, W., & Alan, A. (2012a). Anatomical and scanning electron microscopic investigations of the tongue and laryngeal entrance in the long-legged buzzard (*Buteo rufinus*, cretzschmar, 1829). Microscopy research and technique, 75(9), 1245-1252.

Erdoğan, S., Sağsöz, H., & Akbalik, M. E. (2012b). 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(3), 307-315.

Erdoğan, S., & Iwasaki, S. I. (2013). Function-related morphological characteristics and specialized structures of the avian tongue. Annals of Anatomy-Anatomischer Anzeiger, 196(2):75-87.

Ewald, P. W., & Williams, W. A. (1982). Function of the bill and tongue in nectar uptake by hummingbirds. The Auk, 573-576.

Filshie, B. K., & Rogers, G. E. (1962). An electron microscope study of the fine structure of feather keratin. The Journal of cell biology, 13(1), 1-12.

Fleming, T. H., Muchhala, N. 2008. Nectar-feeding bird and bat niches in two worlds: pantropical comparisons of vertebrate pollination systems. Journal of Biogeography 35 (5), 764-780.

Fujii, Y. (2011). Comparison of Surface Roughness Estimations by X-ray Reflectivity Measurements and TEM observations. In IOP Conference Series: Materials Science and Engineering (Vol. 24, No. 1, p. 012008). IOP Publishing.

Gardner, L. L. (1925). The adaptive modifications and the taxonomic value of the tongue in birds. Washington, D.C: G.P.O.

Ghosh, S., Bowen, J., Jiang, K., Espino, D. M., & Shepherd, D. E. (2013). Investigation of techniques for the measurement of articular cartilage surface roughness. Micron, 44, 179-184.

Gignac, P. M., & Kley, N. J. (2014). Iodine-enhanced micro-CT imaging: Methodological refinements for the study of the soft-tissue anatomy of post-embryonic vertebrates. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution.

Goldman, L., Vahl, J., Rockwell, R. J., Meyer, R., Franzen, M., Owens, P., & Hyatt, S. (1969). Replica Microscopy and Scanning Electron Microscopy of Laser Impacts on the Skin1. Journal of Investigative Dermatology, 52(1), 18-24.

Gould, J. (1861). An introduction to the Trochilidae: Or family of humming-birds. London: Printed by Taylor and Francis.

Guimarães, J. P., Mari, R. D. B., Carvalho, H. S. D., & Watanabe, I. S. (2009). Fine structure of the dorsal surface of ostrich's (*Struthio camelus*) tongue.Zoological science, 26(2), 153-156.

Gussekloo, S. W., & Bout, R. G. (2005). The kinematics of feeding and drinking in palaeognathous birds in relation to cranial morphology. Journal of Experimental Biology, 208(17), 3395-3407.

Hainsworth, F. R. (1973). On the tongue of a hummingbird: its role in the rate and energetics of feeding. Comparative Biochemistry and Physiology Part A: Physiology, 46(1), 65-78.

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. Frontiers in zoology, 10(1), 44.

Hardesty, J. (2009). Using nitrogen-15 to examine protein sources in hummingbird diets. Ornitología Colombiana, 8: 19-28.

Harper, C. J., Swartz, S. M., & Brainerd, E. L. (2013). Specialized bat tongue is a hemodynamic nectar mop. Proceedings of the National Academy of Sciences,110(22), 8852-8857.

Harte, M., Legreneur, P., Pelle, E., Placide, M. A., & Bels, V. (2012). Ballistic food transport in birds: the example of *Casuarius casuarius*. Computer methods in biomechanics and biomedical engineering, 15(sup1), 137-139.

Hayat, M. A. (2000). Principles and Techniques of Electron Microscopy: Biological Applications. Cambridge University Press. pp. 45–61.ISBN 0-521-63287-0.

Herrera, C. M., García, I. M., & Pérez, R. (2008). Invisible floral larcenies: microbial communities degrade floral nectar of bumble bee-pollinated plants.Ecology, 89(9), 2369-2376.

Herrera, C. M., de Vega, C., Canto, A., & Pozo, M. I. (2009). Yeasts in floral nectar: a quantitative survey. Annals of Botany, 103(9), 1415-1423.

Hilty, S. L., & Brown, B. (1986). A guide to the birds of Colombia. Princeton University Press.

Holliday, C. M., Ridgely, R. C., Balanoff, A. M., & Witmer, L. M. (2006). Cephalic vascular anatomy in flamingos (*Phoenicopterus ruber*) based on novel vascular injection and computed tomographic imaging analyses. The Anatomical Record Part A: Discoveries in Molecular, Cellular, and Evolutionary Biology, 288(10), 1031-1041.

Homberger, D. G. (1980). Funktionell-morphologische Untersuchungen zur Radiation der Ernährungs- und Trinkmethoden der Papageien (Psittaci). Zoologisches Forschungsinstitut und Museum Alexander Koenig. Bonn. Zool. Monographien No. 13, 192 pp.

Homberger, D. G. (1986). The lingual apparatus of the African Grey Parrot, *Psittacus erithacus* Linné (Aves: Psittacidae): description and theoretical mechanical analysis (Vol. 32). Amer Ornithologists Union.

Homberger, D. G., & Brush, A. H. (1986). Functional-morphological and biochemical correlations of the keratinized structures in the African Grey Parrot, *Psittacus erithacus* (Aves). Zoomorphology, 106(2), 103-114.

Igwebuike UM, Eze UU. 2010. Anatomy of the oropharynx and tongue of the African pied crow (*Corvus albus*). Vet Archiv 80:523–531.

Igwebuike, U. M., & Anagor, T. A. (2013a). The morphology of the oropharynx and tongue of the muscovy duck (*Cairina moschata*). Veterinarski arhiv, 83(6), 685-693.

Igwebuike, U. M., & Anagor, T. A. (2013b). Gross and Histomorphological Assessment of the Oropharynx and Tongue of the Guinea Fowl (*Numida meleagris*). Animal Research International, 10(2), 1739-1746.

Igwebuike, Udensi M., Ugwuoke, Wilfred I. And Udoumoh, Anietie F. (2013) Histological Features Of The Tongue Of The Common Pigeon (*Columba livia*). Animal Research International 10(3): 1779 – 1785

Iwasaki, S., and Miyata, K. (1990) Fine structure of the dorsal epithelia of the mongoose. J. Anat., 172: 201-212

Iwasaki, S. I., Asami, T., & Wanichanon, C. (1996). Fine structure of the dorsal lingual epithelium of the juvenile hawksbill turtle, *Eretmochelys imbricata bissa*.Anatomical Record, 244(4), 437-443.

Iwasaki, S., Asami, T. & Chiba, A. (1997) Ultrastructural studyof the keratinization of the dorsal epithelium of the tongue of Middendorff's bean goose, *Anser fabalis middendorffii* (Anseres, Antidae). The Anatomical Record, 247: 149–163.

Iwasaki, S. I. (2002). Evolution of the structure and function of the vertebrate tongue. Journal of Anatomy, 201(1), 1-13.

Iwasaki, S. I., & Yoshihara, M. (2003). Histochemical and ultrastructural features of the lingual epithelium of the rat snake (*Elaphe climacophora*). Zoology,106(1), 63-72.

Jackowiak, H., & Godynicki, S. (2005). Light and scanning electron microscopic study of the tongue in the white tailed eagle (*Haliaeetus albicilla*, Accipitridae, Aves). Annals of Anatomy-Anatomischer Anzeiger, 187(3), 251-259.

Jackowiak, H., Andrzejewski, W., & Godynicki, S. (2006). Light and scanning electron microscopic study of the tongue in the cormorant *Phalacrocorax carbo* (Phalacrocoracidae, Aves). Zoological science, 23(2), 161-167.

Jackowiak, H., & Ludwig, M. (2008). Light and scanning electron microscopic study of the structure of the ostrich (*Strutio camelus*) tongue. Zoological Science, 25(2), 188-194.

Jackowiak, H., Skieresz-Szewczyk, K., Kwiecinski, Z., Trzcielinska-Lorych, J., & Godynicki, S. (2010). Functional morphology of the tongue in the nutcracker (*Nucifraga caryocatactes*). Zoological science, 27(7), 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*). The Anatomical Record, 294(9), 1574-1584.

Johnson, D. J., & Sikorski, J. (1965). Alpha-Keratin: Molecular and Fine Structure of α-Keratin (IV). Nature, 205(4968), 266-268.

Kadhim, K. K., Hameed, A. T., & Abass, T. A. (2013a). Histomorphological and Histochemical Observations of the Common Myna (*Acridotheres tristis*) Tongue.ISRN veterinary science, 2013.

Kadhim, K. K., Zuki, A. B. Z., Babjee, S. M. A., Noordin, M. M., & Zamri-Saad, M. (2013b). Morphological and histochemical observations of the red jungle fowl tongue *Gallus gallus*. African Journal of Biotechnology, 10(48), 9969-9977.

Kim, W., Gilet, T., & Bush, J. W. (2011). Optimal concentrations in nectar feeding.Proceedings of the National Academy of Sciences, 108(40), 16618-16621.

Kim, W., Peaudecerf, F., Baldwin, M. W., & Bush, J. W. (2012). The hummingbird's tongue: a self-assembling capillary syphon. Proceedings of the Royal Society B: Biological Sciences, 279(1749), 4990-4996.

Kobayashi, K., Kumakura, M., Yoshimura, K., Inatomi, M., & Asami, T. (1998). Fine structure of the tongue and lingual papillae of the penguin. Archives of histology and cytology, 61(1), 37-46.

Leitner, L. M., & Roumy, M. (1974a). Mechanosensitive units in the upper bill and in the tongue of the domestic duck. Pflügers Archiv, 346(2), 141-150.

Leitner, L. M., & Roumy, M. (1974b). Thermosensitive units in the tongue and in the skin of the duck's bill. Pflügers Archiv, 346(2), 151-155.

Lingham-Soliar, T., Bonser, R. H., & Wesley-Smith, J. (2010). Selective biodegradation of keratin matrix in feather rachis reveals classic bioengineering. Proceedings of the Royal Society B: Biological Sciences, 277(1685), 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(6), e65849.

Louchart, A., Tourment, N., Carrier, J., Roux, T., & Mourer-Chauviré, C. (2008). Hummingbird with modern feathering: an exceptionally well-preserved Oligocene fossil from southern France. Naturwissenschaften, 95(2), 171-175.

Lucas, F. A. (1891). On the structure of the tongue in humming birds. Washington: Smithsonian Institution, U.S. National Museum.

Lucas, F. A. (1896). The taxonomic value of the tongue in birds. New York.

Martin WCL (1833) The Naturalist's Library: A General History of Humming-Birds or the Trochilidae, ed W Jardine (H.G. Bohn, London), Vol 41.

Martinez, M., Stefanini, M. A., Martinez, F. E., Guida, H. L., Pinheiro, P. F. F., Almeida, C., & Segatelli, T. (2003). Morphological study of the tongue of budgerigar (*Melopsittacus undulatus*). International Journal of Morphology, 21(2), 117-122.

Mayr, G. (2004). Old World fossil record of modern-type hummingbirds. Science,304(5672), 861-864.

McGuire, J. A., Witt, C. C., Remsen Jr, J. V., Corl, A., Rabosky, D. L., Altshuler, D. L., & Dudley, R. (2014). Molecular Phylogenetics and the Diversification of Hummingbirds. Current Biology, 24(8), 910-916.

McLelland, J. 1979. Systema digestiorum. Pp. 267-295, In Baumel, J. J., King, A. S., Lucas, A. M., Breazile, J. E., & Evans, H. E. (eds.), Nomina anatomica avium. An annotated anatomical dictionary of birds. Academic Press, London.

Michael, N., & Bhushan, B. (2007). Hierarchical roughness makes superhydrophobic states stable. Microelectronic engineering, 84(3), 382-386.

Metscher, B. D. (2009). MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. BMC physiology, 9(1), 11.

Mobbs, A. J. (1979) Methods used by the Trochilidae hummingbirds when capturing insects. Avicultural Magazine, 851: 26-30

Moore, C. A., & Elliott, R. (1946). Numerical and regional distribution of taste buds on the tongue of the bird. Journal of Comparative Neurology, 84(2), 119-131.

Moreau, R. E., Perrins, M., & Hughes, J. T. (1969). Tongues of the Zosteropidae (white-eyes). Ardea, 57, 29-47.

Nanda, K. K., Sarangi, S. N., & Sahu, S. N. (1998). Measurement of surface roughness by atomic force microscopy and Rutherford backscattering spectrometry of CdS nanocrystalline films. Applied Surface Science, 133(4), 293-297.

Newton, A., Gadow, H., Lydekker, R., Roy, C. S., & Shufeldt, R. W. (1896). A dictionary of birds. A. and C. Black.

Kudo, K.-I., Nishimura, S., & Tabata, S. (2008). Distribution of taste buds in layer-type chickens: Scanning electron microscopic observations. Animal Science Journal,79(6), 680-685.

Nalavade, M. N., & Varute, A. T. (1977). Histochemical studies on the mucins of the vertebrate tongues: XI. Histochemical analysis of mucosubstances in the lingual glands and taste buds of some birds. Acta histochemica, 60(1), 18-31.

Onuk, B., Tütüncü, S., Kabak, M., & Alan, A. (2013). Macroanatomic, light microscopic, and scanning electron microscopic studies of the tongue in the seagull (*Larus fuscus*) and common buzzard (*Buteo buteo*). Acta Zoologica.

Onyango, L. A., Dunstan, R. H., Gottfries, J., von Eiff, C., & Roberts, T. K. (2012). Effect of low temperature on growth and ultra-structure of Staphylococcus spp.PloS one, 7(1), e29031.

Pandian, S., Sundaram, J., & Panchatcharam, P. (2012). Isolation, identification and characterization of feather degrading bacteria. European Journal of Experimental Biology, 2(1), 274-282.

Parchami, A., Dehkordi, R. A. F., & Bahadoran, S. (2010a). Scanning electron microscopy of the tongue in the golden eagle *Aquila chrysaetos* (Aves: Falconiformes: Accipitridae). World Journal of Zoology, 5, 257-263.

Parchami, A., Fatahian, R. A., & Bahadoran, S. (2010b). Fine structure of the dorsal lingual epithelium of the common quail (*Coturnix coturnix*). World Appl Sci J, 10(10), 1185-1189.

Paton, D. C., & Collins, B. G. (1989). Bills and tongues of nectar-feeding birds: A review of morphology, function and performance, with intercontinental comparisons. Australian Journal of Ecology, 14(4), 473-506.

Pauw, A. (1998). Pollen transfer on birds' tongues. Nature, 394(6695), 731-732.

Picoli, L. C., Lopes, R. A., Semprini, M., Sala, M. A., Ogawa, K., and I.-S. Watanabe (2006). Transmission electron microscopy study of the neonatal rat tongue mucosa treated with special attention to the bacteriae on the epithelial cell membrane. Int J Morphol, 24(2), 159-63.

Purwar, R. S. (1975). Anatomical, neurohistological and histochemical observations on the tongue of Francolinus pondicerianus (grey partridge or safed teeter). Cells Tissues Organs, 93(4), 526-533.

Rico-Guevara, A. (2005). Relaciones entre morfología y forrajeo de artópodos en colibríes. Trabajo de grado. Universidad Nacional de Colombia.

Rico-Guevara, A. (2008). Morfología y forrajeo para buscar artrópodos por colibríes altoandinos. Ornitología Colombiana, 7: 43-58.

Rico-Guevara, A. (2014). Morphology and Function of the Drinking Apparatus in Hummingbirds. Doctoral dissertation. University of Connecticut.

Rico-Guevara, A., & Rubega, M. A. (2011). The hummingbird tongue is a fluid trap, not a capillary tube. Proceedings of the National Academy of Sciences,108(23), 9356-9360.

Reynolds, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. The Journal of cell biology, 17(1), 208-212.

Rubega, M. A. (2000). Feeding in birds: approaches and opportunities. Pp. 395-408. In: Feeding: Form, Function and Evolution in Tetrapod Vertebrates. K. Schwenk (ed.). Academic Press, San Diego.

Santos TC, Fukuda KY, Guimara˜es JP, Oliveira MF, Miglino MA, Watanabe L. 2011. Light and scanning electron microcopy study of the tongue in *Rhea americana*. Zool Sci 28:41–46.

Schwenk, K. (2000a) Tetrapod feeding in the context of vertebrate morphology. Pp. 3-20. In: Feeding: Form, Function and Evolution in Tetrapod Vertebrates. K. Schwenk (ed.). Academic Press, San Diego.

Schwenk, K. (2000b) An introduction to tetrapod feeding. Pp. 21-61. In: Feeding: Form, Function and Evolution in Tetrapod Vertebrates. K. Schwenk (ed.). Academic Press, San Diego.

Schwenk, K. (2001). Extrinsic versus intrinsic lingual muscles: a false dichotomy. Bull Mus Comp Zool, 156(1), 219-235.

Schwenk, K.,and M. Rubega (2005) Diversity of vertebrate feeding systems. Pp. 1-41. In: Physiological and Ecological Adaptations to Feeding in Vertebrates. J. M. Starck and T. Wang (eds.). Science Publishers, Enfield, NH.

Scharnke, H. (1931). Beiträge zur Morphologie und Entwicklungsgeschichte der Zunge der Trochilidae, Meliphagidae und Picidae. Journal of Ornithology, 79(4), 425-491.

Shufeldt, R. W. (1900). On the osteology of the woodpeckers. Proceedings of the American Philosophical Society, 578-622.

Skieresz-Szewczyk, K., Prozorowska, E., & Jackowiak, H. (2012). The development of the tongue of the domestic goose from 9th to 25th day of incubation as seen by scanning electron microscopy. Microscopy research and technique, 75(11), 1564-1570.

Smith, M. L., Yanega, G. M., & Ruina, A. (2011). Elastic instability model of rapid beak closure in hummingbirds. Journal of theoretical biology, 282(1), 41-51.

Stiles, F. G. (1995). Behavioral, ecological and morphological correlates of foraging for arthropods by the hummingbirds of a tropical wet forest. Condor, 853-878.

Susi, F. R. (1969). Keratinization in the mucosa of the ventral surface of the chicken tongue. Journal of anatomy, 105(Pt 3), 477.

Ting-Beall, H. P., Zhelev, D. V., & Hochmuth, R. M. (1995). Comparison of different drying procedures for scanning electron microscopy using human leukocytes. Microscopy research and technique, 32(4), 357-361.

Tomlinson, C.A.B. (2000). Pp. 359–394. In: Feeding: Form, Function and Evolution in Tetrapod Vertebrates. K. Schwenk (ed.). Academic Press, San Diego.

Vannette, R. L., Gauthier, M. P. L., & Fukami, T. (2013). Nectar bacteria, but not yeast, weaken a plant–pollinator mutualism. Proceedings of the Royal Society B: Biological Sciences, 280(1752).

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. The Auk, 121(2), 509-514.

Vogel, S. (2011). Surface tension helps a tongue grab liquid. Proceedings of the National Academy of Sciences, 108(23), 9321-9322.

Ward, P., Labandeira, C., Laurin, M., & Berner, R. A. (2006). Confirmation of Romer's Gap as a low oxygen interval constraining the timing of initial arthropod and vertebrate terrestrialization. Proceedings of the National Academy of Sciences, 103(45), 16818-16822.

Weymouth, R. D., Lasiewski, R. C., & Berger, A. J. (1964). The tongue apparatus in hummingbirds. Cells Tissues Organs, 58(3), 252-270.

Wild, J. M. (1990). Peripheral and central terminations of hypoglossal afferents innervating lingual tactile mechanoreceptor complexes in Fringillidae. Journal of Comparative Neurology, 298(2), 157-171.

Williams, C. M., Richter, C. S., Mackenzie, J. M., & Shih, J. C. (1990). Isolation, identification, and characterization of a feather-degrading bacterium. Applied and Environmental Microbiology, 56(6), 1509-1515.

Xue, T., Trent, J. S., & Osseo-Asare, K. (1989). Characterization of nafion® membranes by transmission electron microscopy. Journal of membrane science,45(3), 261-271.

Yanega, G. M., & Rubega, M. A. (2004). Feeding mechanisms: Hummingbird jaw bends to aid insect capture. Nature, 428(6983), 615-615.

Yanega, G. M. (2007). A comparative study of the functional morphology and ecology of insectivory in hummingbirds. Doctoral Dissertation: University of Connecticut. Paper AAI3289529. http://digitalcommons.uconn.edu/dissertations/AAI3289529

Zusi, R. L. (2013). Introduction to the Skeleton of Hummingbirds (Aves: Apodiformes, Trochilidae) in Functional and Phylogenetic Contexts. Ornithological Monographs, 77, 1-94.

Zweers, G. A., Berge, J. V., & Berkhoudt, H. (1997). Evolutionary patterns of avian trophic diversification. Zoology-Analysis of Complex Systems, 100(1-2), 25-57.

**Supplementary Figures**

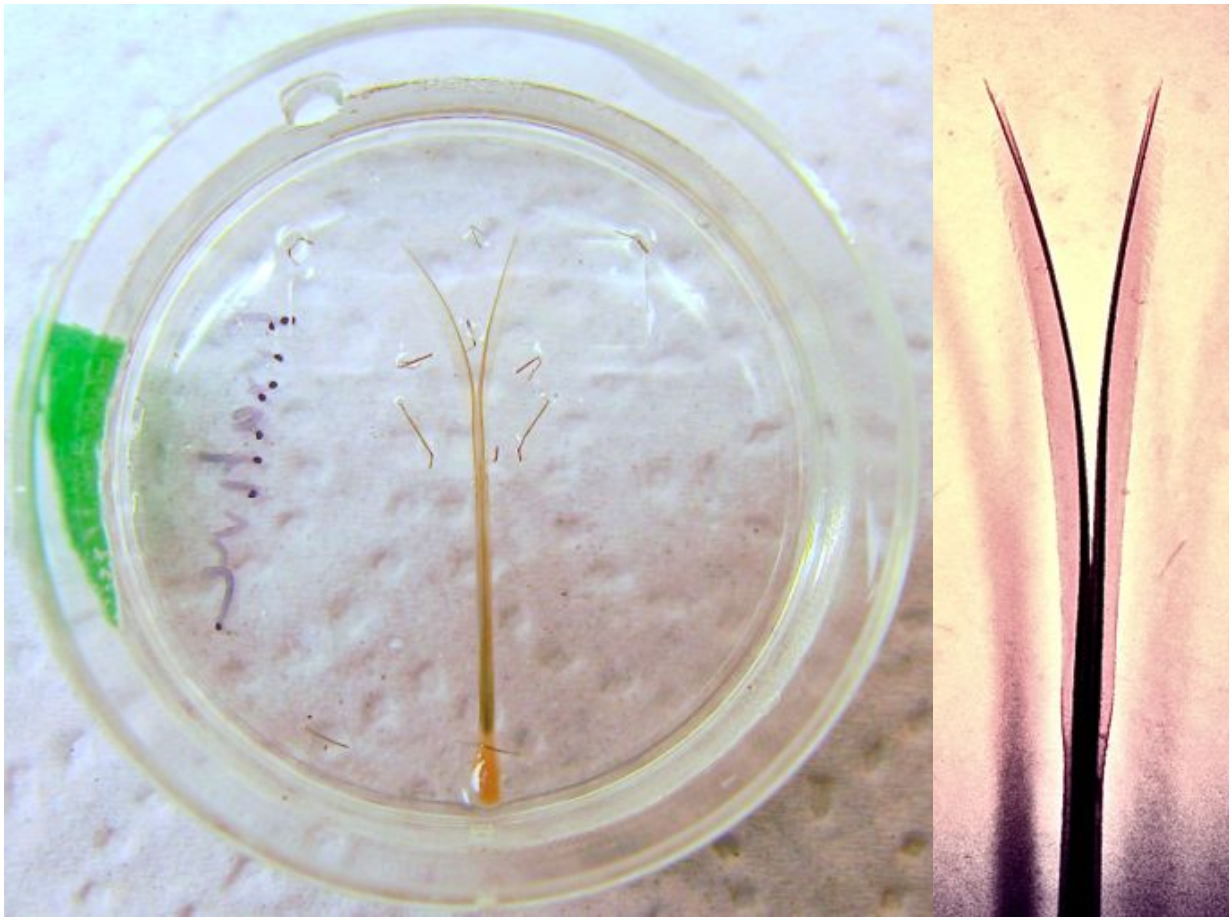


**Figure S1. Flattening of hummingbird tongues.** On the left, a general overview of the mini Preti dish with a gel layer at the bottom to which we fixed the tongues (with their ventral surfaces against the gel) by using micropins. In order to fix the tongue exposing the groove inner surfaces, we gently flattened the grooves open with the help of microslides (cover slides for microscopy) secured to the gel with micropins. On the right, the end result of the fixation with $OsO_4$, the grooves stay open even after releasing the tongue from the gel.

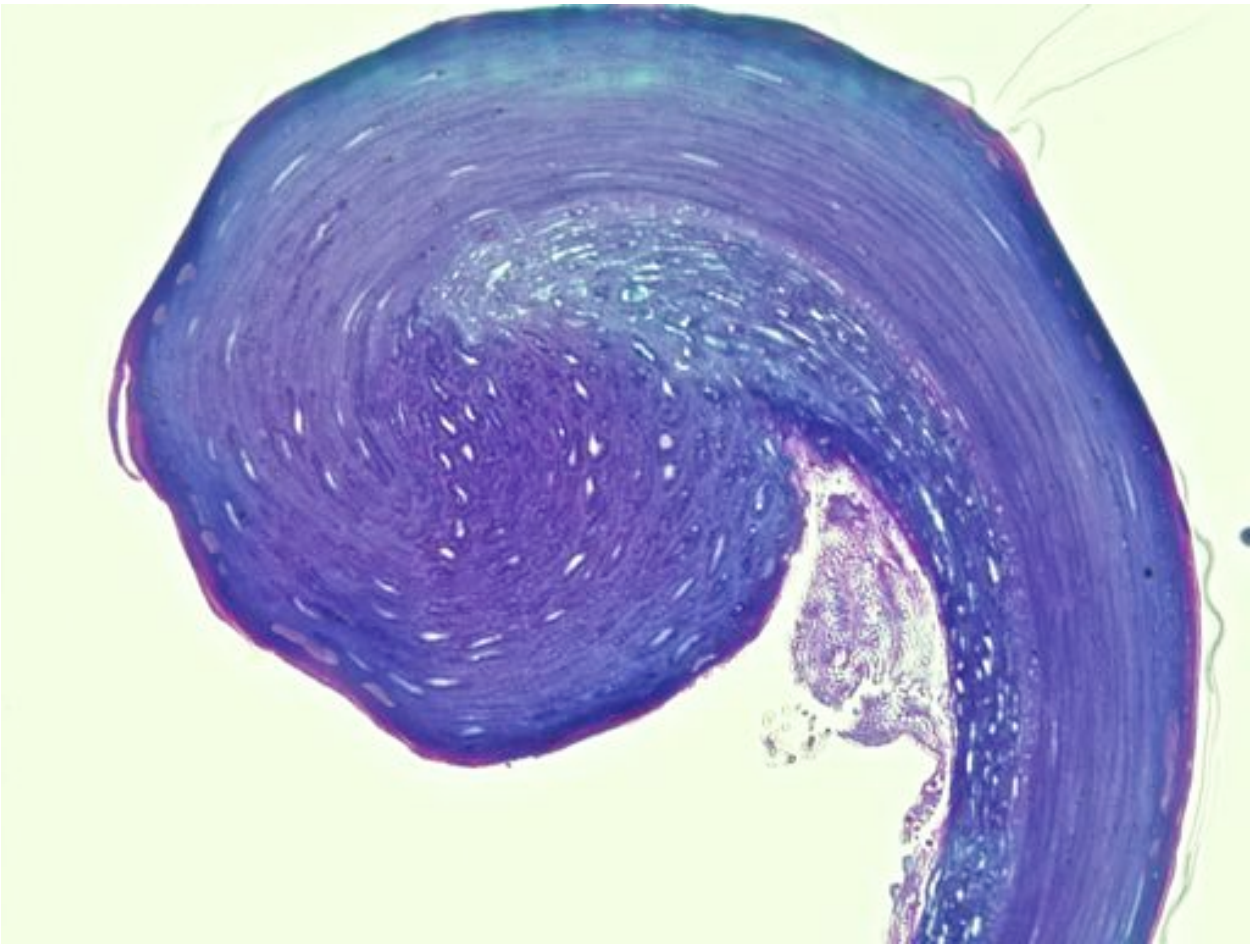


**Figure S3**. **Supporting rod on the distal dorsal portion of a tongue groove.** Light micrograph (1,000x, reconstructed pseudo-planar field in Auto-Montage) showing the stratified epithelium at the dorsal (inner) surface of the groove, and the keratinized band at the ventral (outer) groove surface. Stained with methylene blue/azure II (counterstained with fuchsine).

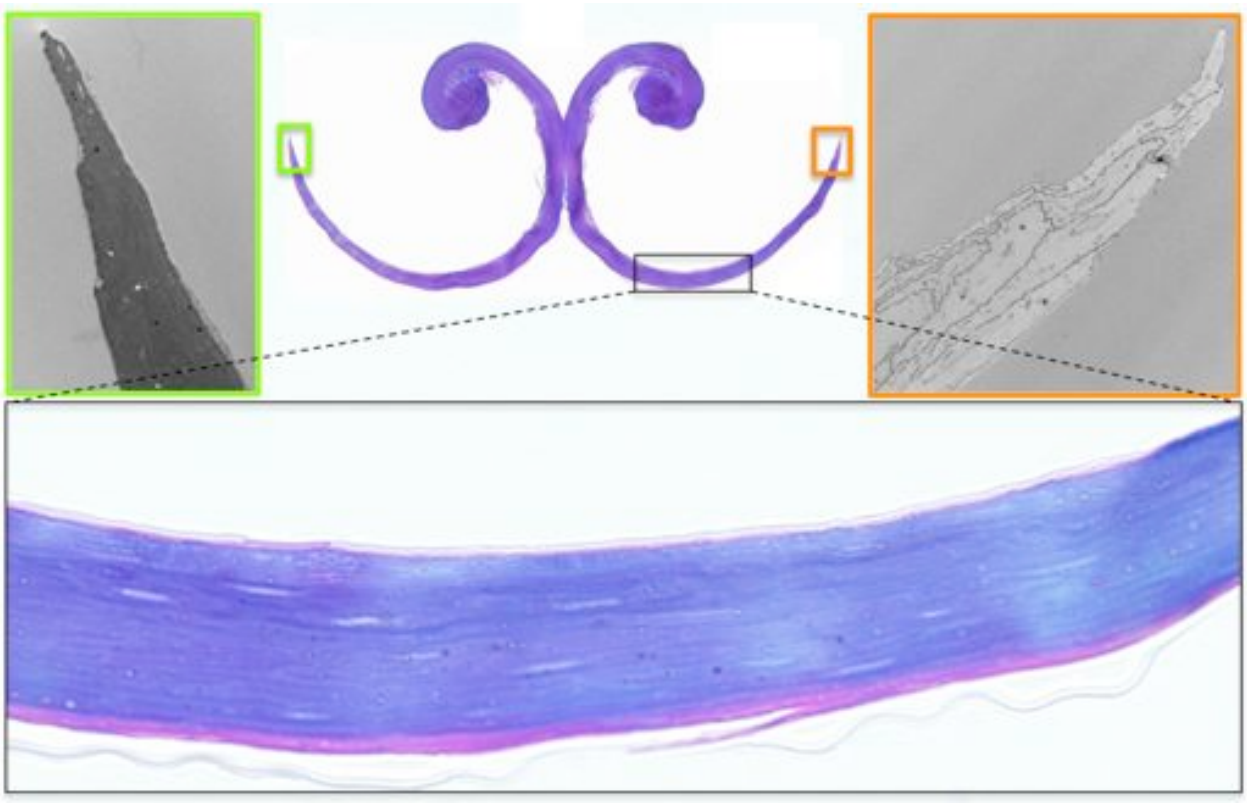


**Figure S4. Cross section close ups of the outward halves of the groove walls, contrasting staining methods.** At the top left, an electron micrograph stained with uranyl acetate and lead citrate. At the top right, an electron micrograph vapor-stained with $RuO_4$. Bottom, light micrograph stained with methylene blue/azure II (counterstained with fuchsine). Note that the most outward halves of the groove wall in cross section are entirely composed of the keratinized band (lack of stratified epithelium).

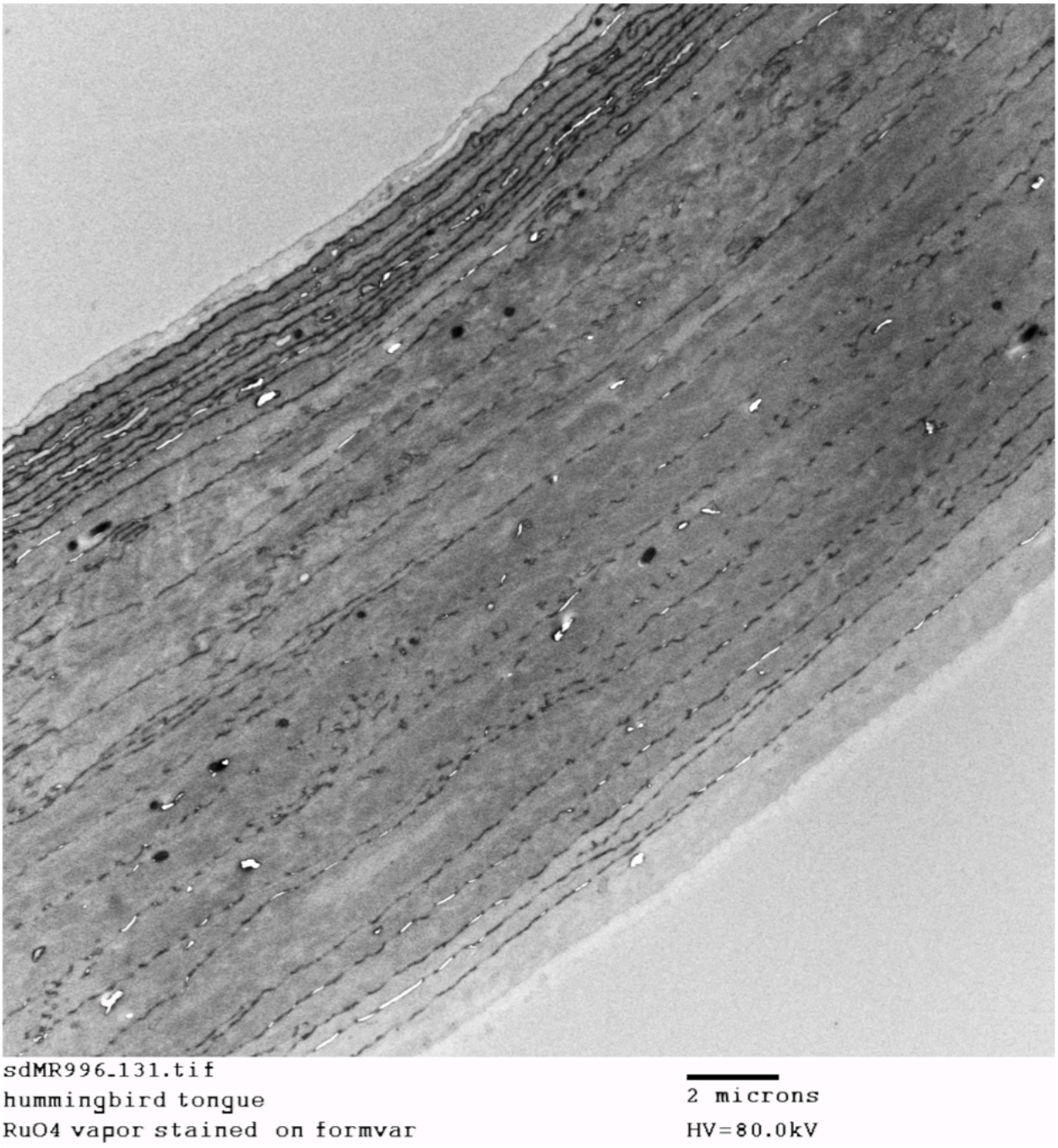



**Figure S5**. **Electron micrograph a hummingbird tongue groove wall.** The top left corner corresponds to the inner (dorsal) surface, and the bottom right to the outer (ventral) surface. Note that the layers of tissue are thinner (closely compacted together) near the inner surface in comparison to the rest of the tissue.

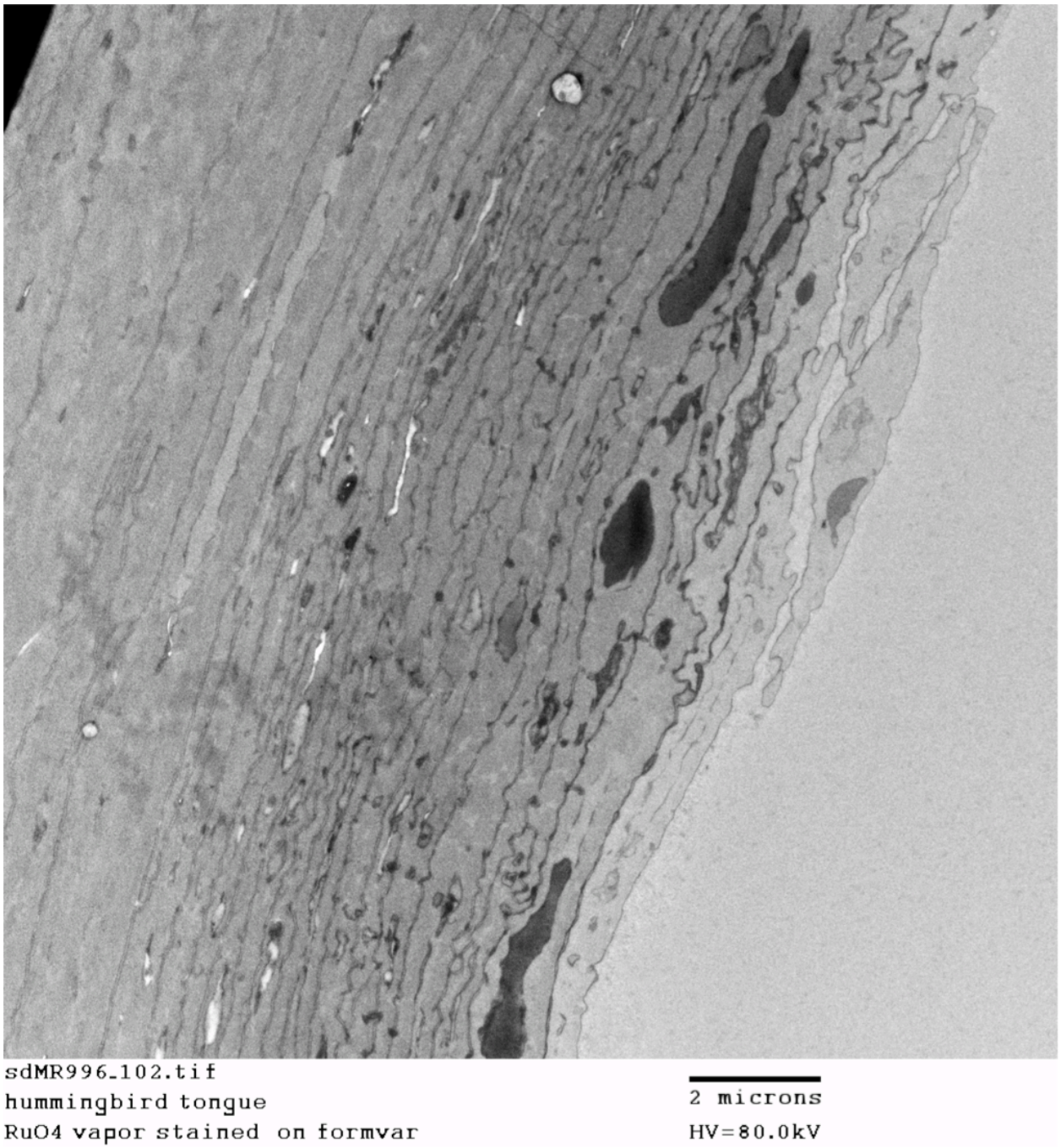



**Figure S6. Electron micrograph a hummingbird tongue groove wall near the supporting rod.** The top left corner corresponds to the outer (ventral) surface, and the bottom right to the inner (dorsal) surface, the relative positions are inverted relative to previous images because of the curling of the tissue near the rod. We observed irregularly elliptical dark spots in the keratinized stratified squamous epithelium near the inner surface, which are likely to be keratohyalin granules.

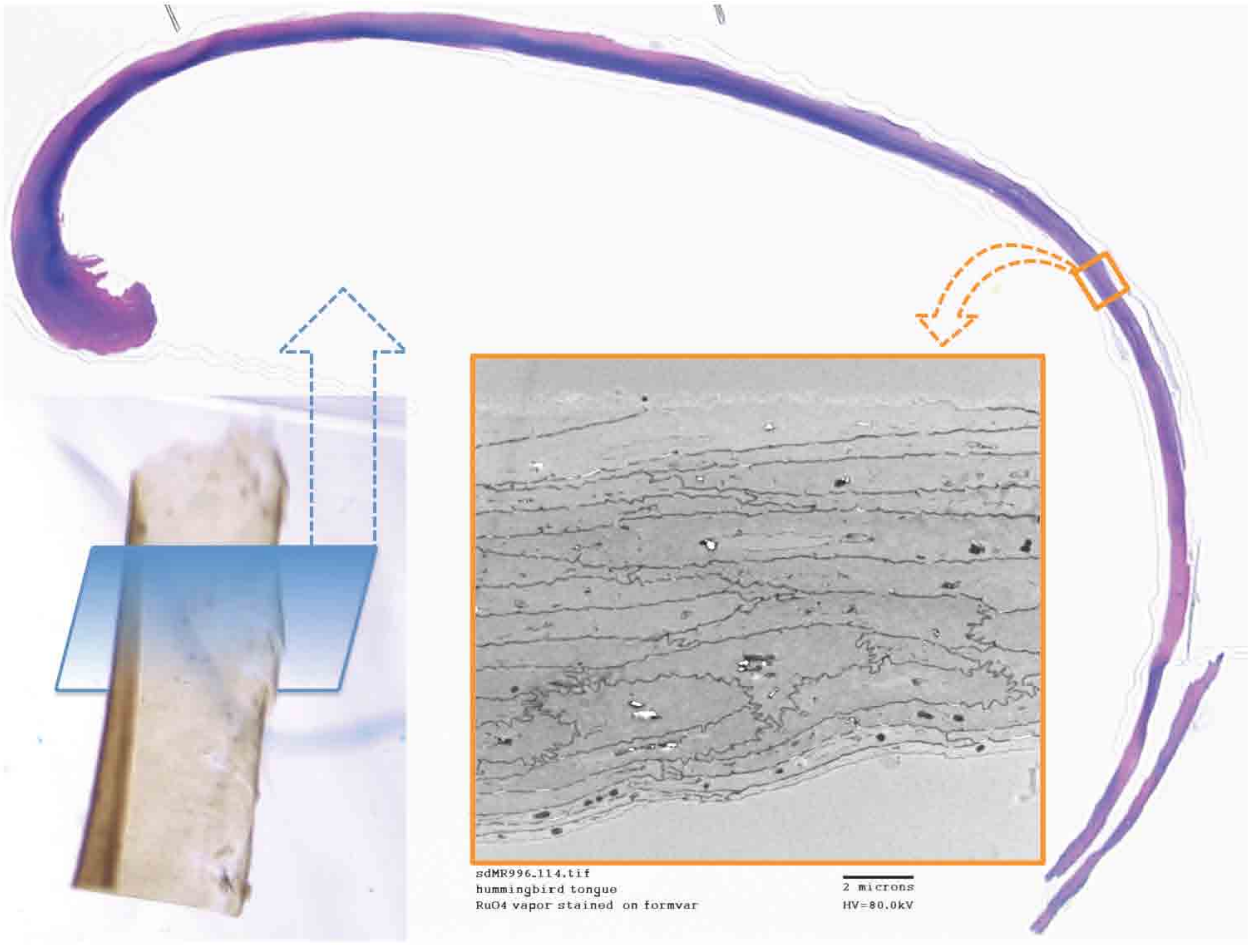



**Figure S7. Hummingbird tongue groove morphology at the most distal portions (near the tip).** On the bottom left, a section of the tongue embedded in resin from which the cross section on top is taken. Cross sections near the tip appear broken at their outward tips because of the presence of diagonal cuts in the tissue (forming the lamellae). Note that this distal portion of the tongue is characterized by a reduction of the supporting rods and a thinning in the keratinized tissue composing the grooves (which at this point is only keratinized band). On the bottom right an electron micrograph showing a close up to the keratinized band tissue.

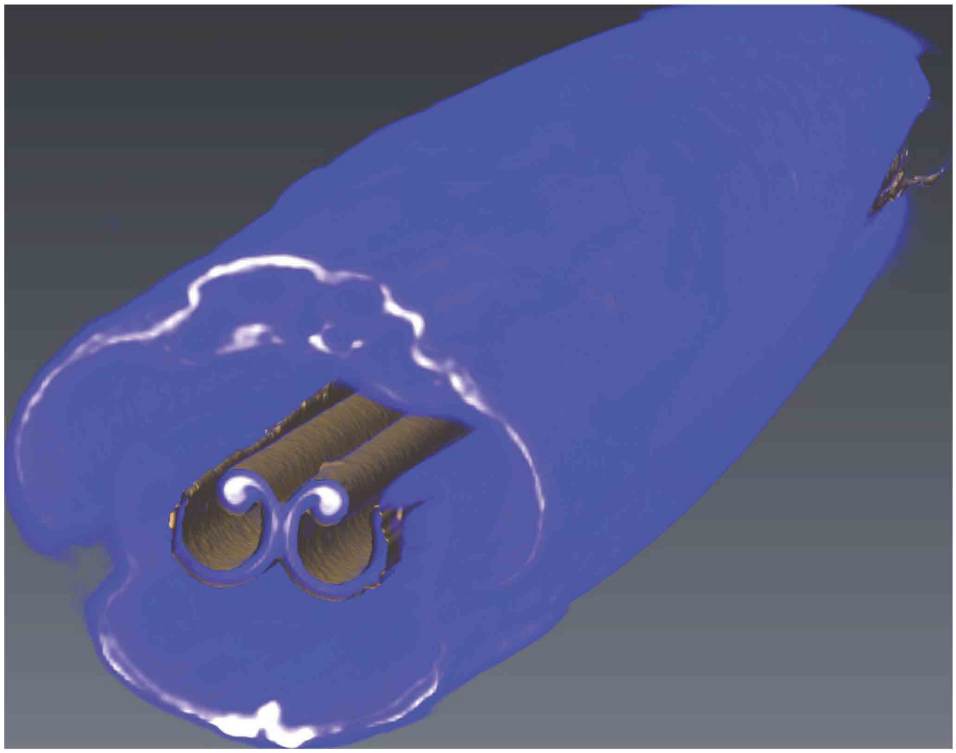


**Figure S8. Three-dimensional digital rendering of a microCT scan of a hummingbird bill stained with $OsO_4$.** We decoupled the tongue from the rest of the bill following a segmentation protocol in Avizo®.

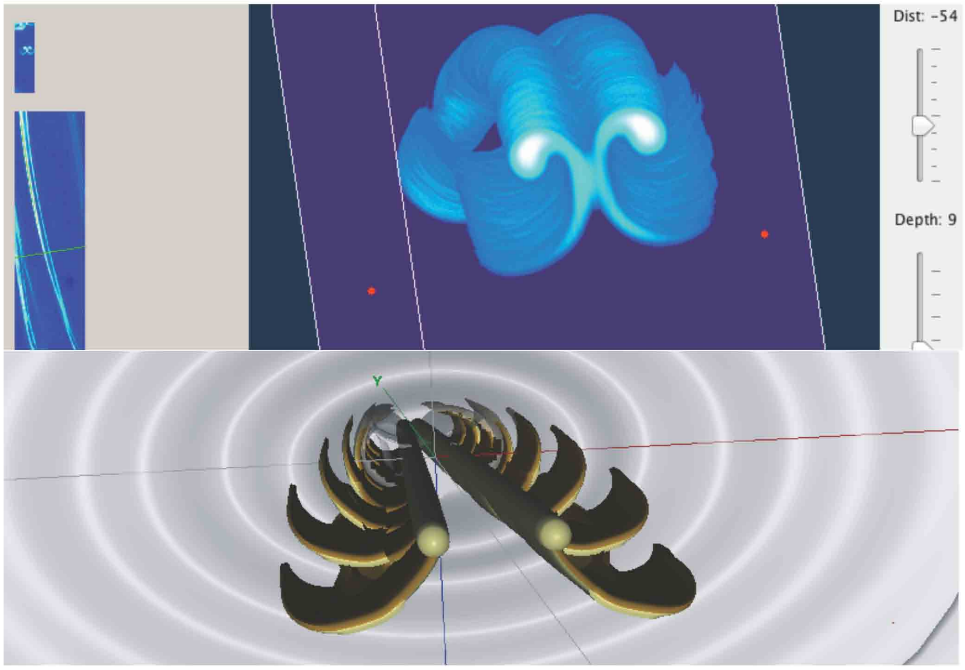



**Figure S9. Reconstructions and models of hummingbird tongues.** On top, a three-dimensional reconstruction of a hummingbird tongue from a microCT scan. Bottom, three-dimensional model of a hummingbird tongue viewed in perspective from the inside of the fluid. Concentric ellipses correspond to waves at the nectar-air interface.

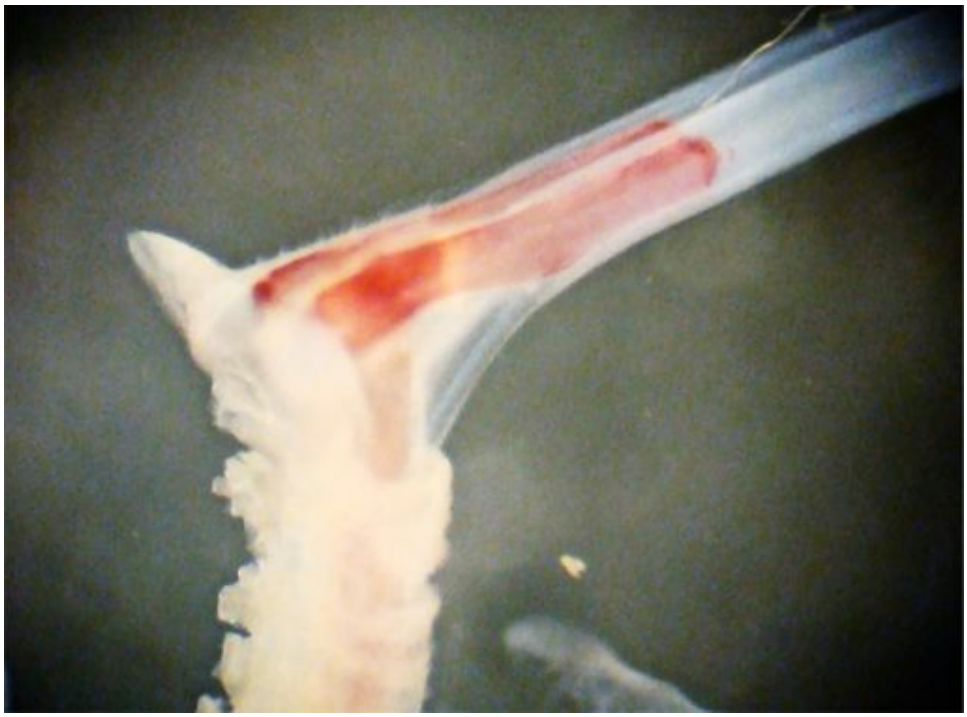


**Figure S10. Dissecting microscope photograph of the tongue base in a cleared and stained specimen.** In this picture of a Black-chinned Hummingbird (*Archilochus alexandri*) the *Os entoglossum* or Paraglossal bones are noticeable. These supporting bones are located only at the basal portion of the tongue tissue chambers.

**Figure S11. Dissecting microscope photograph of the throat region in a dissected specimen.** Featuring a White-necked Jacobin (*Florisuga mellivora*). The accordion-like structure or *tuba elastica* (Zusi 2013) contains the basihyal and ceratobranchial bones allowing them to move independently from the rest of the surrounding tissue and protrude the tongue.

**Figure S12. Macro photograph of the bill and tongue-base of a White-tipped Sicklebill (*Eutoxeres aquila*).** Note the tongue wings at the base of the tongue, which are enlarged in comparison to other hummingbirds.

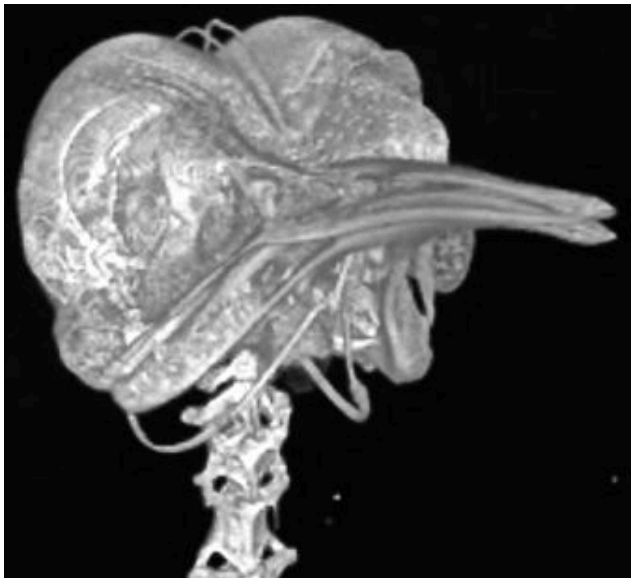


**Figure S13. Three-dimensional digital rendering of a microCT scan of the skull of a Ruby-throated Hummingbird (*Archilochus colubris*).** Note the elongated epibranchials surrounding the skull. A spinning reconstruction makes it possible to follow and visualize the structures (Movie S3).

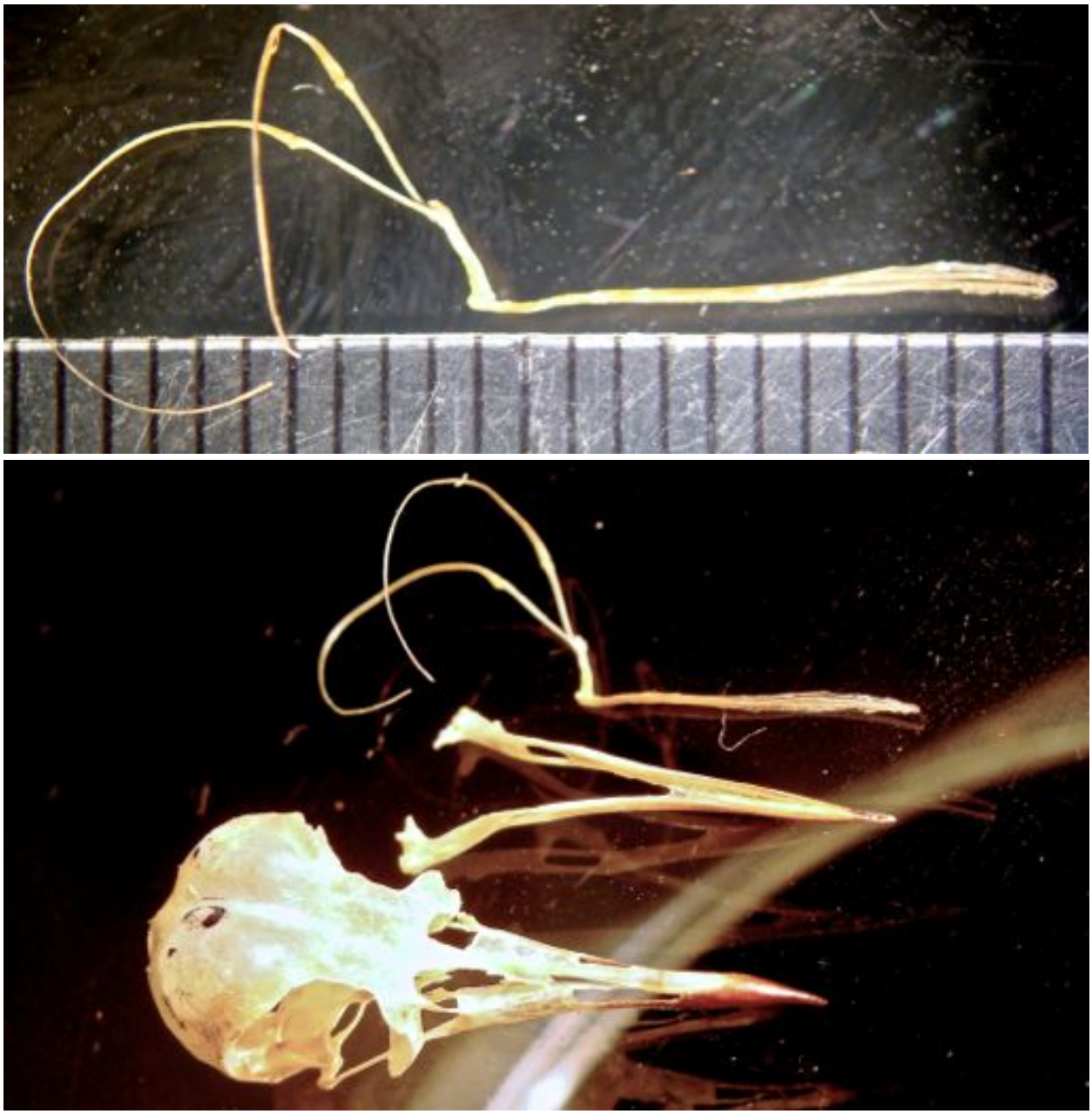


**Figure S14. Photographs of a skull and tongue of a Purple-Backed Thornbill (*Ramphomicron microrhynchum*).** The upper image shows a close up of the tongue and hyobranchial apparatus.

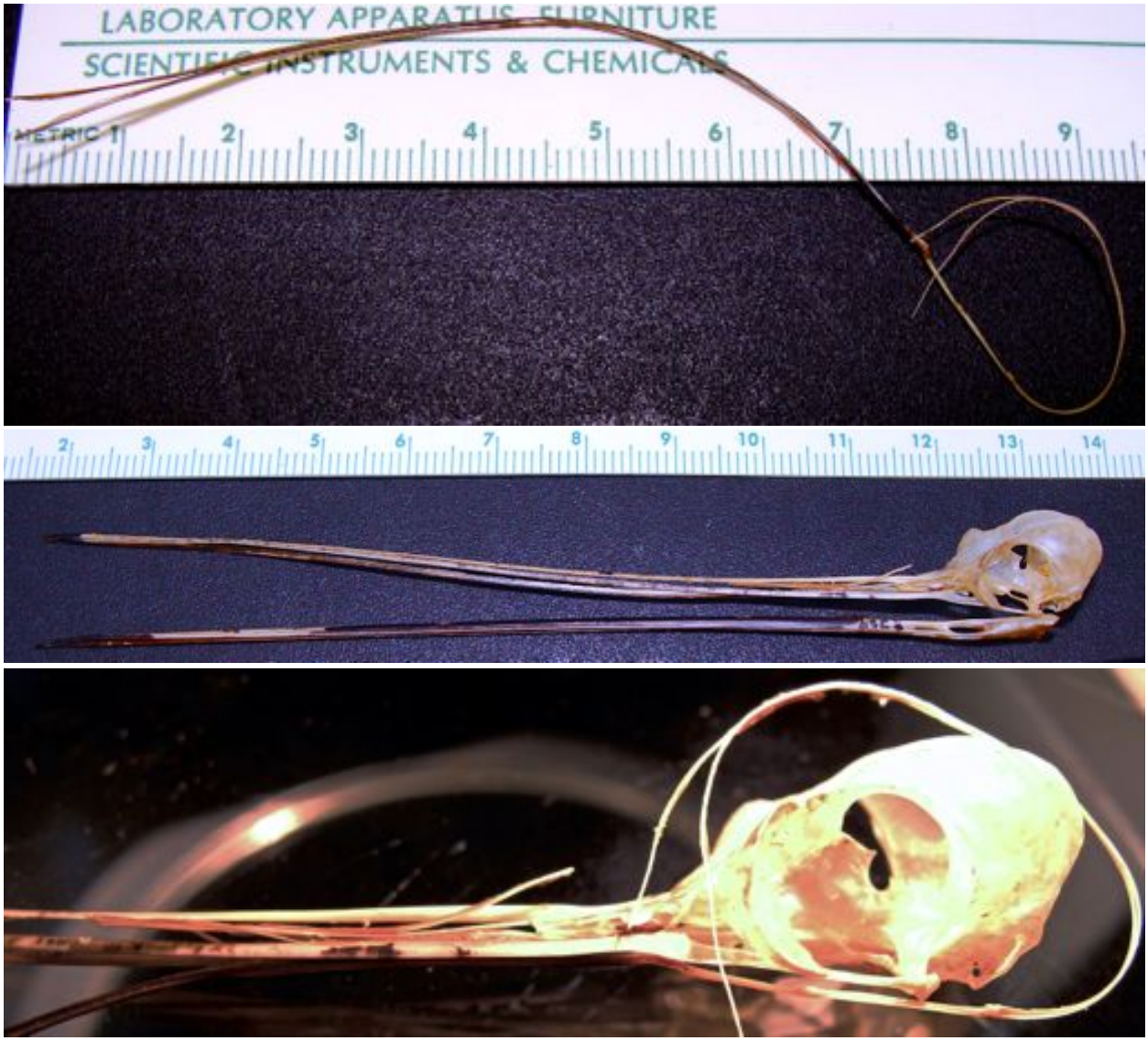
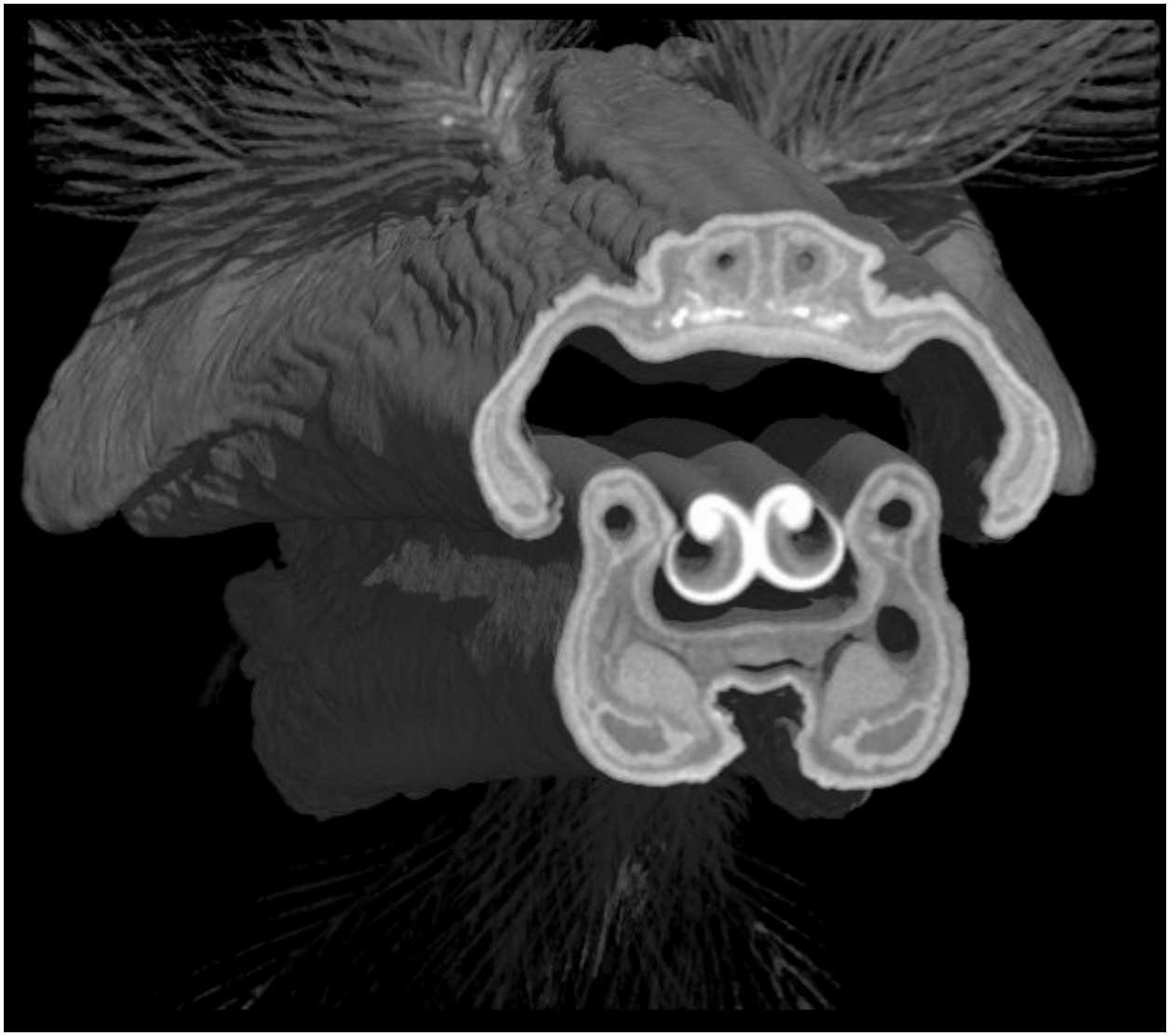


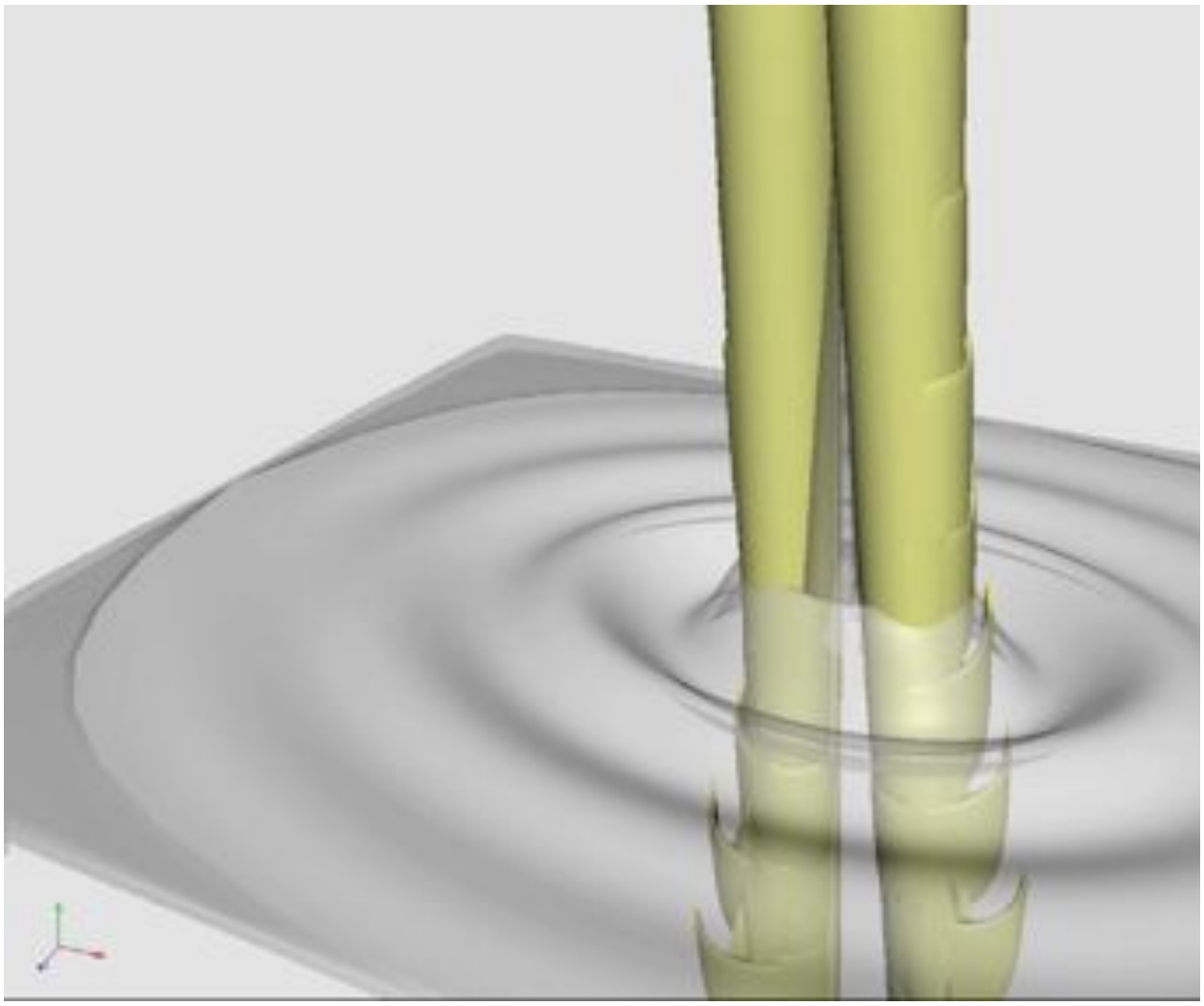

**Figure S15. Photographs of a skull and tongue of a Sword-billed Hummingbird (*Ensifera ensifera*).** The upper image shows a close-up to the tongue and partial (broken epibranchials) hyobranchial apparatus. At the bottom, the rest of the epibranchials are still attached to the top of the maxillary dorsal bone. The attachment between the free ends of the epibranchials and the dorsal maxillary bone in this specimen (~ 25 mm) does not correspond to the predicted maximum insertion of the epibranchials inside of the maxillary rhamphotheca. By matching the tongue inside the mandible, we calculated that the maximum insertion was between 30 and 40 mm.

**Figure S16. Photographs of a skull and tongue of a White-tipped Sicklebill (*Eutoxeres aquila*).** Note how the strong bill curvature is reflected in the tongue shape. We predict that when the tongue is protruded, it would follow a curved trajectory proportional to the bill curvature.

**Supplementary Movies**



**Movie S1. MicroCT rendering (rostro-cranial coronal cross sectioning) of the bill and tongue of an Anna's Hummingbird (*Calypte anna*).** These virtual models of the internal three-dimensional architecture help us to understand the fit between bill and tongue.



**Movie S2. Virtual reconstruction of the tongue based on the MicroCT scans (*cf.* Fig. S9).** We used the morphological information to model the tongue-nectar interaction.

## Supplementary Tables

**Table S1. Tongue measurements for 18 hummingbird species.** Species ordered following the main clades (*e.g.* McGuire *et al.* 2014). Most of these averages per species are displayed in Fig. 1. All the measurements are lengths in millimetres, except for the bill/tongue ratio. See methods for details about the measuring protocol.

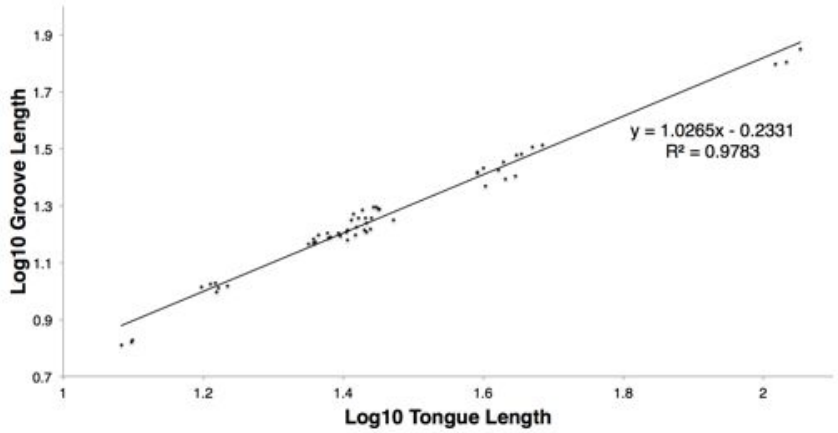
| Clade | Genus | species | Exposed culmen | Total tongue length | Groove length | Fringe region length | Bifurcation length | Tongue thickness | Bill/tongue ratio |
|---|---|---|---|---|---|---|---|---|---|
| Topazes | *Florisuga* | *mellivora* | 20.39 | 26.82 | 19.22 | 6.03 | 14.23 | 0.73 | 0.76 |
| Hermits | *Eutoxeres* | *aquila* | 28.3 | 25.26 | 16.06 | 8 | 16.8 | 0.65 | 1.12 |
| Hermits | *Phaethornis* | *superciliosus* | 35.85 | 41.6 | 28.13 | 8.82 | 24.06 | 0.72 | 0.86 |
| Mangoes | *Doryfera* | *johannae* | 25.7 | 28.1 | 16.8 | 4.9 | 9.4 | 0.49 | 0.91 |
| Coquettes | *Ramphomicron* | *microrhynchum* | 6.36 | 12.4 | 6.6 | 3.5 | 5.9 | 0.35 | 0.51 |
| Coquettes | *Lophornis* | *pavonina* | 9.39 | 16.16 | 10.52 | 4.14 | 6.96 | 0.39 | 0.58 |
| Coquettes | *Oreotrochilus* | *estella* | 18.3 | 26.2 | 15.7 | 6.6 | 9.2 | 0.5 | 0.70 |
| Coquettes | *Sappho* | *sparganura* | 18.7 | 27 | 18.5 | 6.9 | 12.1 | 0.39 | 0.69 |
| Brilliants | *Aglaeactis* | *curpipennis* | 19.7 | 27 | 17.4 | 8.3 | 14 | 0.4 | 0.73 |
| Brilliants | *Pterophanes* | *cyanopterus* | 30.8 | 41.5 | 27.4 | 12.1 | 19.3 | 0.52 | 0.74 |
| Brilliants | *Coeligena* | *violifer* | 33.02 | 42.36 | 24.48 | 14.91 | 22.73 | 0.45 | 0.78 |
| Brilliants | *Ensifera* | *ensifera* | 101.1 | 108 | 66 | 22 | 18.38 | 0.7 | 0.94 |
| Giant | *Patagona* | *gigas* | 36.5 | 46 | 31.1 | 13 | 21.1 | 0.6 | 0.79 |
| Bees | *Mellisuga* | *minima* | 12 | 16.8 | 10.2 | 4.6 | 7.1 | 0.37 | 0.71 |
| Emeralds | *Stephanoxis* | *lalandi* | 16.3 | 22.7 | 14.9 | 5.3 | 10.7 | 0.38 | 0.72 |
| Emeralds | *Amazilia* | *tzacatl* | 20.06 | 23.25 | 15.51 | 8.24 | 15.17 | 0.5 | 0.86 |
| Emeralds | *Thalurania* | *glaucopis* | 16.05 | 24.27 | 15.62 | 6.49 | 12.57 | 0.44 | 0.66 |
| Emeralds | *Eupetomena* | *macroura* | 22.7 | 27.5 | 19.1 | 10.2 | 19 | 0.5 | 0.83 |

**Table S2. Hyobranchial apparatus measurements for 6 hummingbird species.** Basihyale, ceratobranchiale, and epibranchiale lengths were taken for consistency in the right side only, but we did not notice asymmetries. Total hybranchial length is the sum of basihyale, ceratobranchiale, and epibranchiale lengths. All the measurements are lengths in millimetres.

| Genus | species | Total tongue length | Groove length | Fringe region length | Bifurcation length | Tongue thickness | Basihyale | Ceratobranchiale | Epibranchiale | Total hyobranchial |
|---|---|---|---|---|---|---|---|---|---|---|
| *Florisuga* | *mellivora* | 26.82 | 19.22 | 6.03 | 14.23 | 0.73 | 4.25 | 4.43 | 27.33 | 36.01 |
| *Eutoxeres* | *aquila* | 25.26 | 16.06 | 8 | 16.8 | 0.65 | 3.31 | 5.18 | 24.25 | 32.74 |
| *Doryfera* | *johannae* | 26.1 | 16.8 | 4.9 | 9.4 | 0.49 | 3.05 | 3.73 | 22.49 | 29.27 |
| *Ensifera* | *ensifera* | 108 | 66 | 22 | 18.38 | 0.7 | 5.54 | 7.27 | 53.74 | 66.55 |
| *Ramphomicron* | *microrhynchum* | 12.4 | 6.6 | 3.5 | 5.9 | 0.35 | 2.05 | 3.44 | 14.1 | 19.59 |
| *Coeligena* | *violifer* | 42.36 | 24.48 | 14.91 | 22.73 | 0.45 | 3.94 | 5.47 | 31.71 | 41.12 |

**Table S3. Isometric scaling test for tongue *vs.* groove length.** All data (3 specimens/species x 18 species) are used in the log-log plot and in the analyses. The observed slope of 1.0 in the log-log plot indicates isometry. The 95% confidence interval contains this isometric scaling slope (1.0), therefore the tongue length – groove length slope for this relationship is not significantly different from 1.0. A statistically significant result is also obtained when using averages per species (*cf.* Fig. 1), we conclude that this scaling among species is isometric.

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.989 |
| R Square | 0.978 |
| Adjusted R Square | 0.978 |
| Standard Error | 0.032 |
| Observations | 54 |




ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 2.333 | 2.333 | 2344.610 | **6.20858E-45** |
| Residual | 52 | 0.052 | 0.001 | | |
| Total | 53 | 2.385 | | | |

| | *Coefficients* | *SE* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* |
|---|---|---|---|---|---|---|
| Intercept | -0.233 | 0.031 | -7.485 | 8.35634E-10 | -0.296 | -0.171 |
| X Variable 1 | **1.026** | 0.021 | 48.421 | 6.20858E-45 | **0.984** | **1.069** |

# Chapter 2

Appears as published in: Rico-Guevara, A., & Rubega, M. A. (2011). The hummingbird tongue is a fluid trap, not a capillary tube. Proceedings of the National Academy of Sciences, USA, 108(23), 9356-9360.

## The hummingbird tongue is a fluid trap, not a capillary tube

**Abstract**

Hummingbird tongues pick up a liquid, calorie-dense food that cannot be grasped, a physical challenge that has long inspired the study of nectar-transport mechanics. Existing biophysical models predict optimal hummingbird foraging on the basis of equations that assume that fluid rises through the tongue in the same way as through capillary tubes. We demonstrate that the hummingbird tongue does not function like a pair of tiny, static tubes drawing up floral nectar via capillary action. Instead, we show that the tongue tip is a dynamic liquid-trapping device that changes configuration and shape dramatically as it moves in and out of fluids. We also show that the tongue–fluid interactions are identical in both living and dead birds, demonstrating that this mechanism is a function of the tongue structure itself, and therefore highly efficient because no energy expenditure by the bird is required to drive the opening and closing of the trap. Our results rule out previous conclusions from capillarity-based models of nectar feeding and highlight the necessity of developing a new biophysical model for nectar intake in hummingbirds. Our findings have ramifications for the study of feeding mechanics in other nectarivorous birds, and for the understanding of the evolution of nectarivory in general.

We propose a conceptual mechanical explanation for this unique fluid-trapping capacity, with far-reaching practical applications (*e.g.*, biomimetics).

*Keywords:* biomechanics, fluid dynamics, nectar trapping, surface tension

## Introduction

Phenomena driven by surface tension are important in a variety of biological systems (1, 2), and in recent years the importance of working with living organisms to test theoretical biophysical models [e.g., trees (3, 4), arthropods (5–8), and birds (9, 10)] has become evident. Exploration of natural solutions to specific fluid dynamics challenges has provided conceptual tools fostering practical advances in a wide array of fields (11, 12). Discovery of new biophysical mechanisms opens doors to new applied research lines [e.g., biomimicry (13, 14)]. We report here on a previously undescribed mechanism of fluid capture and transport in nature, performed by the tongue of hummingbirds.

The tetrapod tongue evolved to facilitate feeding on land, and in many taxa its primary function is to transport captured food to where it can be swallowed (15). Nectarivores, however, have evolved specialized tongues that function as their primary food-capturing device (Fig. 1A). Hummingbirds are the most specialized nectar-feeding vertebrates (16, 17); thus, we would expect them to possess a highly efficient liquid extraction system.

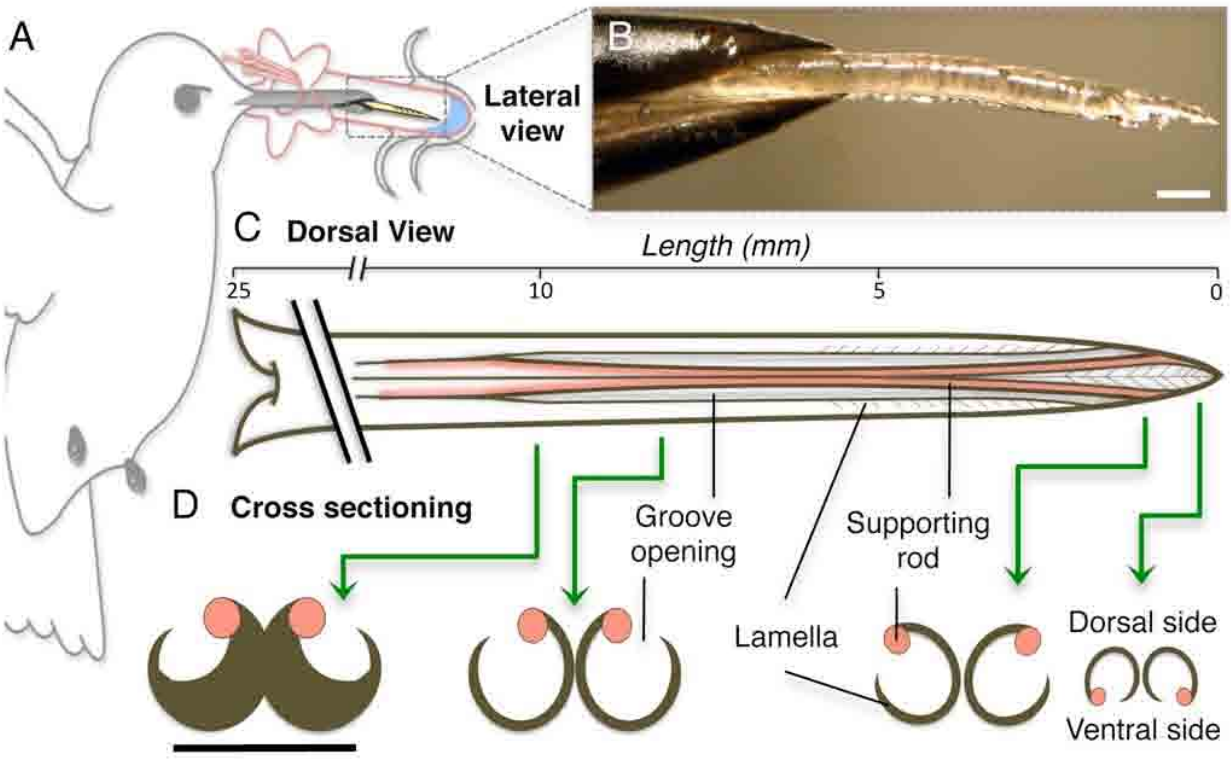



**Figure 1. Hummingbird tongues.** (A) Nectarivores use their tongue (yellow) as their primary food-gathering tool. (B) Lateral picture of a *post mortem* Ruby-throated Hummingbird (*Archilochus colubris*) tongue tip protruding from the bill tip. (C) Dorsal view of the morphology of a hummingbird tongue (approximate dimensions for *A. colubris*) showing length of the entire tongue, open-sided grooves, and the fringed (lamellar) region of the tip (distal approximately 6 mm). Base of the tongue is on the left; tip on the right. (D) Cross-sectioning shows the structural arrangement along the distal region of the tongue; green arrows identify the placement of the cross-sections. Black lines indicate the same structures in dorsal and cross-sectional views. Note the change in position of supporting rods from the base of the grooves to the tongue tip. Unlabeled scale bars, 0.5 mm.

Since first proposed in 1833, it has been believed that the tongue tips of hummingbirds are loaded with nectar by means of capillary rise (18). Detailed biophysical models of nectar-feeding strategies developed almost 30 y ago were based on this idea (19, 20). Since then, all models of foraging strategy (including those predicting concentration preferences) and energy balance in hummingbirds have calculated calorie intake rate under the assumption that the tongue tip is in the form of a pair of semi-cylindrical grooves that fill passively via capillarity when in contact with the nectar (21–26).

The notion that fluid is drawn toward the mouth from the tongue tip and along the lingual grooves through the action of capillarity is currently widely accepted (19–28). However, if capillarity were responsible for tongue loading, the aid of gravity should increase nectar-uptake rates at pendulous (downward facing) flowers, yet empirical work in recent years has failed to demonstrate any consistent correlation between nectar extraction rates and flower position (26, 29). Similarly, according to the parameters of the capillarity models (19, 20), maximum energy intake is predicted to occur with nectar at low sugar concentrations [20–40% (mass/mass)]. Nonetheless, in experimental studies, hummingbirds offered a range of nectar concentrations (spanning those found in wild flowers) preferred higher values [45–65% (21, 24, 30–32)]. Such inconsistencies suggest that a mechanism other than capillarity is involved during tongue loading.

Here, we provide evidence for a different nectar-uptake mechanism and offer a biophysical hypothesis for our observations of tongue–nectar interactions. We found that, contrary to the capillarity models, hummingbird tongue tips dynamically trap nectar by rapidly changing their shape during feeding (Fig. 2 and Movies S1 and S2). High-speed video observations show that an entire tongue transformation cycle occurs in as little as $1/20^{th}$ of a second (cf. ref. 33). This oscillating transformation is driven by fluid and atmospheric forces acting directly on morphological elements of the tongue tips. This description of a (highly efficient) dynamic liquid collecting mechanism has implications for the development of capillary-driven self-assembly of flexible structures (34, 35), and may be useful in microfluidic (36, 37) and microelectromechanical (34, 38) systems with a broad range of applications [*e.g.*, micropliers (39)].

**Methods**

*Morphological Survey of the Tongue Tips.* We examined the tongues of 20 species (three adults/sex/species, for a total of 120 specimens) representing the nine major clades of hummingbirds (Table S1) at magnifications up to 90x. We scrutinized the hummingbird tongues, focusing on their distal region and characterizing the three-dimensional arrangement of their different structures (grooves, supporting rods, lamellae).

In our survey, we included morphologically extreme species (e.g., White-tipped Sicklebill *Eutoxeres aquila*, with a strongly decurved bill) as well as the species with the longest and shortest tongues (Sword-billed Hummingbird *Ensifera ensifera*, and Purple-backed Thornbill *Ramphomicron microrhynchum*, respectively). We used whole, alcohol-preserved specimens from: the Instituto de Ciencias Naturales, Universidad Nacional de Colombia; the Vertebrate Research Collection, University of Connecticut; the National Bird Collection, Smithsonian Institution; and the Department of Ornithology, American Museum of Natural History.

*In Vivo Filming of the Tongue–Nectar Interactions*. We worked at three different elevations (1700, 2400, 2800mabove sea level) in the Andes mountains in Colombia, South America. We filmed free-living hummingbirds of 10 species (three individuals per species; Table S1) feeding at flat-sided (as opposed to tubular, to minimize image distortion) transparent feeders filled with artificial nectar (18.6% mass/mass sucrose concentration). We filmed the tongue–fluid interactions with high-speed cameras (PhantomMiro eX4, monochrome and color) with macro lenses (Nikon 105 mm f/2.8) running at 1,260 frames/s (Fig. 4 and Movies S1 and S2). Laboratory (Post Mortem) Filming of the Tongue–Nectar Interactions.

We used whole tongues of five recently deceased individuals (salvaged specimens) of four species (*Archilochus colubris*, *Colibri coruscans*, *Eriocnemis vestita*, and *Metallura tyrianthina*).We fixed each tongue in place and then slid a drop of artificial nectar (18.6% sucrose concentration) on a glass microscope slide onto and off of the tongue tip (Figs. 2A and 3A and Movies S3 and S4). We filmed the tongue–fluid interaction by coupling high-speed cameras (TroubleShooter HR and Phantom Miro eX4) running up to 2,400 frames/s to a dissecting microscope (Olympus SZX-12) at magnifications up to 50x (Movie S4). We also coupled a digital camera (Casio EX-FH20) to the dissecting microscope to take high-resolution (7 Megapixels) still pictures at 40 frames/s (Fig. 2A).

*Animal Welfare Statement*. All hummingbird filming 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.

**Results**

*Hummingbird Tongue Morphology.* Earlier studies have shown that the distal portion of a hummingbird tongue is bifurcated, with each side forming a groove (by the sides furling inward) when the structures are wet, and that the tongue tips have membranous edges that are fringed with lamellae (18, 40–42). We provide here previously uncharacterized morphological details. We examined the fringed (lamellar) region of the tongue tip of 120 specimens in 20 species of hummingbirds (Table S1). We found that the last approximately 6 mm of the tongue (regardless of its total length) is structured in a previously undocumented arrangement (Fig. 1 B–D).

The lamellae are supported longitudinally by rods (*cf*. ref. 40), and we found that these structures change their relative position both anatomically (along the tongue's length; Fig. 1 C and D) and dynamically (during the process of feeding; Fig. 2). The change in orientation of the supporting rods in resting position, from the dorsal (proximally) to the ventral side (distally, at the tips) of the tongue (Fig. 1 C and D), allows the rotation of the tongue tips when they are withdrawn from the nectar (Fig. 2B and Movie S3), which in turn could improve liquid collection in shallow nectar layers (a common condition in horizontal flowers).

*Mechanics.* We used high-speed video, at rates up to 2,400 frames/s, to document the mechanics of whole, unaltered hummingbird tongues moving in and out of nectar. We filmed 30 free-living birds (10 species; Table S1) attracted to a modified feeder; hereafter, we refer to these results as in vivo observations. To improve visualization of the mechanics, and to assess the degree of control of the mechanism that birds might exert via tongue muscles, we also used 20 tongues removed from salvaged carcasses of dead hummingbirds (4 species; Table S1). We emulated position and movements of the tongue and air–nectar interface under controlled laboratory conditions. The results from these salvaged specimens are hereafter referred to as post mortem observations.

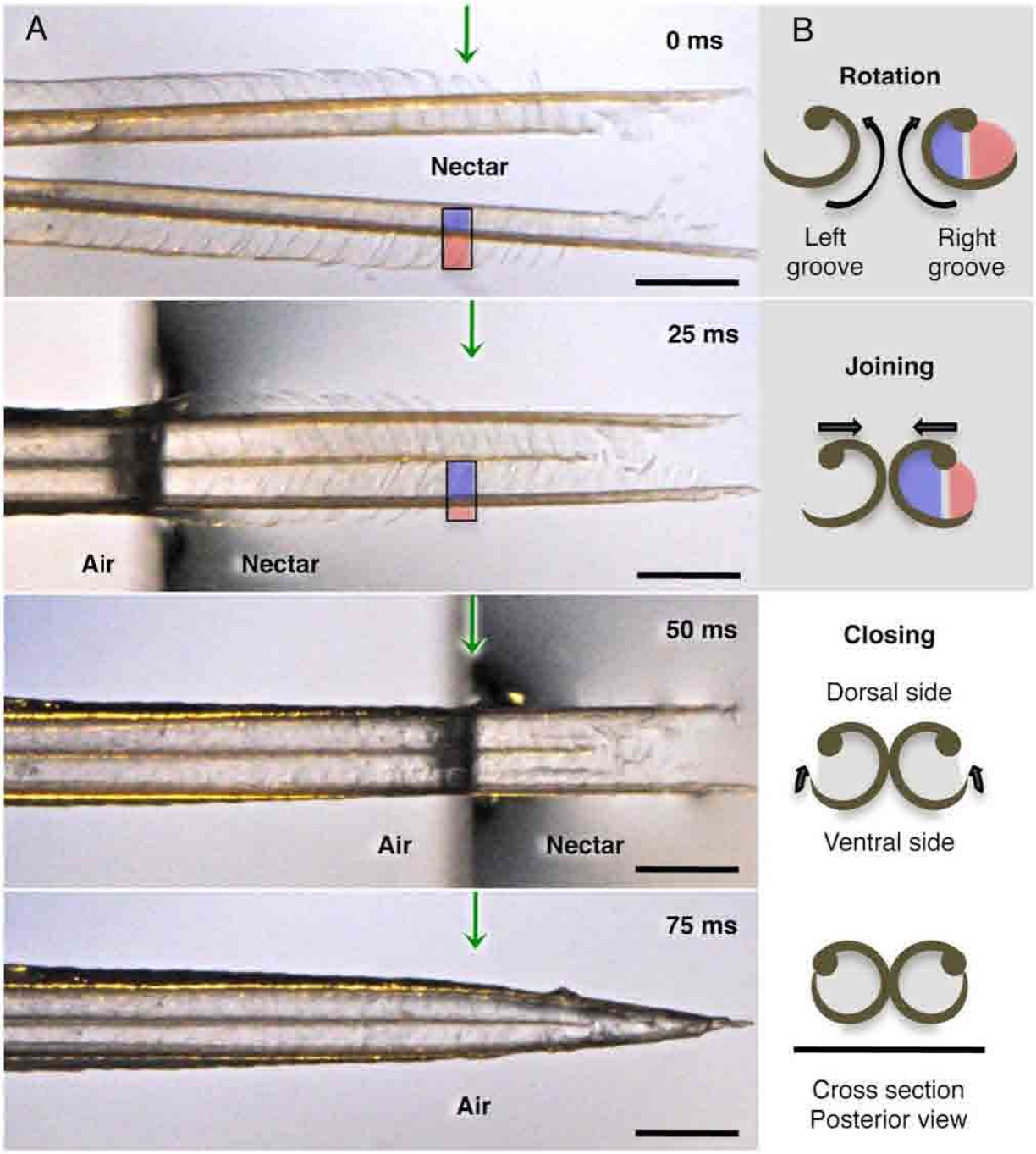



**Figure 2. Hummingbird tongue trapping nectar.** (A) Dorsal view of a post mortem tongue tip (*A. colubris*) leaving nectar, from totally immersed (Top photograph) at 0 milliseconds (ms), to outside the liquid (Bottom photograph). Green arrows mark the same reference point on the tongue in each image. (B) Cross-sectional diagrams (right margin) indicate the changes in position of lamellae at the reference point over time. From top to bottom: inside rotation of the entire structure (blue and red colors represent portions of visible lamellae along each side of the rod), tongue tips joining, and lamellae closing. In the first two diagrams, lamellae are inside the nectar; in the last two, lamellae have been withdrawn but contain nectar trapped inside the grooves. Scale bars, 0.5 mm.

Both the in vivo and post mortem observations reveal that before entering the fluid the tongue is wet (with some nectar inside) and the lamellae are tightly furled in a flattened tube-like conformation, with the tongue tips adhering to each other, forming a pointed, unitary structure (Fig. 1 B and C). Upon contact with fluid, the lamellae immediately unfurl and the tips separate (shown in vivo in Movies S1 and S2). At full immersion, the tongue tips are completely bifurcated and the lamellae entirely extended (Fig. 2, 0 ms). As the tongue is withdrawn from the fluid, the lamellae roll inward, trapping the nectar (shown post mortem in Movie S3). In vivo observations were wholly consistent with the higher-resolution visualization provided by manipulated post mortem tongues.

Post mortem observations were particularly useful in observing the details of the tongue furling process because they could be made under the highest magnification and the highest filming rate. As the tongue is withdrawn from the nectar, each lamella begins closing just before it passes the air–nectar interface, and is fully closed by the interface itself (shown post mortem in Movie S4). This implies that physical forces at the nectar surface are involved in the liquid collection (Fig. 3). We also noted that the progressively smaller lamellae toward the tongue tip (Figs. 1D and 2A) impart a conical shape, distally closed, at the furled tip when the tongue is withdrawn from the nectar (shown post mortem in Movie S3). We surmise that this creates a "lingual seal", preventing fluid from dripping out of the tongue during the transit from the nectar chamber to the interior of the beak; avoiding nectar leakages could be especially important at high licking rates [approximately 17 Hz (33)] when inertial forces would tend to dislodge fluid from the tongue tip.

Our in vivo videos show that hummingbirds maintain a wider opening between the bill tips while retracting their nectar-loaded tongues than during protrusion (compare Fig. 4A *vs.* Fig. 4E; *cf.* ref. 33). We have observed in live birds that during tongue protrusion the bill is opened only at the tip, and apparently only enough to allow the tongue to squeeze past the upper and lower bill tips (cf. ref. 33 and Movie S1). These observations confirm that the distal portion of the tongue is furled, and compressed dorso-ventrally during tongue protrusion, and that the compression is caused by the bill tips that are held closer together at this time (Fig. 4 A and B, frames in first column) than during retraction (Fig. 4 D and E).

**Discussion**

Our observation of rapid lamellar unfurling rules out the idea that the hummingbird tongue tip acts as a set of static capillary tubes during nectar feeding (18–28, 41). The tongue does not passively draw floral nectar up into the grooves via capillarity when its tips contact the liquid; rather, it is dynamically trapping nectar within the lamellae while the tips leave the fluid. Our work with dead specimens demonstrates that neither the unfurling nor the furling of the lamellae requires any muscular work; the process of nectar trapping results purely from the structural configuration of the tongue tips. We are unaware of any other biological mechanism for fluid trapping that is similarly dynamic, yet requires no energy expenditure to drive the opening and closing of the fluid trap.

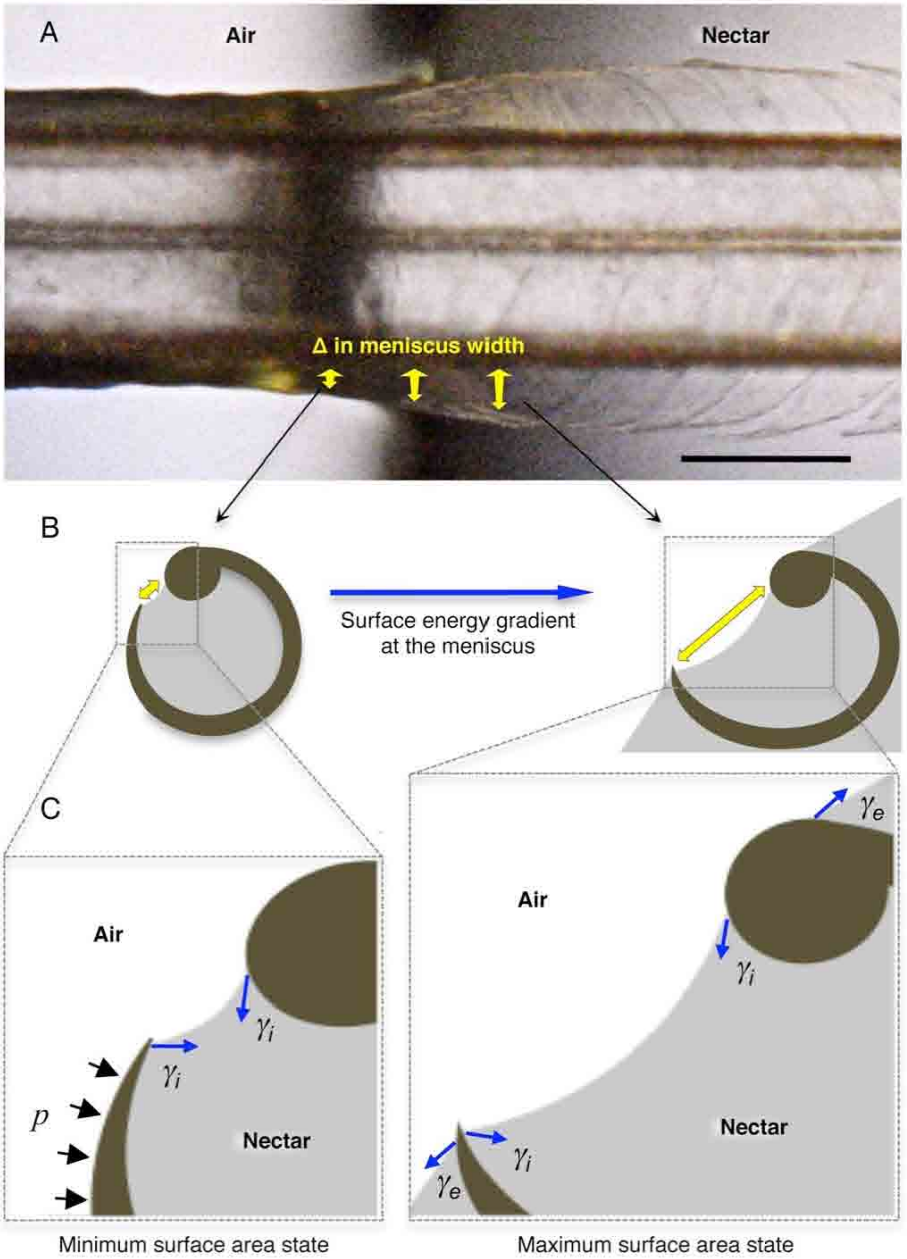



**Figure 3. Conceptual hypothesis of the forces involved in lamellar closing.** Blue arrows indicate the force exerted by surface tension ($\gamma$). Black arrows represent the Laplace pressure (p). (A) Dorsal view of a post mortem tongue (*A. colubris*) interacting with the air–nectar interface, showing the change in lamellar position with respect to the change in meniscal width (sagittally inclined yellow arrows). (B) Cross-section diagrams indicating the surface energy gradient on the internal menisci outside the nectar (Left) and at the beginning of the interface (Right). Yellow arrows depict meniscal width matching the points in the Upper panel. (C) Conceptual representation of the main forces acting on each lamella. Note that the minimum surface area state is achieved outside the nectar (Left) and the maximum surface area state is reached at the beginning of the interface (Right). When the tongue is leaving the nectar and the fluid no longer covers the outer wall of the lamella, the external component of the surface tension ($\gamma_e$) stops operating on the structure, Laplace pressure ($p$) begins to act and the surface area tends to be reduced by the internal component of surface tension ($\gamma_i$). The net result is the bending of the flexible lamella over the stiffer rod. Scale bar, 0.5 mm.

Discovery of this dynamic nectar-trapping mechanism defies a consensus almost two centuries old, and has broad implications for our understanding of the evolution (16, 23, 43), energy budgets (24, 29, 44), foraging behavior (25, 26, 45), feeding mechanics (33, 41, 42), and morphology of the feeding apparatus (18, 46, 47) of hummingbirds. Our morphological survey documented the existence of the structures necessary for dynamic nectar trapping in species of hummingbirds representing all nine main clades in the family (*cf*. ref. 48). Thus, it is reasonable to assume, on the basis of the anatomical evidence, that the dynamic nectar-trapping mechanism documented here is present in every species of hummingbird. We suggest that dynamic nectar trapping is likely to be a component of the feeding mechanics of other nectarivorous birds with convergent tongue morphologies (26, 28, 41, 49, 50). Mechanistically, dynamic trapping appears likely to be functionally superior to simple capillarity in two ways: (I) the tongue-loading rate is not limited by the nectar displacement inside the tongue grooves (which makes it potentially faster) and, perhaps more importantly, (ii) the tongue tip can capture fluid successfully (filling its entire capacity) even in thin layers of nectar. This should allow hummingbirds to take full advantage of even the smallest quantity of resource offered in the shortest amount of time, which also has implications for the minimum volume of nectar a flower must offer in order to attract pollination services.

From a practical point of view, further understanding of this highly efficient liquid collecting mechanism may be useful in bionics (or biomimicry); for instance, in the development of low energy mechanisms for trapping, transporting, and depleting fluids at high production rates, including surface interactions at the microscale (*e.g*., refs. 34–39) with industrial (*e.g.* refs. 36 and 37) and biomedical (*e.g*., ref. 51) applications.

But for these practical applications to be realized, it will be important to answer the question: How does it work? We offer below, as a hypothesis to be tested, an initial biophysical explanation of the nectar-trapping mechanism. This conceptual model can serve to generate testable predictions. Some qualitative predictions can be addressed with observations from this study, but most will require a deeper mathematical treatment to generate quantitative predictions that are testable with measurements of the tongue action under a variety of conditions.

*Biophysical Hypothesis.* We hypothesize that the dynamic nectar trapping process we have observed results from the interplay among surface tension, Laplace pressure, and the elastic properties of the keratinous materials making up the tongue tip (Figs. 3 and 4). We define the start of nectar feeding as the point at which the bird first approaches and inserts its beak into a flower, with the tongue inside the closed bill. The bird protrudes its tongue through a small aperture of the bill tips (*cf*. ref. 33), and past this point the tongue continues to be flattened (Fig. 4 A and B, frames on first column). We posit that at this point (past the compression point of the bill tips) the cohesive and adhesive forces of liquid previously trapped inside the tongue and Laplace pressure keep the lamellae, and hence the grooves, at the tongue tip furled and in a dorso-ventrally flattened configuration (Fig. 4A, cross-section diagram).

In further support of the idea that physical forces (acting on the fluid trapped inside the tongue) are responsible for keeping the lamellae furled, we have observed in post mortem specimens that when the tongue is completely dry the lamellae open and the grooves lose their cylindrical shape.

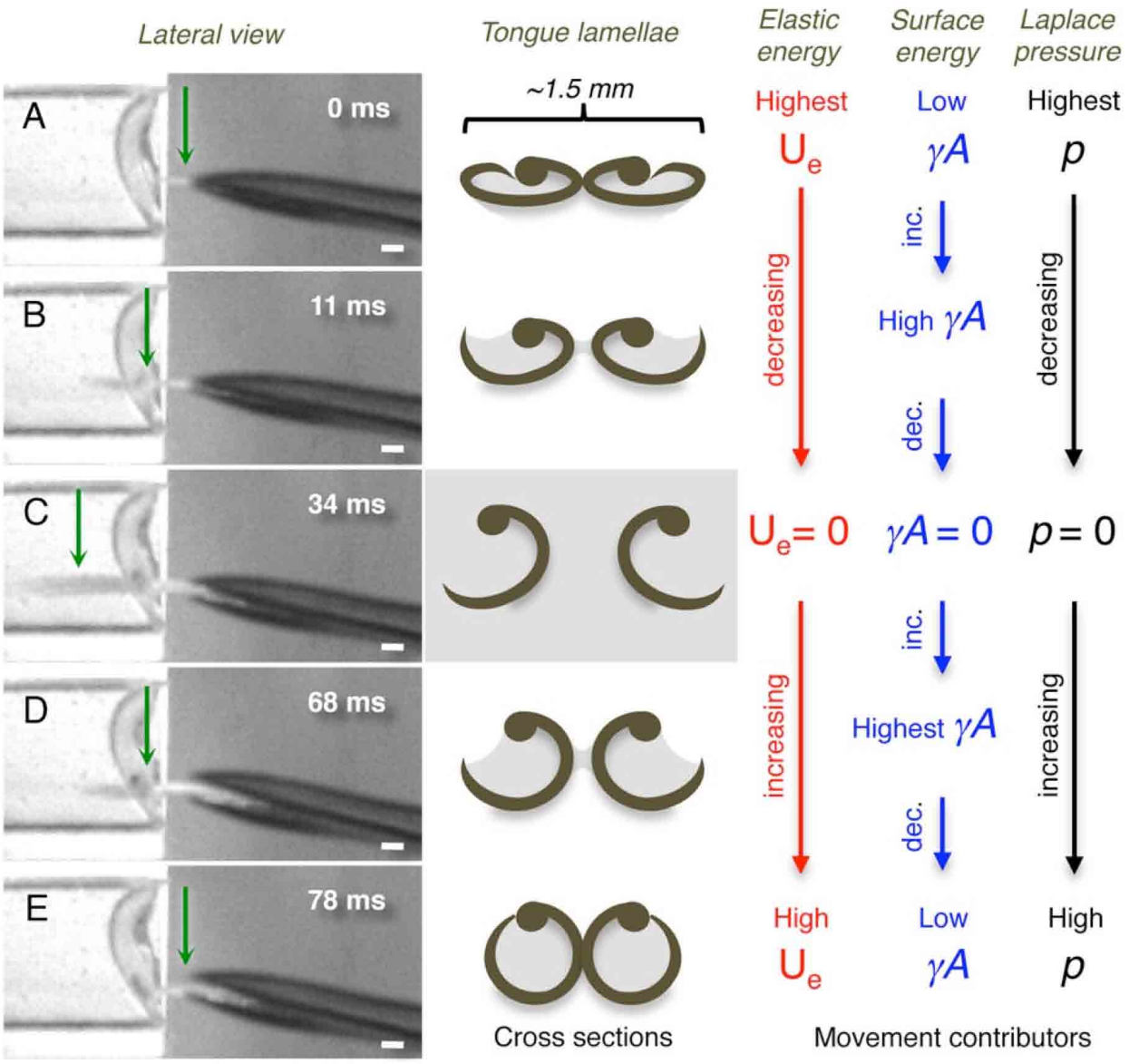



**Figure 4. Conceptual hypothesis for lamellar movements during the licking (tongue) cycle**. (Left column) Frames from the high-speed videos showing a lateral view of the bill tip and the tongue of a living Indigo-capped Hummingbird (*Amazilia cyanifrons*). Green arrows identify the cross-sections denoted in the middle column. (Center column) Cross-sections of the tongue tip showing the shape of the lamellae on each frame in the Left column. (Right columns) Conceptual depiction of the hypothesized relative contributions of the most important contributors to lamellar movements on each shape of the lamellae. Red stands for elastic potential energy ($U_e$), blue for surface energy ($\gamma A$), and black for Laplace pressure ($p$). (A) The cycle begins when the tongue is protruded through a narrow space left when the bill tips are separating from each other. (B) Tongue penetrating the nectar located in the artificial feeder on the Left. (C) Maximum protrusion distance of the tongue in this licking cycle. (D) Tongue leaving the fluid while being retracted inside the bill. (E) Tongue almost fully retracted inside the bill; when the bill closes the cycle starts again. Scale bars, 1 mm.

Thus lamellar furling stores potential energy by bending the flexible lamellae. We suggest that this elastic potential energy is then transformed into kinetic energy when the lamellae unfurl as the tongue enters the nectar. This occurs because as the lamellae are immersed (with liquid on both the outside and the inside of the tongue), opposing surface tension forces at the air–nectar interface cancel each other out (Fig. 3C), allowing the lamellae to open. Thus, inside the liquid, the tongue structures should be released from the forces acting on them outside the nectar pool (Fig. 4C). Two of our observations are consistent with our hypothesis of the forces acting on the lamellae. First, as each lamella crosses the air–nectar interface, it unfurls (Fig. 4B); second, as the tongue penetrates further, the tongue tips separate (Movie S2).

We have also observed (both in vivo and post mortem) that when the tongue is withdrawn from the liquid, each lamella refurls as it reaches the air–nectar interface, thereby trapping nectar. We hypothesize that surface tension at the tongue–fluid interface and Laplace pressure combine to refurl the structure using the supporting rod as a closing and rotational axis (Fig. 3 B and C and Movies S3 and S4). In this model, the surface energy acting on each lamella is expected to build up when the structure approaches the air–nectar interface and should decrease with the subsequent lamellar furling (Fig. 4 D and E). The combination of surface tension along the contact line (the change in meniscal width represented by the three-dimensionally inclined yellow arrows in Fig. 3A) and Laplace pressure should be sufficient to overcome the bending force opposing the lamellar closing (Figs. 3 B and C and 4E). The magnitude of the bending force involved will be quantifiable only through an understanding we currently lack of the physical properties of the keratinized tongue tissue.

Finally, we have observed that when the tongue is entirely free from the nectar pool, the forked tongue tips stick together again; we hypothesize that this results from the cohesive and adhesive forces of the liquid layer between them (Fig. 2A, 25 ms, and Fig. 4D, cross-section diagram).

*Future Directions.* Now that we have shown how nectar is captured at the tongue tip, the next step is to document the mechanics and path of nectar transport along the portions of the tongue that remain outside the nectar and inside the beak. In order to complete the cycle and initiate the nectar-ingestion process, the bird must retract the tongue within the bill and offload the trapped nectar, using an as-yet undocumented process; thereafter the cycle can start again.

Our videos showing that the tongue is dorso-ventrally compressed during protraction (*cf.* ref. 33, Movie S1), suggest that nectar offloading might be accomplished during the tongue protrusion phase by the beak tips "squeezing" nectar off the tongue and into the interior of the bill. It is worth noting that we expect this nectar offloading to clear fluid only from the distal-most portion of the tongue at the start of every tongue cycle. However, the portion of the tongue (and attendant grooves) that remains inside the bill would still be filled with nectar and would also need somehow to be offloaded. Furthermore, after the final lick and tongue retraction at a given flower, the whole tongue would still be loaded with nectar. This hypothesis, that hummingbirds are squeezing nectar from the tongue by protracting it through narrowly opened bill tips, is consistent with the common observation that wild hummingbirds continue cycling their tongues, with a much greater protraction distance than would be necessary inside a flower, even after the tongue has been withdrawn from it.

To actually consume the nectar, the bird must transport the offloaded nectar into the pharynx, where it can be swallowed. The mechanics of this crucial last step of nectar feeding is completely unknown, and the understanding of this process requires further study. Capillary transport of nectar in tongue grooves alone cannot account for transport of nectar from the tongue into the pharynx. In the absence of any additional forces, once the tongue grooves are fully loaded the system should reach equilibrium, and the nectar should cease to move any further. We suspect that a variety of mechanisms (such as suction, surface tension transport, and hydraulic pressure) are mediated by bill–tongue interactions actively controlled by the bird in order to move nectar to the pharynx and thence into the esophagus. Achieving an understanding of this intraoral transport system is likely to be challenging, because the process cannot be observed directly through the bill.

The conceptual hypothesis we offer here for the observed dynamic nectar trapping is in agreement with the empirical data available on hummingbird foraging preferences (21, 24, 26, 29–32). Because the force of gravity should be negligible in comparison to other forces during the lamellar closing process (Figs. 3 and 4), no variation in the extraction rate is expected when varying flower position [in contrast to the capillarity models in which gravity is a determinant (19, 20)] and in fact, none is consistently seen in experiments with living birds (21, 26). Similarly, given the Reynolds number (approximately 1–10) for the different interactions at the tongue–fluid boundary, any drag due to viscosity [also a determinant in the capillarity models (19, 20)] should be overcome by Laplace pressure and surface tension (Figs. 3 and 4).

Higher nectar concentrations are not, therefore, expected to limit fluid intake rate [nectar volume uptake (μL/s)]. Hence, the optimal sugar concentration for a foraging hummingbird should not be limited by the loading portion of the lingual cycle. In contrast, the capillarity models predict that optimal sugar concentrations should be in the range of 20–40% (mass/mass) because those models assume that tongue loading is the rate-limiting step of uptake (19, 20). Instead, concentrations preferred by living birds [45–65% (21, 24, 30–32)] are more likely to be determined by mechanisms of intraoral transport yet to be investigated, or by physiological constraints on uptake and metabolism of the sugars in the nectar (52, 53).

Our 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. We believe that investigations of the physical basis of dynamic nectar trapping can also lead to new tools for the development of engineering applications in microfluidics.

**Acknowledgments**

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## Literature cited

1. Vogel S (1988) Life's Devices (Princeton Univ Press, Princeton, NJ), pp 82–129.

2. Denny MW (1993) Air and Water (Princeton Univ Press, Princeton, NJ).

3. Tributsch H, Nadeždina N, Čermák J (2006) Infrared images of heat fields around a linear heater in tree trunks: What can be learned about sap flow measurement? Ann For Sci 63:1–8.

4. Tyree MT (2003) Plant hydraulics: The ascent of water. Nature 423:923.

5. Suter RB, Rosenberg RB, Loeb S, Wildman H, Long JH (1997) Locomotion on the water surface: Propulsive mechanisms of the fisher spider Dolomedes triton. J Exp Biol 200:2523–2538.

6. Suter RB, Gruenwald J (2000) Predator avoidance on the water surface? Kinematics and efficacy of vertical jumping by Dolomedes (Araneae: Pisauridae). J Arachnol 28:201–210.

7. Hu DL, Bush JWM (2005) Meniscus-climbing insects. Nature 437:733–736.

8. Hu DL, Bush JWM (2010) The hydrodynamics of water-walking arthropods. J Fluid Mech 644:5–33.

9. Rubega MA, Obst BS (1993) Surface-tension feeding in phalaropes: Discovery of a novel feeding mechanism. Auk 110:169–178.

10. Rubega MA (1997) Surface tension prey transport in shorebirds: How widespread is it? Ibis 139:488–493.

11. Blossey R (2003) Self-cleaning surfaces: Virtual realities. Nat Mater 2:301–306.

12. Song W, Veiga DD, Custódio CA, Mano JF (2009) Bioinspired degradable substrates with extreme wettability properties. Adv Mater 21:1830–1834.

13. Parker AR, Lawrence CR (2001) Water capture by a desert beetle. Nature 414:33–34.

14. Zheng Y, *et al.* (2010) Directional water collection on wetted spider silk. Nature 463:640–643.

15. Schwenk K, Rubega MA (2005) Diversity of vertebrate feeding systems. Physiological and Ecological Adaptations to Feeding in Vertebrates, eds JM Starck and T Wang (Science Publishers, Enfield, NH), pp 1–41.

16. Stiles FG (1981) Geographical aspects of bird-flower coevolution, with particular reference to Central America. Ann Mo Bot Gard 68:323–351.

17. 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.

18. Martin WCL (1833) The Naturalist's Library: A General History of Humming-Birds or the Trochilidae, ed W Jardine (H.G. Bohn, London), Vol 41, pp 65–68.

19. Kingsolver JG, Daniel TL (1983) Mechanical determinants of nectar feeding strategy in hummingbirds: Energetics, tongue morphology, and licking behavior. Oecologia 60:214–226.

20. Heyneman AJ (1983) Optimal sugar concentrations of floral nectars: Dependence on sugar intake efficiency and foraging costs. Oecologia 60:198–213.

21. Tamm S, Gass CL (1986) Energy intake rates and nectar concentration preferences by hummingbirds. Oecologia 70:20–23.

22. Stromberg MR, Johnsen PB (1990) Hummingbird sweetness preferences: Taste or viscosity? Condor 92:606–612.

23. Gass CL, Roberts WM (1992) The problem of temporal scale in optimization: Three contrasting views of hummingbird visits to flowers. Am Nat 140:829–853.

24. Roberts WM (1996) Hummingbirds' nectar concentration preferences at low volume: The importance of time scale. Anim Behav 52:361–370.

25. Bateson M, Healy SD, Hurly TA (2003) Context-dependent foraging decisions in rufous hummingbirds. Proc Biol Sci 270:1271–1276.

26. Collins BG (2008) Nectar intake and foraging efficiency: Responses of honeyeaters and hummingbirds to variations in floral environments. Auk 125:574–587.

27. Renvoisé P, Bush JWM, Prakash M, Quéré D (2009) Drop propulsion in tapered tubes. Europhys Lett 86:64003.

28. Köhler A, Leseigneur CDC, Verburgt L, Nicolson SW(2010) Dilute bird nectars: Viscosity constrains food intake by licking in a sunbird. Am J Physiol Regul Integr Comp Physiol 299:1068–1074.

29. Montgomerie RD (1984) Nectar extraction by hummingbirds: Response to different floral characters. Oecologia 63:229–236.

30. Stiles FG (1976) Taste preferences, color preferences, and flower choice in hummingbirds. Condor 78:10–26.

31. Van Riper W (1958) Hummingbird feeding preferences. Auk 75:100–101.

32. Pyke GH, Waser NM (1981) The production of dilute nectars by hummingbird and honeyeater flowers. Biotropica 13:260–270.

33. Ewald PW, Williams WA (1982) Function of the bill and tongue in nectar uptake by hummingbirds. Auk 99:573–576.

34. Guo X, *et al.* (2009) Two- and three-dimensional folding of thin film single-crystalline silicon for photovoltaic power applications. Proc Natl Acad Sci USA 106:20149–20154.

35. Liu JL, Nie ZX, Jiang WG (2009) Deformation field of the soft substrate induced by capillary force. Phys B Condensed Matter 404:1195–1199.

36. Vestad T, Marr DWM, Oakey J (2004) Flow control for capillary-pumped microfluidic systems. J Micromech Microeng 14:1503–1506.

37. Luo JK, *et al.* (2009) Moving-part-free microfluidic systems for lab-on-a-chip. J Micromech Microeng 19:054001.

38. Bico J, Roman B, Moulin L, Boudaoud A (2004) Elastocapillary coalescence in wet hair. Nature 432:690.

39. Py C, *et al.* (2007) Capillary origami: Spontaneous wrapping of a droplet with an elastic sheet. Phys Rev Lett 98:156103.

40. Weymouth RD, Lasiewski RC, Berger AJ (1964) The tongue apparatus in hummingbirds. Acta Anat 58:252–270.

41. Scharncke H (1931) Contribution to the morphology and developmental evolution of the tongue of the Trochilidae, Meliphagidae and Picidae (Beiträge zur Morphologie und Entwicklungsgeschichte der Zunge der Trochilidae, Meliphagidae und Picidae). J Ornithol 79:425–491 (in German).

42. Hainsworth FR (1973) On the tongue of a hummingbird: Its role in the rate and energetics of feeding. Comp Biochem Physiol 46:64–78.

43. Stiles FG (1985) Seasonal patterns and coevolution in the hummingbird-flower community of a Costa Rican subtropical forest. Ornithol Monogr 36:757–787.

44. Weathers WW, Stiles FG (1989) Energetics and water balance in free-living tropica hummingbirds. Condor 91:324–331.

45. Baum KA, Grant WE (2001) Hummingbird foraging behavior in different patch types: Simulation of alternative strategies. Ecol Modell 137:201–209.

46. Berns CM, Adams DC (2010) Bill shape and sexual shape dimorphism between two species of temperate hummingbirds: Black-Chinned hummingbird (*Archilochus alexandri*) and Ruby-Throated hummingbird (*A. colubris*). Auk 127:626–635.

47. Temeles EJ, Roberts WM (1993) Effect of sexual dimorphism in bill length on foraging behavior: An experimental analysis of hummingbirds. Oecologia 94:87–94.

48. McGuire JA, Witt CC, Remsen JV, Jr, Dudley R, Altshuler DL (2009) A higher-level taxonomy for hummingbirds. J Ornithol 150:155–165.

49. 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.

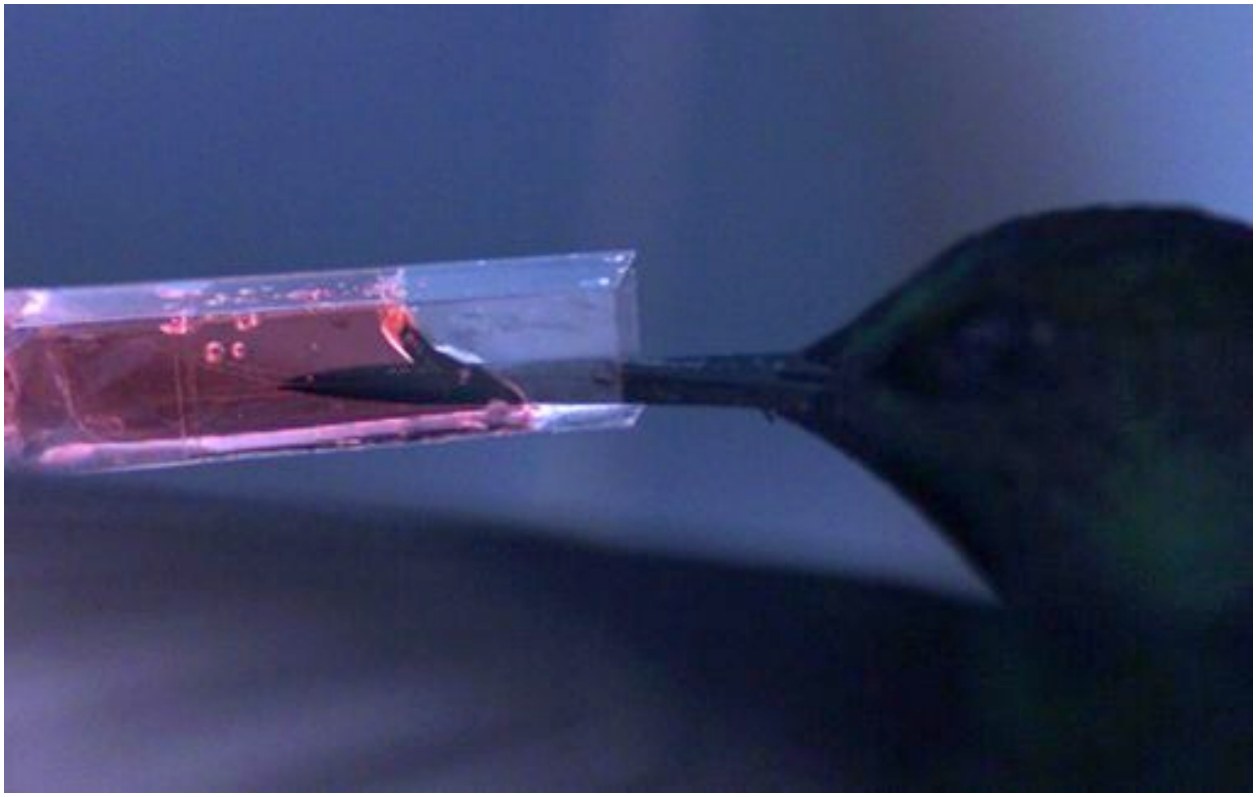
50. Downs CT (2004) Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. Ostrich 75:169–175.

51. Sohn YS, *et al.* (2005) A microbead array chemical sensor using capillary-based sample introduction: Toward the development of an "electronic tongue". Biosens Bioelectron 21:303–312.

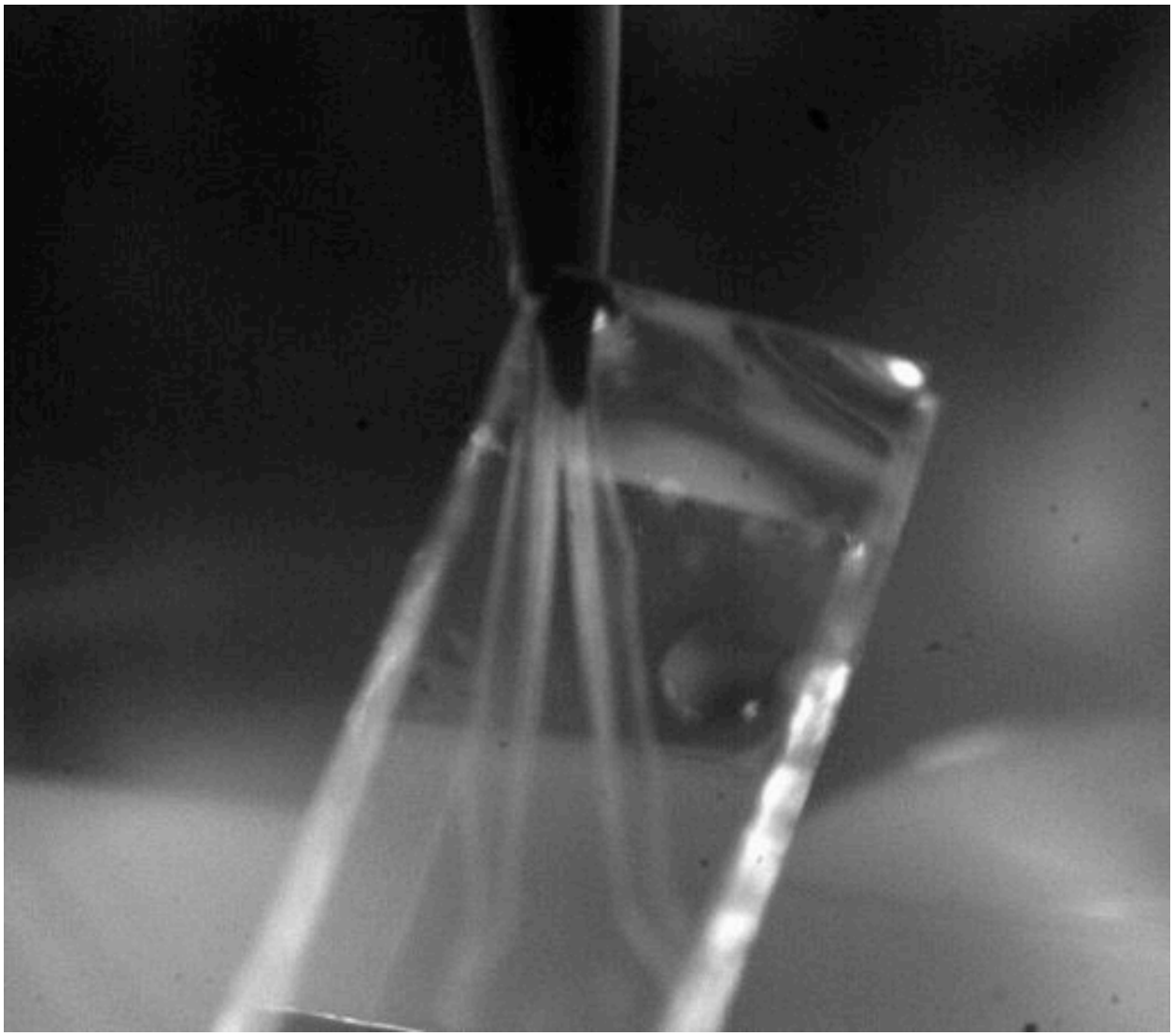
52. Diamond JM, Karasov WH, Phan D, Carpenter FL (1986) Digestive physiology is determinant of foraging bout frequency in hummingbirds. Nature 320:62–63.

53. McWhorter TJ, Martínez del Rio C (2000) Does gut function limit hummingbird food intake? Physiol Biochem Zool 73:313–324.

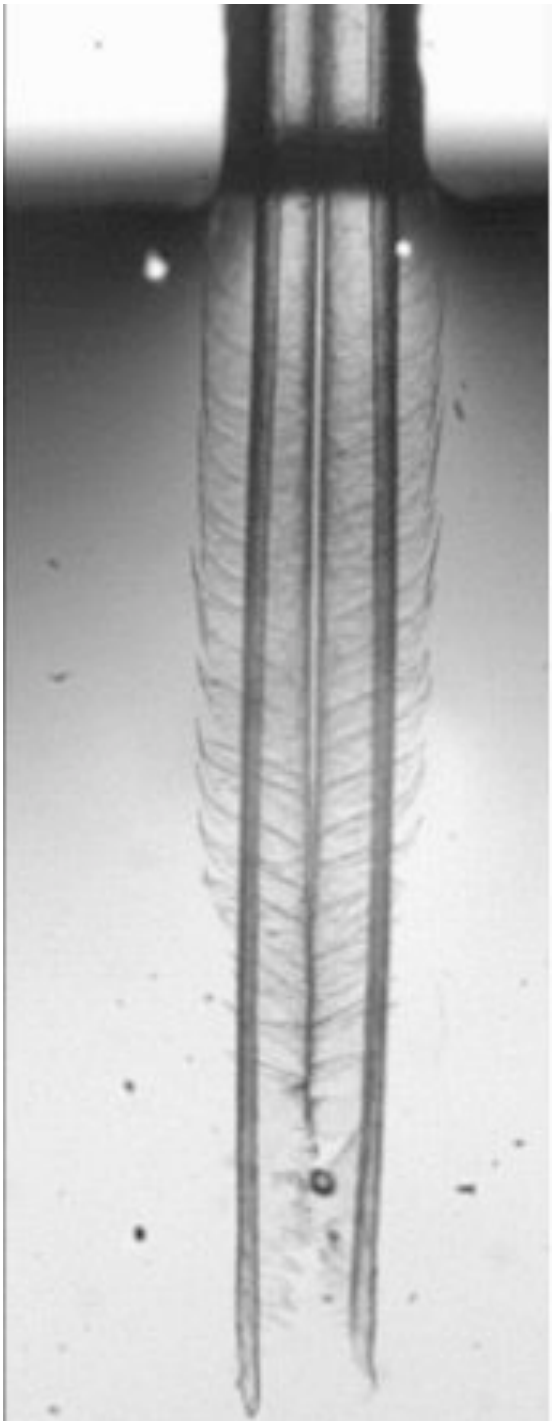
**Supplementary Movies**



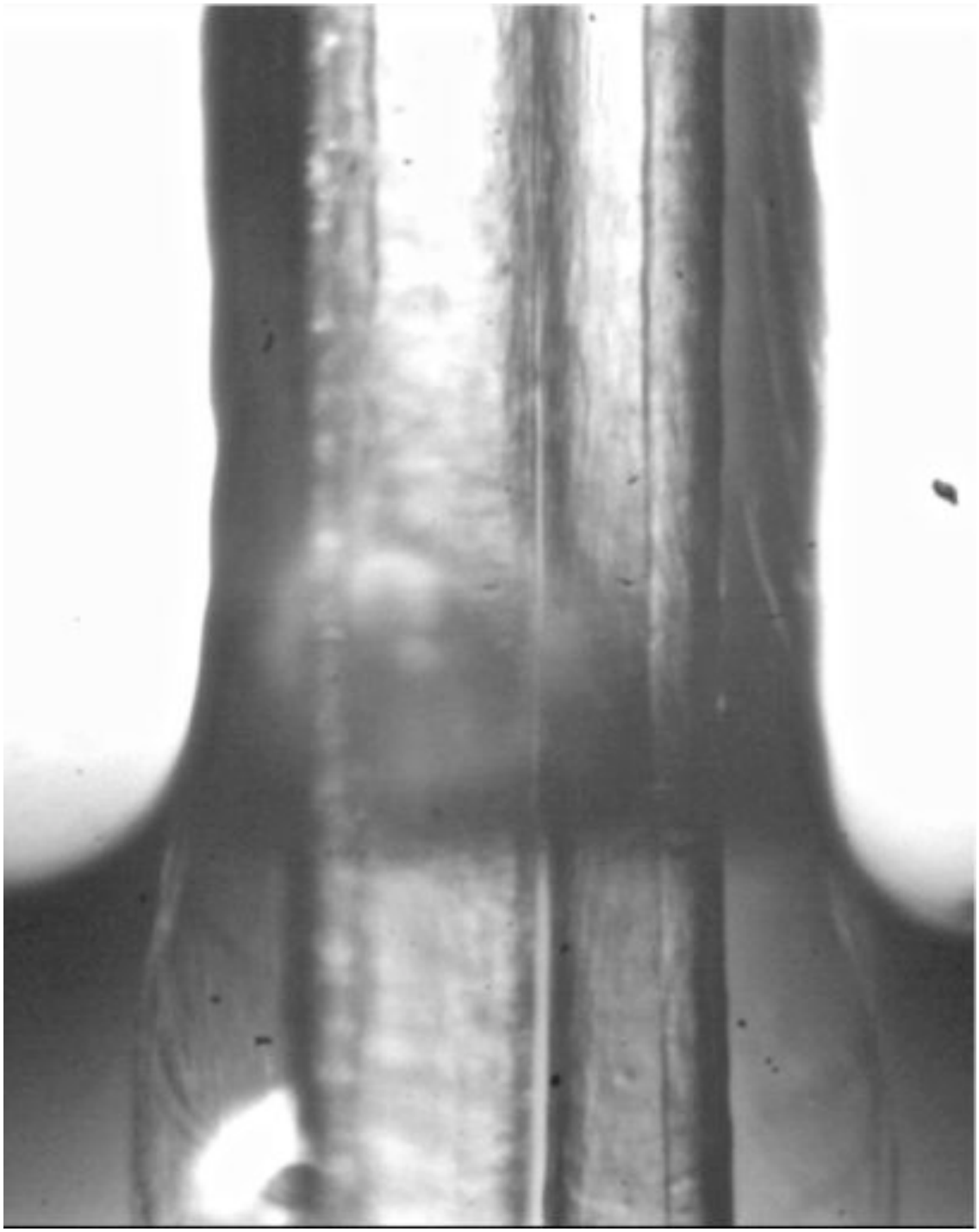
**Movie S1. Hummingbird licking nectar.** A slow motion (165 times slower than real time) video of the lateral view of a Glowing Puffleg (*Eriocnemis vestita*) hovering and feeding on artificial nectar. Note the bifurcation of the tongue as soon as it contacts the liquid. The footage was taken at 500 frames per second (fps), and the timer is displaying in milliseconds.



**Movie S2. Hummingbird licking nectar (close-up).** A slow motion (165 times slower than real time) video of the dorsal view of a Buff-tailed Coronet (*Boissonneaua flavescens*) clinging and feeding on artificial nectar. Note the lamellae opening and rotating as the tongue goes in and out of the fluid. The footage was taken at 500 fps, and the timer is displaying in milliseconds.



**Movie S3. Hummingbird tongue trapping nectar.** A 30× magnification, slow motion (280 times slower than real time) dorsal view video of the post mortem tongue of a Ruby-throated Hummingbird (*Archilochus colubris*) being retracted from a drop of artificial nectar. A spread drop of fluid (thin layer) is drawn along the stationary tongue. Note the rotation of the lamellae before they reach the interface, and that lamellae close and both sides of the tongue tip stick together when the tongue leaves the fluid. The footage was taken at 1000 fps, and the timer is displaying in milliseconds.



**Movie S4. Hummingbird tongue trapping nectar (close-up).** A 50×magnification, slow motion (330 times slower than real time) dorsal view video of a section of the post mortem tongue of a Ruby-throated Hummingbird (*A. colubris*) being retracted from a drop of artificial nectar. A spread drop of fluid (thin layer) is drawn along the stationary tongue. Note how each lamella curves closed and traps fluid as soon as it passes through the air-liquid interface. The footage was taken at 2,400 fps, and the timer is displaying in milliseconds.

**Supplementary Tables**

**Table S1. Hummingbird species sampled for tongue morphology (M) and performance (P).**

| | M | P | | M | P |
|---|---|---|---|---|---|
| *Florisuga mellivora* (0) | ✓ | | *Boissonneaua flavescens* (4) | ✓ | ✓ |
| *Eutoxeres aquila* (1) | ✓ | | *Ensifera ensifera* (4) | ✓ | |
| *Phaethornis longirostris* (1) | ✓ | | *Patagona gigas* (5) | ✓ | |
| *Colibri coruscans* (2) | ✓ | ✓ | *Lampornis amethystinus* (6) | ✓ | |
| *Anthracothorax nigricollis* (2) | ✓ | ✓ | *Chaetocercus mulsanti* (7) | ✓ | ✓ |
| *Rhamphomicron microrhynchum* (3) | ✓ | | *Archilochus colubris* (7) | ✓ | |
| *Metallura tyrianthina* (3) | ✓ | ✓ | *Calypte anna* (7) | ✓ | |
| *Eriocnemis vestita* (4) | ✓ | ✓ | *Thalurania colombica* (8) | ✓ | ✓ |
| *Lafresnaya lafresnayi* (4) | ✓ | | *Chalybura buffonii* (8) | ✓ | ✓ |
| *Coeligena bonapartei* (4) | ✓ | ✓ | *Amazilia cyanifrons* (8) | ✓ | ✓ |

Numbers in parentheses following each species indicate the main clades:

0=Topazes

1=Hermits

2=Mangoes

3=Coquettes

4=Brilliants

5=Giant

6=Mt. Gems

7=Bees

8=Emeralds

# Chapter 3

Rico-Guevara, A., Fan T-H, & Rubega, M. A. Hummingbird tongues combine fluid trapping and expansive filling to function as elastic micropumps. *In prep.*

## Hummingbird tongues are elastic micropumps


### Abstract

A proper comprehension of the feeding mechanics of an organism is the foundation over which a detailed understanding of its foraging behavior, patterns of resource use, and niche limitations can be built. In hummingbirds, downstream calculations of the rates at which they can obtain nectar under different conditions will inform how and where the limits of nectar uptake have shaped their distribution, ecology and evolution. Here we present a hitherto undocumented mechanism of fluid transport through the tongue; we show it works as an elastic micropump in which fluid trapping at the tips is complemented by tongue filling driven by expansion, or "expansive filling", in the section of the tongue between the nectar surface and the bill tip. Using high-speed cameras and artificial feeders that simulated natural conditions, we filmed 18 species of hummingbirds that belong to seven out of the nine main hummingbird clades. We observed expansive filling in all of the species filmed and report detailed calculations for five species. We found that expansive filling loads the tongue five times faster than capillary filling, allowing hummingbirds to extract nectar at higher rates than previously expected. We rule out capillary filling as an important drinking mechanism in free-living hummingbirds, thus previous ecological models and inferences based on estimated extraction rates require re-evaluation.



*Keywords:* Capillarity | Feeding Mechanism | Fluid Dynamics | Hummingbird Foraging

## Significance

Hummingbirds have remarkably high metabolic rates, amazing aerodynamic control, and are classic examples of coevolution with flowering plants. These facts result from hummingbirds having evolved to efficiently exploit small, scattered nectar pools. We describe the processes by which the tongue collects this nectar. Instead of capillarity, long and mistakenly thought to be responsible for tongue loading, we found a surprising mechanism of elastic expansion of the tongue that loads nectar five times faster than capillarity. We present a biophysical model that mathematically describes the expansive filling process, and rules out capillarity as an important drinking mechanism in free-living hummingbirds. This discovery will help us re-evaluate our understanding of how hummingbirds' ability to efficiently extract nectar molds their ecology and evolution.

## Introduction

Pumps are ubiquitous components of living organisms and human technology; they encompass the preferred means to move fluids from the macro to the micro scales [1, 2]. Along this scale gradient, there is an astonishing diversity of mechanisms, from evaporative pumps in the xylem tracheids of over one hundred meter tall conifers [3] all the way to thrust-producing devices like propulsive jets in miniature jellyfish ephyrae [4]. At this micro-scale end we find capillary pumps [2, 5] believed to be important for nectar-feeding birds since the early eighteen hundreds [6, 7, 8] until today [9, 10, 11, 12]. The applicability of the capillary pump mechanism to tongue function, or “capillarity hypothesis”, has been a subject of recent controversy [12, 13, 14, 15].

The importance of accepting or rejecting the capillarity hypothesis as an explanation for nectar-transport has overarching implications. Capillarity equations have been used to infer optimal concentrations in the nectar produced by bird-pollinated plants [14, 16, 17], optimization in drinking behaviors of nectar-feeding animals [18], and fluid transport optimality in a variety of natural and artificial systems [19]. Hummingbirds have remarkably high metabolic rates, amazing speed, superb aeronautic control, and exhibit extreme examples of coevolution with flowering plants [20]. All of these traits relate to a single fact: hummingbirds feed on nectar efficiently enough to make a living out of this sparse resource, and to afford fueling their extreme lifestyles. Therefore the way in which they feed on nectar (their efficiency, preferences and limits) will determine the peaks and ranges of their maximal performance, and thus their behavior (and evolutionary trajectory), across a range of environments. Accordingly, the details of their ecological and evolutionary patterns have been the subject of intense study for over 40 years (*e.g.* [21, 22, 23, 24, 25, 26] and many others). Yet, our recent work demonstrates that we have been making false assumptions about the most basic aspects of how they extract nectar from flowers [13, 15]. Half a century's worth of coevolutionary theory, as understood through hummingbirds as an example, depends on obtaining an empirical, and biologically relevant, mechanistic understanding of the nectar collection process.

Departing from the use of capillarity equations, which are at the base of a long chain of calculations, would generate a domino effect yielding previous inferences and conclusions (*e.g.* optimal concentrations) spurious. The idea that capillarity plays an important role in tongue-loading was an inference arising from the structure of hummingbird tongues, which feature paired longitudinal grooves running from near the tip to mid-tongue (Fig. 1).

Capillarity has been considered important for feeding because it readily occurs when manipulating spirit specimens [6] and may even occur in living hummingbirds under particular experimental settings [12]. Thus, capillary filling is a physically plausible phenomenon given the structure of hummingbird tongues. Nevertheless, we shall focus on a more important question: Is capillarity biologically relevant for hummingbird feeding? Here we report strong evidence to rule out the capillarity hypothesis [6, 12] as the method of feeding in hummingbirds. We describe a new mechanism of tongue filling, which, along with fluid trapping [13], accounts for the total volume of nectar that hummingbirds extract from flowers with every lick.

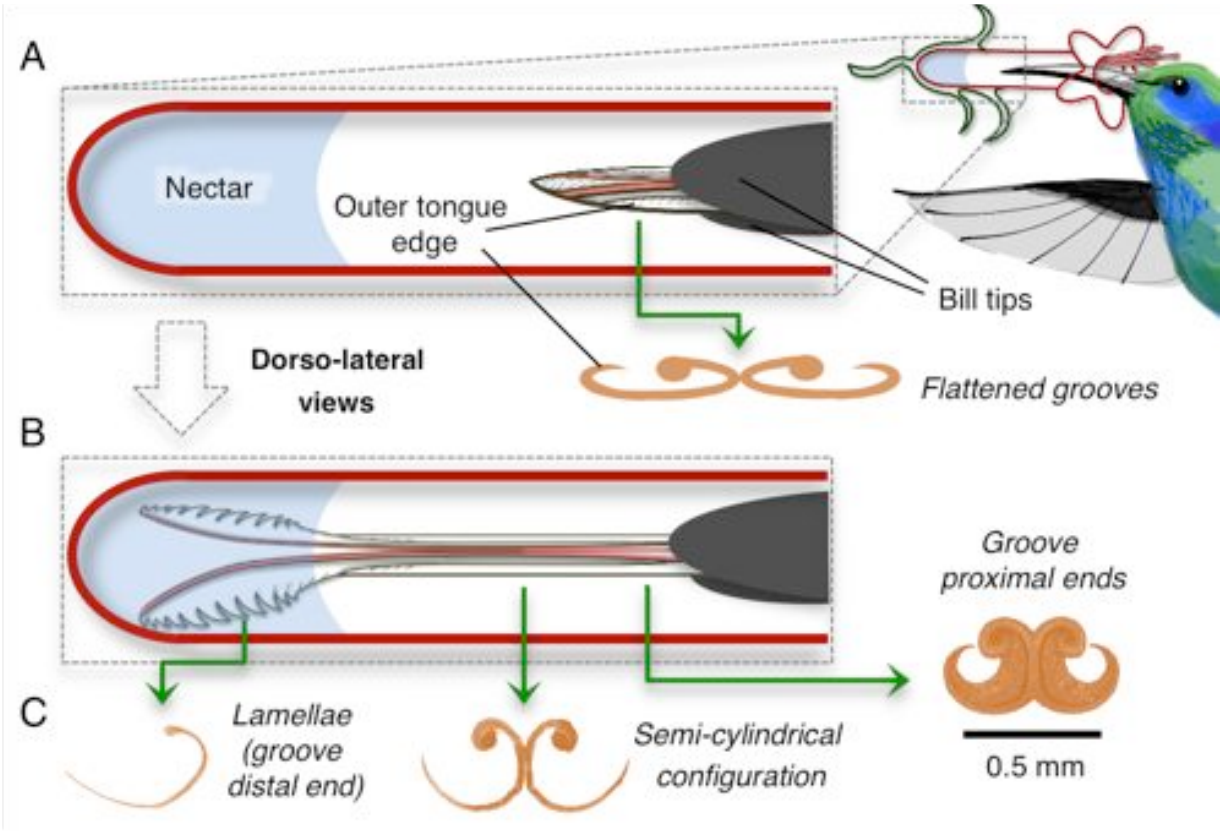



**Figure 1. The hummingbird tongue is structured to fill with nectar even when only the tip is immersed.** A) Hummingbirds frequently drink from flowers with corollas longer than their bills. The bird extends its bifurcated, longitudinally grooved tongue to reach nectar at the bottom of the flower, compressing it dorso-ventrally as it passes through the bill tips. This results in flattening of the grooves (shown in cross-section). B) Upon reaching the nectar, the tongue tips fringed with lamellae roll open and spread apart, but some or all of the grooved portions of the tongue will never contact the nectar pool. For these grooves to fill with nectar, they must return to their uncompressed, semi-cylindrical configuration. C) Cross sections from light microscopy photographs explaining the anatomy of the tongue.

We have previously documented liquid collection through fluid trapping, a mechanism that explains how the tongue tip encapsulates nectar drops using flexible lamellae bent primarily by the action of surface tension [13]. Nectar trapping accounts for the fluid that adheres to the tongue tip when it is submerged in the nectar, however this trapping mechanism does not explain the filling of the portion of the tongue that, in some cases, remains outside the fluid during a licking cycle. When flowers are deep enough that the bill tip never touches the nectar pool, and the extruded tongue has to bridge this gap, the basal portion of the tongue grooves may never contact the liquid (Fig. 1). We studied the process by which this basal portion of the tongue is filled with nectar. Instead of capillary filling, we found a surprising mechanism of elastic expansion of the tongue that accounts for the complete filling of the basal portion of the grooves with nectar.

It has been shown that as the tongue is extruded from the bill (*i.e.* whilst squeezing the nectar off the grooves inside the bill), it is compressed dorso-ventrally along the grooves' entire length [13, 27]. While the tongue is being extruded, at the compression point before emerging (bill tip), the grooves exhibit a flattened configuration (Fig. 1A). Once a given portion of the grooves passes the compression point, there are two possible, mutually exclusive, scenarios (Fig. S1): 1) In the absence of the compression force imposed by the bill tip over the structure, the grooves could recover their cylindrical configuration, yielding two empty cylinders soon after the tongue emerges and before the tongue contacts the nectar, 2) Alternatively the grooves could remain folded while traversing the air, possibly with the force at the compression point being transferred along the length of the tongue walls and/or with the thin layer of liquid remaining inside the folded grooves acting as an adhesive (*cf.* capillary adhesion [5, 28], Fig. S2).

The first scenario in which the grooves regain their cylindrical shape as soon as they emerge from the bill is compatible with the capillary filling hypothesis. In this scenario the tongue would reach the nectar surface as two empty cylinders, and the grooves will fill by capillary action (*i.e.* a meniscus will form and then move proximally, filling the entire length of the grooves). Conversely, in the second scenario the tongue tip would contact the nectar while the grooves are still flattened and retain a thin layer of fluid inside of them; the grooves would then expand dorso-ventrally (recovering their cylindrical shape) only after the tongue tip has contacted the nectar. In this scenario, the grooves would expand as they fill with nectar; the fluid layer that initially kept the grooves flat would increase its volume without allowing air to penetrate the structure, thus preventing bubble formation. Given that there would never be empty spaces inside the grooves while they fill, menisci could be never formed; therefore this second scenario is incompatible with the capillary filling hypothesis, and the tongue, by definition, would be filling by a different mechanism.

In the course of previous work studying fluid trapping in hummingbirds, it appeared to us that the second scenario prevailed, and tongue reshaping occurred only after nectar contact. We provide a hydrodynamic model that incorporates the elastic recovering force on the grooves and the physical properties affecting the fluid dynamics of the nectar. Using high-speed video of free-living hummingbirds we measured nectar and tongue dynamics and empirically tested predictions arising from our model with data drawn from videos. We demonstrate that tongue filling in hummingbirds is achieved by elastically-driven tongue expansion.

*Elastohydrodynamic model*

The licking cycle starts when the tongue is dorsoventrally flattened upon protrusion; this is achieved by pushing the tongue through a small aperture between the bill tips (Fig. S3a). We suggest that while squeezing nectar off the tongue during protrusion, the bird is collapsing the grooves and loading elastic energy into the groove walls that will be subsequently used to pump nectar into the grooves. While the tongue is being extruded, a thin layer of nectar remains inside the grooves, acting as an adhesive overcoming the elastic recovering force and maintaining the dorsoventrally flattened configuration. This stable flattened configuration is conserved during the trip of the tongue across the space between the bill tip to the nectar pool. Once the tongue tip contacts the nectar surface, the free supply of fluid eliminates the adhesive-cohesive forces that were holding the tongue in the flattened configuration, allowing the grooves to expand.

The release of the elastic energy (expansion of the grooves) pulls nectar inside the grooves until they fill completely; hereafter we refer to this previously undocumented mechanism as “expansive filling”. The liquid column has a progressive front within the tongue *h(t)* (Fig. S3b). We model a single uptake, periodic, event by a simplified tube configuration with a sealed end at the groove base (Fig. S3b), and deduce that the fluid motion and uptake rate can be characterized by short- and long-time processes (Fig. S3c). The local effects of gravity are negligible due to the small dimension of the system. The collapsed state is maintained by the adhesion or negative excess pressure applied on the groove structure. The elastic recovery occurs as nectar rushes into the tube, and the fluid motion and uptake rate can be characterized by the balance of inertial, elastic, viscous, and local transmural pressure forces.

Using preliminary observations, we determined that the peak flow velocity is on the order of 1 m/s. The tube inner diameter is estimated to be around 0.3 to 0.4 mm from the tongue thickness of the species studied, which leads to a Reynolds number on the order of 100. Considering a long time process, the inertia of the tube is neglected, and the viscous incompressible fluid motion within the tube can be modeled as a quasi-steady pressure driven flow. The quasi-Poiseuille flow provides the volumetric flow rate based on a linear relation with the pressure gradient,

$$\dot{Q}(z,t_L) \simeq -\frac{\pi R(z,t_L)^4}{8\mu}\frac{\partial p(z,t_L)}{\partial z}, \qquad \textbf{[1]}$$

where $t_L$ indicates the long-time process in which the inertia of the tube and the fluid are neglected and the elastic recovery of the tube is quasi-static, $R$ is the apparent local radius of the tube, $\mu$ is dynamic viscosity, $p$ is pressure, and the traveling distance of the liquid column is defined by $0 \leq z \leq L$ where $L$ is the tube length (much larger than the tube diameter). The flow rate and various apparent radii along the tube satisfy the quasi-1D continuity equation:

$$\pi\frac{\partial R(z,t_L)^2}{\partial t_L} + \frac{\partial \dot{Q}}{\partial z} \simeq 0 \qquad \textbf{[2]}$$

The negative excess pressure is assumed proportional to the change of the cross sectional area,

$$p(z,t_L) = E\left[\frac{R(z,t_L)^2}{R_f^2} - 1\right]. \qquad \textbf{[3]}$$

where $R_f$ is the fully recovered tube radius and $E$ is the apparent area modulus of the tube. Both $p$ and $E$ influence the strength and time for the elastic recovery. Combining the momentum, continuity, and the elastic equations leads to a nonlinear diffusive pressure equation [29]:

$$\frac{\partial p}{\partial t_L} = \frac{E R_f^2}{8\mu} \frac{\partial}{\partial z} \left[ \left( \frac{p}{E} + 1 \right)^2 \frac{\partial p}{\partial z} ) \right], \qquad \textbf{[4]}$$

which can be used to characterize a collapsible tube as in blood flow and other physiological scenarios [30, 31, 32].

In the low Reynolds number regime, the elasticity of *e.g.* blood vessels determines the degree of compliance of the tube wall due to the pressure drop, while in the tongue of hummingbirds the elasticity of the groove walls plays an active pumping role during the recovery of the collapsed configuration. Accordingly, the characteristic length and diffusive time scale are $L$ and $8\mu L^2/(R_f{}^2 E)$, respectively. Here we define an initial condition for the negative excess pressure $p(z, 0) = -p_a$ where $p_a$ is a positive constant to be determined. The boundary condition at the inlet ($z = 0$) has zero gage pressure, while the pressure gradient vanishes at the sealed end ($z = L$).

At the very beginning of the process, because acceleration is important when the fluid is suddenly drawn into the tube, the above quasi-steady approximation is no longer valid in this short time regime. The elastic relaxation is considered fast and the motion of the tube boundary is much slower than the accelerated momentum transport.

We therefore decompose the velocity field for the whole process into transient $v_z(r, z, t)$ and quasi-steady $\tilde{v}_z(r, z, t_L)$ contributions, which correspond to the short-time and long-time processes respectively. In the short-time regime, the quasi-linear transient flow can be expressed by the diffusive momentum equation,

$$\frac{\partial v_z}{\partial t} \simeq \frac{\mu}{\rho}\left(\frac{\partial^2 v_z}{\partial r^2} + \frac{1}{r}\frac{\partial v_z}{\partial r}\right), \qquad \textbf{[5]}$$

in which the pressure effect vanishes due to the balance with the viscous effect. The initial condition is $v_z(r, z, 0) = -\tilde{v}_z(r, z, 0)$. The boundary conditions are finite velocity $v_z(0, z, t)$ along the axial line, and no-slip condition at the wall $v_z(R(z, t_L), z, t) = 0$. The analytical solution for the transient velocity field can be derived and expressed as

$$v_z \simeq -2\sum_{m=1}^{\infty} \exp\left(-\beta_m^2 \frac{t}{\rho R_f^2/\mu}\right) \frac{J_0\left(\beta_m r/R_f\right)}{J_1^2\left(\beta_m R/R_f\right)} \times \int_0^R \frac{r J_0\left(\beta_m r/R_f\right)\tilde{v}_z}{R^2} dr, \qquad \textbf{[6]}$$

where $J_0$ and $J_1$ are the zeroth and first-order Bessel functions, respectively, and $\beta m$ are the eigenvalues given by the no-slip condition $J_0(\beta m R/R_f) = 0$. As a further correction for the leading-order approximation, the initial condition in the integral term is given by the long-time velocity from the pressure equation, expressed as

$$\tilde{v}_z(r, z, t = t_L) \simeq \frac{1}{4\mu}\frac{\partial p(z, t_L)}{\partial z}\left[r^2 - R^2(z, t = t_L)\right]. \qquad \textbf{[7]}$$

Finally the complete velocity is the summation of both transient and quasi-steady components, $v_z + \tilde{v}_z$. The characteristic short time scale is on the order of 1 ms, while the long time scale is about 10 to 20 ms. The local flow rate is obtained from the apparent radius and pressure gradient given by Eq. [1]. Here the nonlinear pressure equation is solved numerically before substituting into the integral solution for the velocity field, Eq. [6]. The traveling distance and the velocity of the progressive moving front of the liquid column can therefore be tracked by a simple Lagrangian integration.

## Methods

*Fieldwork*

At field sites with existing feeders in seven countries throughout the Americas, we filmed free-living, never handled, hummingbirds feeding at modified transparent feeders simulating the nectar volumes and concentrations of hummingbird-pollinated flowers. We measured 96 foraging bouts of 32 focal birds belonging to 18 species from seven out of the nine main hummingbird clades (Table S1). We used artificial nectar (18.6% m/m sucrose concentration), and focused on recording the tongue-fluid interaction using high-speed cameras (TroubleShooter HR and Phantom Miro ex4) with macro lenses (Nikon 105mm f/2.8 VR) running up to 1260 frames/s (1280 x 512 pixels). We positioned red flat plastic sheets (to minimize lateral view obstruction) cut in flower shapes at the entrance of the feeders. The purpose of the flat flowers was two-fold: To attract and guide wild hummingbirds into our feeders, and to allow us to control the relative position of the flower with respect to the nectar chamber.

While filming wild hummingbirds we noted that every individual, after a couple of exploratory visits, would insert its beak as far as possible into the feeder in order to reach the nectar. At real flowers, corolla length limits how close the bird can place the tip of its beak to the surface of the nectar pool. Controlling the position of the flat flower with respect to the nectar reservoir, we achieved videos in which there is enough realistic distance between the bill tip and the nectar surface to study the filling of the tongue portions that never enter the liquid (*e.g.* Video S4). To improve visualization of the filling front, while filming Amazilia Hummingbirds (*Amazilia amazilia*) in Ecuador, we used hummingbird nectar concentrate (Petco®), which comes tinted red, and we diluted it (down to 18.6% m/m concentration).

All filming activities were reviewed and authorized by the Institutional Animal Care and Use Committee at the University of Connecticut; Exemption Number E09-010.

*Velocity and thickness measurements*

We limited our measurements to the instances in which we could confidently track the tongue tip and the groove bases throughout the entire lick. We calculated tongue tip velocity through time and estimated fluid displacement velocities using ImageJ [66]. To measure groove thickness at regular length intervals, we delineated the contour of the tongue in tpsDig 2.16 [67]. Subsequently we limited these outlines between the tip and the base of the grooves and resampled dorsal and ventral outlines into 20 semilandmarks [68, 69].

These semilandmarks allowed us to calculate groove thickness at equally spaced points along the tongue through the licking cycle, and to quantitatively test predictions from the mutually exclusive possible outcomes (Fig. S8). Having good estimates of the thickness of the tongue at the groove tip and base is important to provide accurate measurements of expansion at comparable points (semilandmarks, *e.g.* Fig. S9) across licks, individuals and species. For these comparative measurements we used the semilandmark #12 (near the middle of the grooves), and calculated the percentage, out of the final thickness of the groove, that the expansion represents (Table S1).

**Results**

Our high-speed video data from 96 foraging bouts (hundreds of licks) of 32 individual birds of 18 species confirm that the extruded tongue of a hummingbird does not reshape immediately after passing the compression point (bill tip). Instead, it remains flattened until the tongue tip contacts the nectar surface (Video S1); after contact the whole tongue expands dorso-ventrally, filling completely with nectar (Fig. 2). Given that the tongue does not reshape after passing the compression point, capillary filling cannot occur since there are no "empty cylinders" and menisci are never formed. Measured licks in living birds followed the same pattern: Tongue thickness started increasing when it contacted the nectar and reached a maximum approximately when the tongue started to be retracted. After loading, the grooves filled with nectar were brought back inside the bill and squeezed for the next cycle.

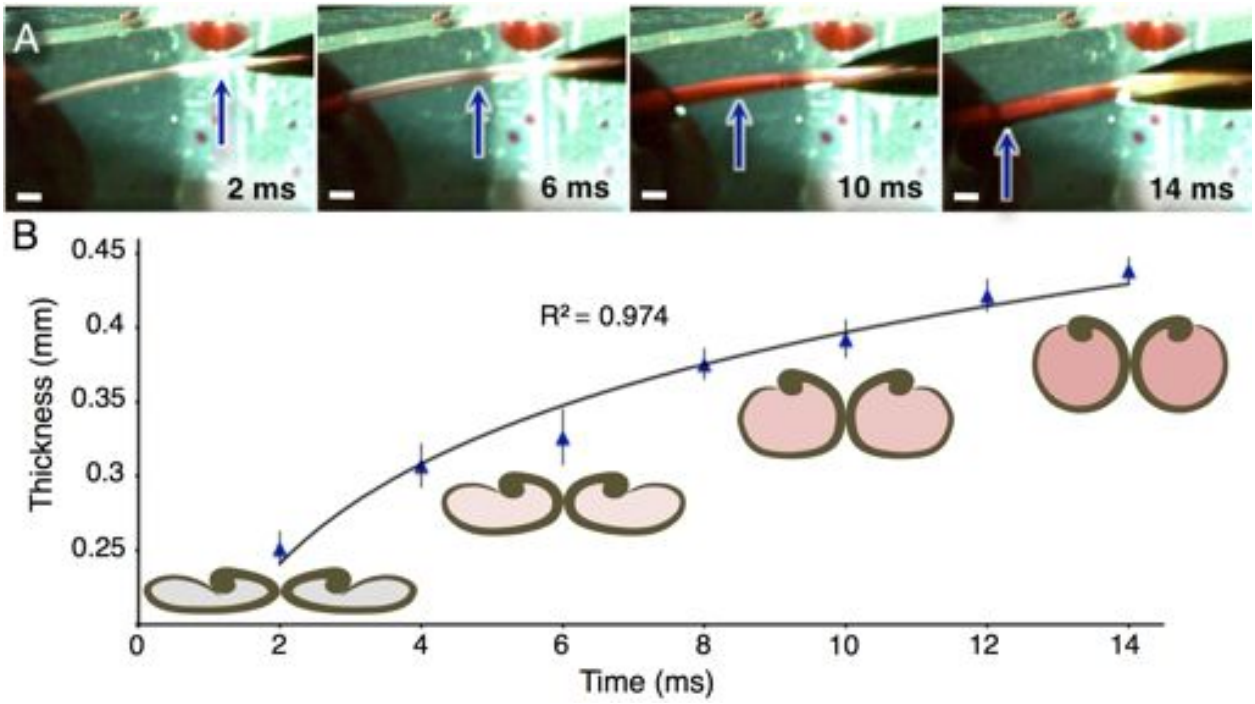



**Figure 2. Expansive filling of hummingbird tongues.** (A) Video frames showing a lateral view of an Amazilia Hummingbird's tongue being protruded and contacting dyed red nectar. Upward (*blue*) arrows point at a particular semilandmark, following it through time. Scale bars (*white*) = 0.4 mm. (B) Temporal change in dorso-ventral thickness of the tongue, measured at the given semilandmark. The increase in thickness is congruent with the expansive filling hypothesis. Diagrams of hypothetical cross sections correspond to the frames above and to the data points in the graph. Shades represent fluid inside the grooves, their color transitions from transparent to red as the tongue is filled with the red nectar. A logarithmic regression describes the expansive trend in the data. Vertical bars correspond to 95% CIs based on 5 repeated measurements of this particular sequence.

Expansive filling occurs in a wave-like fashion, in which the portions of the tongue that are closer to the nectar expand first and the expansion extends proximad sequentially until the entire tongue is filled. We define this displacement of the wedge-shaped most distal portion of the fluid as the "wave front" (Fig. 3). During the first few milliseconds, the increase in thickness is faster than towards the end of the filling process.

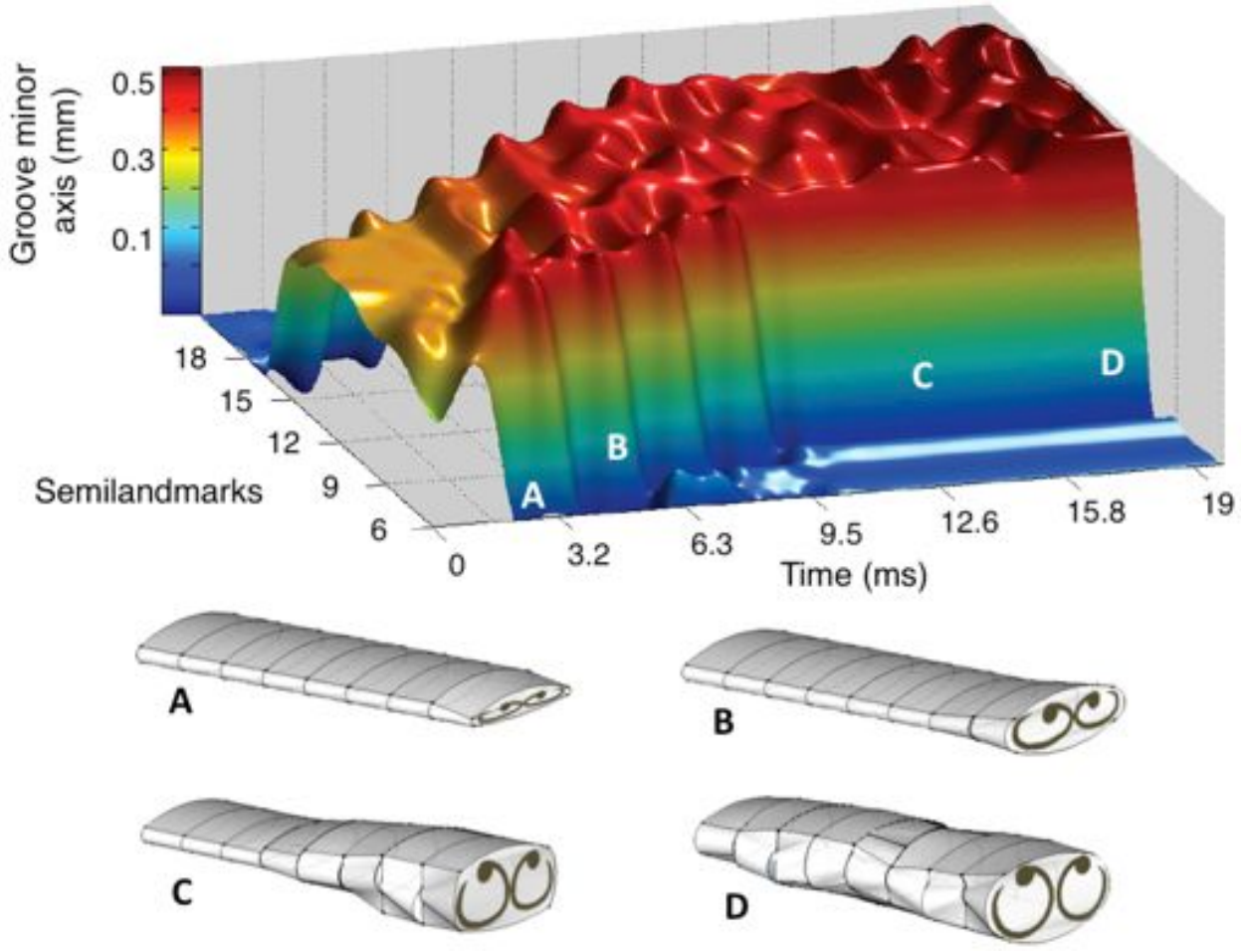



**Figure. 3. Expansive front of nectar during tongue filling.** 3-D surface represents the change in groove thickness at several semilandmarks through time. Groove minor axis (dorso-ventral diameter) shows measurements every 790.5 $\mu$s. The color gradient symbolizes differential groove diameters; blue tones (<0.1 mm) represent areas in which tongue thickness could not be measured, *i.e.* once the tongue enters the fluid or before it exits the bill. The *z* axis denotes semilandmarks along the tongue that range from 0 (tip = zero thickness) to 20 (base of the grooves). In the graph, semilandmarks start at 6 because the distal portion of the tongue is immersed in the nectar. Plotted in Matlab with smoothing spline function. White letters (A-B) in the graph above correspond to the 3D models of the tongue below (with conjectural cross sections of the grooves) showing the expansive front extracted from the data.

The fluid dynamics of expansive filling involves two regimes; in the short-time scale, we observed a super-linear increase of the filling length and sub-linear increase of the velocity (Fig. 4), *i.e.* the filling length $\propto t^n$ and filling velocity $\propto t^{n-1}$ where $1.0 < n < 1.5$, indicating that the elasticity-induced negative excess pressure balances the inertial force on the fluid drawn.

We compared the model against the empirical results using the following parameters: $R_f$ $\simeq$ 0.2 mm, $\mu \simeq$ 0.00181 Pa s, $\rho \simeq$ 1080 kg/m$^3$, $pa \simeq$ 3.3 kPa, $E \simeq$ 4 kPa (Fig. 4). The inertia effect is more significant than the result obtained from the inertia-dominated capillary rise model where $n$ = 1 [33], and of course than the quasi-steady case where $n$ = 0.5 based on the Lucas-Washburn model [34]. The short-time scale is on the order of 1 ms, while the long-time scale is about 10 to 20 ms. The Reynolds number is up to an order of 100 at the peak velocity. The results show a sub-linear increase of the observable front velocity (Fig. S4a) and a super-linear increase of the filling length (equivalent to the front position $h(t)$, Fig. S4b). In the long-time scale we infer a diffusive pressure wave regime, with a quasi-linear and then exponentially decayed velocity to reach the maximum filling length, indicating that the elasticity-induced negative excess pressure eventually balances the viscous force (Fig. 4).

The capillary filling model applied to the hummingbirds is not sufficient to describe the transition of the short-to-long time behaviors that characterize the local variation of the tongue thickness or the influence of elasticity, which is especially reflected in a much higher empirical peak velocity (on the order of 1 m/s) compared with the value (10 to 20 cm/s) underpredicted by the capillary model. Capillarity fails to match the magnitude of change observed and the peak velocities. Capillary rise would provide a zipping force to narrow the tongue groove, narrower at the tip and wider near the base of the grooves during fluid uptake, as shown in the elastocapillary effect [12]; our extensive experimental work however, shows an opposite trend: during the filling process the grooves are wider near the liquid and thinner near the bill tip (Fig. 3). The latter is compatible with our expansive filling explanation.

The inertia effect is important near the initial stage upon contact with nectar (4). The duration for the transition from super-linear to sub-linear increase of the filling length is relatively long compared with the short-time scale; this is perhaps why others have been led to believe that capillarity ($n$ = 1) is the primary driving mechanism. This conclusion is understandable because capillarity, regardless of the contact angle, provides a pulling force very similar to a pressure drop across the liquid column.

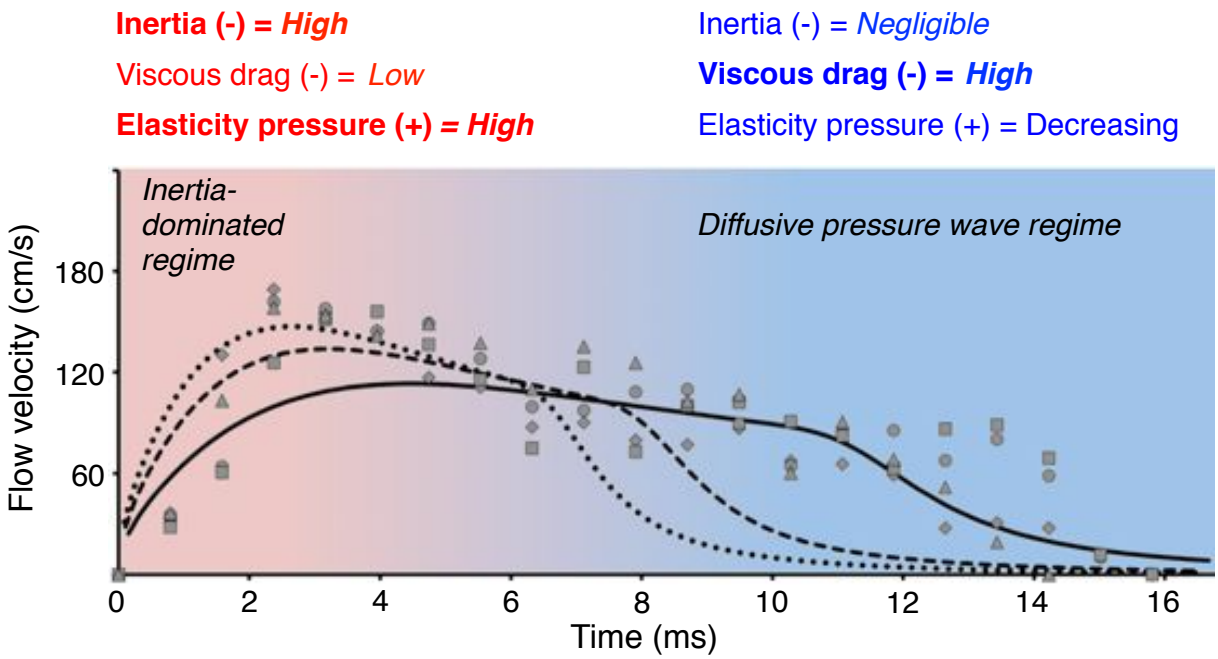



**Figure 4. Flow regimes during expansive filling**. Symbols (*grey*) correspond to independent licks/foraging bouts. Flow velocities are corrected by tongue velocity in each bout. Lines are the predicted results using the elastohydrodynamic model for 10 (dotted), 11 (dashed), and 13 (solid) mm of filled length. *Left* shade (*red*) in the graph designates the inertia-dominated regime: Quick expansion (rapid increase in thickness) of the distal portions of the tongue and high flow acceleration. *Right* shade (*blue*) covers the diffusive pressure wave: slower expansion in which the distal portions reach a maximum and the proximal portions keep filling slowly. In the first regime, elasticity-induced negative excess pressure balances the inertial force. The second regime, with an almost linear deceleration, indicates that the elasticity-induced negative excess pressure balances the viscous force. Plus and minus symbols denote the corresponding force directions to the nectar, *i.e.* towards the beak (+) or towards the nectar pool (−).

The negative excess pressure or the overall adhesion energy that keeps the tongue in a flattened configuration, even after passing the compression point at the bill tip, is explained by at least two factors: 1) the surface tension at the three-phase (tongue-nectar-air) contact line, and 2) the net pressure force between the thin liquid film of nectar remaining in the grooves after compression and ambient pressure. Adhesion forces such as van-der-Waals and steric effects may provide additional contributions to balance the elasticity.

Results from the elastohydrodynamic model we developed on the basis of our observations of the expansive filling process, match our empirical measurements (Figs 4, 5). The concordance between the mathematical model and our data supports our conceptual explanation for the micropumping mechanism. The model predicts top speeds (inertia-dominated regime) more accurately at shorter filling lengths, and the deceleration (diffusive pressure wave regime) more accurately at longer filling lengths. In addition, to validate our conceptualization of the expansive filling process, the mathematical model offers the opportunity to make testable predictions for different species and feeding efficiency under a variety conditions.

We recorded expansive filling in 20 species that belong to seven out of the nine main hummingbird clades (Table S1). Additionally, we report dorso-ventral expansion measurements at semilandmark #12 for five species of hummingbirds in which we could track tongue tip and groove bases. We calculated the percentage, out of the final thickness of the groove, that the expansion represents (Table S1). This percentage ranged from 48 to 60% among species, which demonstrates the importance of expansive filling as an important fluid uptake mechanism.

**Discussion**

Fluid trapping is the predominant process by which hummingbirds achieve nectar collection at small bill tip-to-nectar distances, wherein tongue grooves are wholly immersed in nectar, or when the nectar is found in very thin layers [13]. We demonstrate here that expansive filling accounts for nectar uptake by the portions of a hummingbird's tongue that remain outside the liquid. The relative contributions of the two synergistic mechanisms (fluid trapping [13] and expansive filling) to the rate and volume of nectar ultimately ingested will be determined by the distance from the bill tip to the nectar surface during the licking process (Fig. S5). The amount of nectar collected per lick by means of expansive filling can be calculated by assuming an elliptical to cylindrical transformation in the tongue grooves (*e.g.* Fig. S6, Video S2). The expansive filling mechanism we have documented here excludes capillarity as the main process for loading the portions of the tongue that are not filled through nectar trapping. The combination of fluid trapping and expansive filling gives rise to an elastic micropump with superior nectar gathering efficiency (because of greater tongue-filling speed) than that expected for a capillary pump [12, 16].

The combination of nectar trapping and expansive filling predicts greater nectar gathering efficiency (because of increased tongue-filling speed) than expected under the capillarity hypothesis of nectar uptake and associated predictions (Kingsolver & Daniel 1983, Kim *et al.* 2012). Given my results for tongue velocity at average licking rates (~10Hz) and calculations for the rate of filling using capillarity vs. expansive filling, more nectar should be collected if the latter is operating, due to faster loading times (Figs. S5, S7).

Traditional capillarity equations predict that increasing *bill tip to nectar surface* (*BT-NS*) distance results in a strong decrease in collection efficiency (or a compensating reduction in lick rate). The BT-NS gap is bridged by the tongue grooves outside the bill that will be filled with nectar; the increase in distance translates into a longer portion of the groove to be filled, and ultimately into a longer meniscus displacement time. Because this increases filling time, the bird would need to decrease licking rate to maintain the same amount of nectar collected/lick, or it would collect less nectar/lick while maintaining licking rate constant: both result in lower efficiency. This reduced nectar collection efficiency holds for both, capillarity alone (*cf.* Kingsolver & Daniel 1983) and for nectar trapping combined with capillarity (*cf.* Kim *et al.* 2012). However, I predict that trapping plus expansive filling would result in a much smaller reduction in the nectar collected per lick (and little reduction in licking rate) when the bill is farther away from the nectar (due to faster groove loading time given that there is no meniscus displacement), thereby minimizing loss of efficiency across a range of BT-NS lengths (Fig. S5). In either scenario, hummingbirds will face diminished collection efficiency when feeding at flowers in which BT-NS distances are longer, but a sharper decrease is expected under the capillarity hypothesis than under predictions based on my proposed mechanisms.

We observed capillary filling in only a single groove during only one lick out of hundreds filmed (Video S3, Fig. S7). In this event, one side of the tongue contacted, and adhered to, the feeder wall before the tip reached the surface of the nectar pool. As the bird continued to slide the tongue forward along the feeder wall, the resulting bending of the tongue appeared to pull the flattened groove on that side of the tongue open, while the groove on the side of the tongue not touching the feeder remained flattened (closed).

The end result of this unusual accident was that we filmed one of the two grooves being filled by expansive filling (as usual) and the other being filled by capillary filling, offering a fortuitous opportunity to directly compare the two mechanisms (Video S3). Our results from this single instance show that expansive filling is five times faster than capillarity when all other factors are equal (Fig. S7).

To provide a broader comparison between capillary and expansive filling, we took published data of capillary tongue filling under laboratory conditions [12], and contrasted them against five sequences in which we tracked the front of the filling wave during expansive filling (Fig. 5). It is noteworthy that at filling lengths as short as 4 millimeters, the capillary filling time is twice as long in comparison to expansive filling.

At real flowers, where full tongue immersion is prohibited by corolla length, filling lengths are equivalent to the distances between the bill tip and the nectar surface. Our data show that the greater the bill-nectar gap, the larger the performance difference between capillary and expansive filling (Fig. 5). Given that it is only at great filling lengths (when the bill tip is far from the nectar and full tongue immersion is precluded) that a tongue filling mechanism besides nectar trapping would contribute a significant portion of the total load per lick, we rule out capillary filling as an important drinking mechanism in free-living hummingbirds.

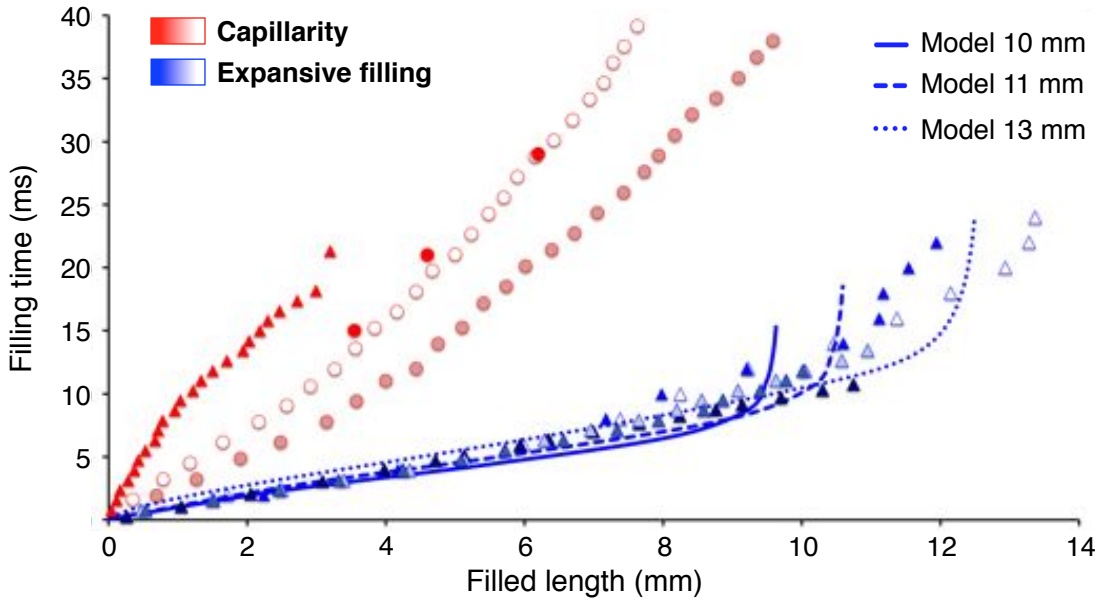



**Figure 5. Free-living hummingbirds use expansive filling during drinking.** Time (Y-axis) to fill a given length (X-axis) of the tongue. Circles (*red*) represent vertical capillary tongue filling from three licks by captive Ruby-throated Hummingbirds reported by Kim *et al.*[12]. Triangles (Amazilia Hummingbirds) on the left (*red*) correspond to our only observation of capillary filling (only one side of the tongue, in a single lick). Triangles (*blue*) denote expansive filling data from five of 110 drinking sequences of free-living hummingbirds. Lines (*blue*) are predictions from our elastohydrodynamic model.

Kim *et al.* [12] reported three licks from two captive individuals of a single species, one feeding while hovering and the other one feeding while handheld. The average filling speed in the capillary filling events they documented was around 20 cm/s, which is about five times slower than the average speed of expansive filling 93 cm/s (Fig. 5). The slow speed of capillary filling limits the hummingbird's licking rate. This is manifest in the time interval for the one full lick cycle reported by Kim *et al.* (Fig. 2b in *op. cit.*): 200 ms. At this rate, the licking frequency is only 5 Hz, while the licking rates under more realistic conditions are around 14 Hz (*cf.*[15]). If the tongue normally worked as a capillary pump in free-living hummingbirds, we would not observe the high licking rates that have been reported in the field (up to 17 Hz [27]).

Instead, the elastic micropump we describe here allows for tongue loading at rates that are compatible with the reported licking rates. The discrepancy between our results and Kim *et al.*'s [12] highlights: 1) The importance of using realistic experimental conditions to make biological inferences, 2) an adequate sample size of individuals, trials, and reported data, and, most importantly, 3) a sufficient sampling of species from different clades if the aim is to generalize across large taxonomic groups.

Updated information on feeding efficiency estimates could provide insights critical to current evolutionary debates (*e.g.* optimal concentrations [14, 17, 35, 36]) and to understanding broad scale ecological patterns (*e.g.* species range limits and competition in hummingbird assemblages [26], and phenological shifts with conservation implications [37]). As a particular example, a reappraising of preference experiments along gradients of nectar concentrations [38, 39, 40, 41, 42, 43] using the new nectar intake models, and applying recent advances on gustatory discrimination [44], could shed new light on coevolutionary enigmas. Convergently, several plant lineages have transitioned from an ancestral insect-pollinated condition to vertebrate-pollination [45, 46, 47, 48], and vertebrate-pollinated flowers tend to have more dilute nectars than the insect-pollinated ones [35, 49, 50, 51]. Accurate modeling of feeding mechanisms would lead us to test the hypothesis that physical constraints are the main determinants of the relation between pollinator type and nectar concentration, and guide us through alternative hypotheses (reviewed in [51]).

Several scaling models and applications have been developed on the basis of recent discoveries of biological phenomena and underlying physical explanations [52, 53].

Within the last three years, detailed and well-supported studies of the mechanics of drinking in some animal taxa (*e.g.*[13, 54, 55, 56]) have opened the doors to new biomimicry avenues. And some amazing animal tongues have inspired biomimetic projects (*e.g.* manipulators [57]). Our description of the functioning of this elastic micropump in hummingbird tongues may be relevant to applications and the study of flow in elastic-walled (flexible) tubes in both biological [58, 59] and artificial [60, 61, 62, 63, 64, 65] systems.

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## Literature cited


1. LaBarbera M (1990) Principles of design of fluid transport systems in zoology. *Science* 249:992–1000.

2. Vogel S (2007) Living in a physical world X. Pumping fluids through conduits. *J Biosci* 32:207–222.

3. Domec J-CJ, Lachenbruch BB, Meinzer FCF, Woodruff DRD, Warren JMJ, McCulloh KAK (2008) Maximum height in a conifer is associated with conflicting requirements for xylem design. *Proc Natl Acad Sci USA* 105(33):12069–12074.

4. Herschlag G, Miller L (2011) Reynolds number limits for jet propulsion: a numerical study of simplified jellyfish. *J Theor Biol* 285(1):84–95.

5. Vogel S (2003) *Comparative biomechanics: life's physical world* (Princeton University Press, New Jersey).

6. Martin WCL (1833) *The Naturalist's Library: A General History of Humming-Birds or the Trochilidae*, ed W Jardine (H.G. Bohn, London), Vol 41, pp 65–68.

7. Lucas FA (1891) On the structure of the tongue in hummingbirds. *Proc US Nat Mus* 14:167–172.

8. Scharncke H (1931) Contribution to the morphology and developmental evolution of the tongue of the Trochilidae, Meliphagidae and Picidae (BeitrÃ⁄ge zur Morphologie und Entwicklungsgeschichte der Zunge der Trochilidae, Meliphagidae und Picidae). *J Ornithol* 79:425–491 (in German).

9. Weymouth RD, Lasiewski RC, Berger AJ (1964) The tongue apparatus in hummingbirds. *Acta Anat* 58:252–270.

10. 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.

11. Collins BG (2008) Nectar intake and foraging efficiency: responses of honeyeaters and hummingbirds to variations in floral environments. *Auk* 125(3):574–587.

12. Kim W, Peaudecerf F, Baldwin MW, Bush JWM (2012) The hummingbird's tongue: a self-assembling capillary syphon *Proc R Soc Lond B Biol Sci* 279(1749):4990–4996.

13. Rico-Guevara A, Rubega MA (2011) The hummingbird tongue is a fluid trap, not a capillary tube. *Proc Natl Acad Sci USA* 108(23):9356–9360.

14. Kim W, Gilet TT, Bush JWM (2011) Optimal concentrations in nectar feeding. *Proc Natl Acad Sci USA* 108(40):16618–16621.

15. Rico-Guevara A, Rubega MA (2012) Hummingbird feeding mechanics: Comments on the capillarity model. *Proc Natl Acad Sci USA* 109(15):E867.

16. Kingsolver JG, Daniel TL (1983) Mechanical determinants of nectar feeding strategy in hummingbirds: Energetics, tongue morphology, and licking behavior. *Oecologia* 60:214–226.

17. Heyneman AJ (1983) Optimal sugar concentrations of floral nectars: Dependence on sugar intake efficiency and foraging costs. *Oecologia* 60:198–213.

18. Kim W, Bush JWM (2012) Natural drinking strategies. *J Fluid Mech* 705:7–25.

19. Jensen KH, Kim W, Holbrook NM, Bush JWM (2012) Optimal concentrations in transport systems. *J R Soc Interface* 10(83):20130138–20130138.

20. Stiles FG (1981) Geographical aspects of bird-flower coevolution, with particular reference to Central America. *Ann Mo Bot Gard* 68:323–351.

21. Snow BK, Snow DW (1972) Feeding niches of hummingbirds in a Trinidad valley. *J Anim Ecol* 41(2):471–485.

22. Feinsinger P, Colwell RK (1978) Community organization among Neotropical nectar-feeding birds *Am Zool* 18(4):779–795.

23. Brown JH, Bowers MA (1985) Community organization in hummingbirds: relationships between morphology and ecology. *Auk* 102(2):251–269.

24. Stiles FG (1985) Seasonal patterns and coevolution in the hummingbird-flower community of a Costa Rican subtropical forest. *Ornithol Monogr* 36:757–787.

25. Rahbek C, Graves GR (2000) Detection of macro-ecological patterns in South American hummingbirds is affected by spatial scale. *Proc R Soc Lond B Biol Sci* 267(1459):2259–2265.

26. Graham CH, Parra JL, Tinoco BA, Stiles FG, McGuire JA (2012) Untangling the influence of ecological and evolutionary factors on trait variation across hummingbird assemblages. *Ecology* 93(sp8):S99–S111.

27. Ewald PW, Williams WA (1982) Function of the bill and tongue in nectar uptake by hummingbirds. *Auk* 99(3):573–576.

28. Denny MW (1993) *Air and water: the biology and physics of life's media* (Princeton University Press, New Jersey).

29. Middleman S (1997) *An Introduction to Fluid Dynamics: Principles of Analysis and Design* (Wiley, New York).

30. Womersley JR (1955) Method for the calculation of velocity, rate of flow and viscous drag in arteries when the pressure gradient is known. *J Physiol* 127:553–563.

31. Katz AI, Chen Y, Moreno AH (1969) Flow through a collapsible tube. *Biophys J* 9:1261–1279.

32. Rubinow SI, Keller JB (1972) Flow of a viscous fluid through an elastic tube with applications to blood flow. *J Theor Biol* 35:299–313.

33. Quéré D (1997) Inertial capillarity. *Europhys Lett* 39(5):533–538.

34. Zhmud BV, Tibert F, Hallstensson K (2000) Dynamics of capillary rise. *J Colloid Interface Sci* 228:263–269.

35. Pyke GH, Waser NM (1981) The production of dilute nectars by hummingbird and honeyeater

flowers. *Biotropica* 13(4):260–270.

36. Johnson SD, Nicolson SW (2008) Evolutionary associations between nectar properties and specificity in bird pollination systems. *Biol Lett* 4(1):49–52.

37. McKinney AMA, CaraDonna PJ, Inouye DW, Barr BB, Bertelsen CDC, Waser NMN (2012) Asynchronous changes in phenology of migrating Broad-tailed Hummingbirds and their early-season nectar resources. *Ecology* 93(9):1987–1993.

38. Hainsworth FR, Wolf LL (1976) Nectar characteristics and food selection by hummingbirds. *Oecologia* 25(2):101–113.

39. Stiles FG (1976) Taste preferences, color preferences, and flower choice in hummingbirds. *Condor* 78(1):10–26.

40. Tamm S, Gass CL (1986) Energy intake rates and nectar concentration preferences by hummingbirds. *Oecologia* 70(1):20–23.

41. Roberts WM (1996) Hummingbirds' nectar concentration preferences at low volume: The importance of time scale. *Anim Behav* 52:361–370.

42. Schondube JE, Mart´ınez-Del Rio C (2003) Concentration-dependent sugar preferences in nectar-feeding birds: mechanisms and consequences. *Funct Ecol* 17:445–453.

43. Guzman WA, Wilson P (2012) Hummingbirds at artificial flowers made to resemble ornithophiles versus melittophiles. *Journal of Pollination Ecology* 8(10):67–78.

44. Nachev V, Stich KP, Winter Y (2013) Weber's law, the magnitude effect and discrimination of sugar concentrations in nectar-feeding animals. *PLoS One* 8(9):e74144.

45. Thomson JD, Wilson P (2008) Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *Int J Plant Sci* 169(1):23–38.

46. Fleming T, Geiselman C, Kress W (2009) The evolution of bat pollination: a phylogenetic perspective. *Ann Bot* 104(6):1017–1043.

47. van der Niet T, Johnson SD (2012) Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends Ecol Evol* 27(6):353–361.

48. Barrett SCH (2013) c *Proc R Soc Lond B Biol Sci* 280(1765):20130913–20130913.

49. Baker HG (1975) Sugar concentrations in nectars from hummingbird flowers. *Biotropica* 7(1):37–41.

50. Nicolson SW (2002) Pollination by passerine birds: why are the nectars so dilute? *Comp Biochem Physiol B Biochem Mol Biol* 131(4):645–652.

51. Nicolson SW, Fleming PA (2003) Nectar as food for birds: the physiological consequences of drinking dilute sugar solutions. *Plant Syst Evol* 238(1-4):139–153.

52. Vogel S (2011) Surface tension helps a tongue grab liquid. *Proc Natl Acad Sci USA* 108(23):9321–9322.

53. Ishii D, Horiguchi H, Hirai Y, Yabu H, Matsuo Y, Ijiro K, Tsujii K, Shimozawa T, Hariyama T, Shimomura M (2013) Water transport mechanism through open capillaries analyzed by direct surface modifications on biological surfaces. *Sci Rep* 3:3024.

54. Crompton AW, Musinsky C (2011) How dogs lap: ingestion and intraoral transport in *Canis familiaris*. *Biol Lett* 7(6):882–884.

55. Cundall D, Brainerd EL, Constantino J, Deufel A, Grapski D, Kley NJ (2012) Drinking in snakes: resolving a biomechanical puzzle. *J Exp Zool* 317(3):152–172.

56. Harper CJ, Swartz SM, Brainerd EL (2013) Specialized bat tongue is a hemodynamic nectar mop. *Proc Natl Acad Sci USA* 110(22):8852–8857.

57. Debray A (2011) Manipulators inspired by the tongue of the chameleon. *Bioinspir Biomim* 6(2):026002.

58. Grotberg JB, Jensen OE (2004) Biofluid mechanics in flexible tubes. *Annu Rev Fluid Mech* 36(1):121–147.

59. Hazel AL, Heil M (2005) Surface-tension-induced buckling of liquid-lined elastic tubes: a model for pulmonary airway closure. *Proc R Soc Lond A Math Phys Sci* 461(2058):1847–1868.

60. Sun D, Shu D, Ji M, Liu F, Wang M, Gong X (2004) Pressure-induced hard-to-soft transition of a single carbon nanotube. *Phys Rev B Condens Matter* 70(16):165417.

61. Marzo A, Luo XY, Bertram CD (2005) Three-dimensional collapse and steady flow in thick-walled flexible tubes. *J Fluid Struct* 20(6):817–835.

62. Yang Y, Gao YF, Sun DY, Asta M, Hoyt JJ (2010) Capillary force induced structural deformation in liquid infiltrated elastic circular tubes. *Phys Rev B Condens Matter* 81(24):241407.

63. Whittaker RJ, Heil M, Waters SL (2011) The energetics of flow through a rapidly oscillating tube with slowly varying amplitude. *Philos Trans R Soc Lond A* 369(1947):2989–3006.

64. Nahar S, Jeelani SAK, Windhab EJ (2012) Influence of elastic tube deformation on flow behavior of a shear thinning fluid. *Chem Eng Sci* 75:445–455.

65. Nahar S, Jeelani SAK, Windhab EJ (2013) Prediction of velocity profiles of shear thinning fluids flowing in elastic tubes. *Chem Eng Commun* 200(6):820–835.

66. Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9:671-675.

67. Rohlf FJ (2010) *TPSDig2 Version 2.16* (Department of Ecology and Evolution, Stony Brook University, New York).

68. Bookstein FL (1991) *Morphometric tools for landmark data: geometry and biology* (Cambridge University Press, Cambridge).

69. Mitteroecker P, Gunz P (2009) Advances in geometric morphometrics. *Evol Biol* 36(2):235–247.

**Supplementary Figures**

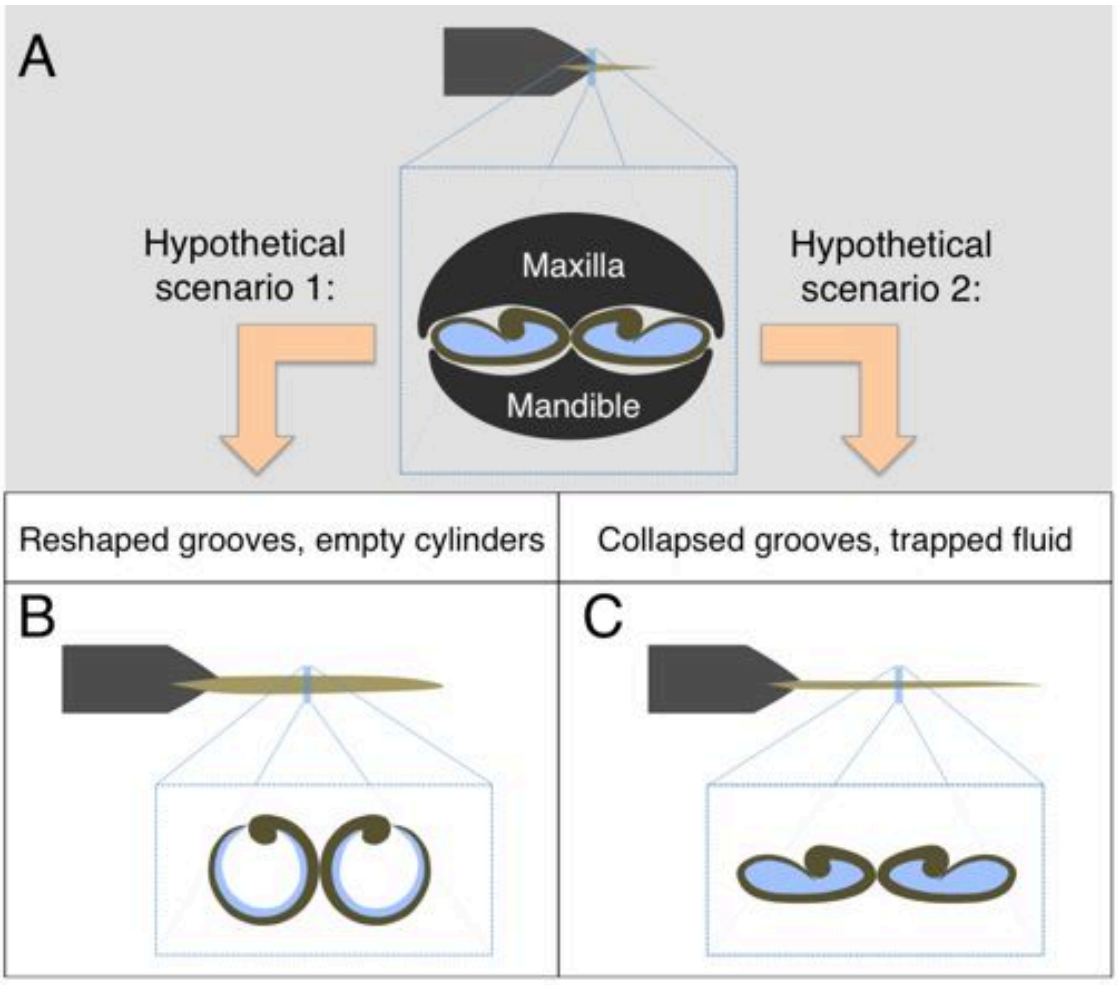



**Figure S1. Schematic representation of the mutually exclusive possibilities of tongue configuration during nectar uptake.** (A) All hummingbirds compress their tongues with their bill tips squeezing the nectar inside the bill. Passing this compression point, there are two possibilities: (B) Passing the compression point the grooves would immediately regain their cylindrical shape yielding two empty cylinders approaching the nectar. (C) Alternatively, passing the compression point the grooves would stay flattened due to adhesive forces of the thin layer of liquid trapped inside of each one of them. The grooves would only reshape when contacting the nectar and there would never be empty cylinders.

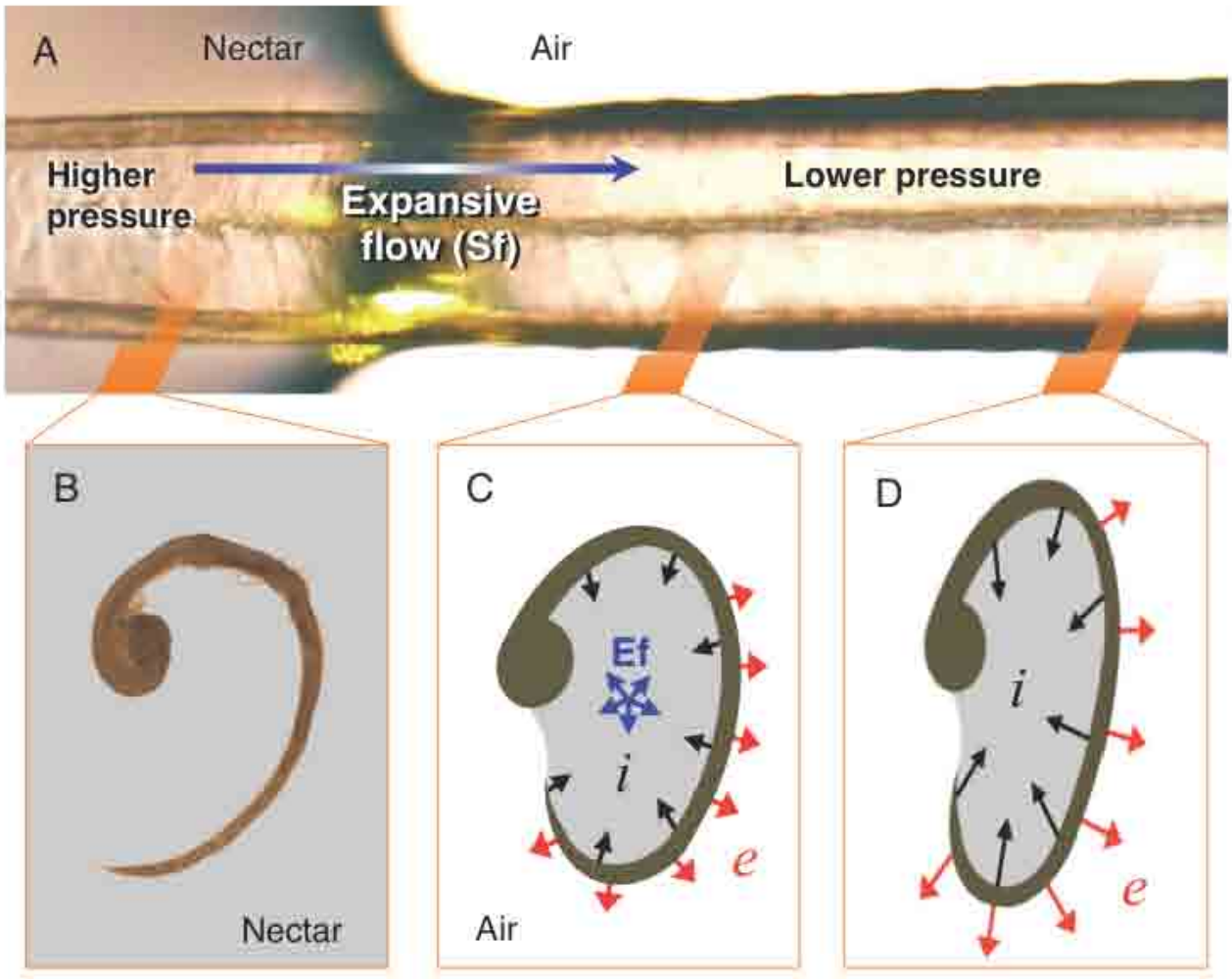



**Figure S2. Hypothesis for the physical forces involved in expansive filling.** (A) Dorsal view of a hummingbird tongue tip just after contacting the nectar surface. Given the flattened configuration of the bent grooves on the right, there would be more elastic energy stored which creates a slight pressure gradient that turns into suction flow. (B) Cross section (light microscope photograph) of a hummingbird tongue in its "relaxed" configuration. (C) Hypothetical cross section showing the interaction among the flow (blue), internal forces (*i*) between the nectar and groove walls (adhesion and cohesion, black), and elastic potential energy (red). (D) Hypothetical cross section for a portion of the tongue not yet affected by the expansive flow (*cf.* expansive front, Fig. 3). Note that the elastic potential energy (red) is larger when the bending of the walls is more pronounced, creating an increase in internal forces (black) to counteract it, and therefore the possibility of pressure differential.

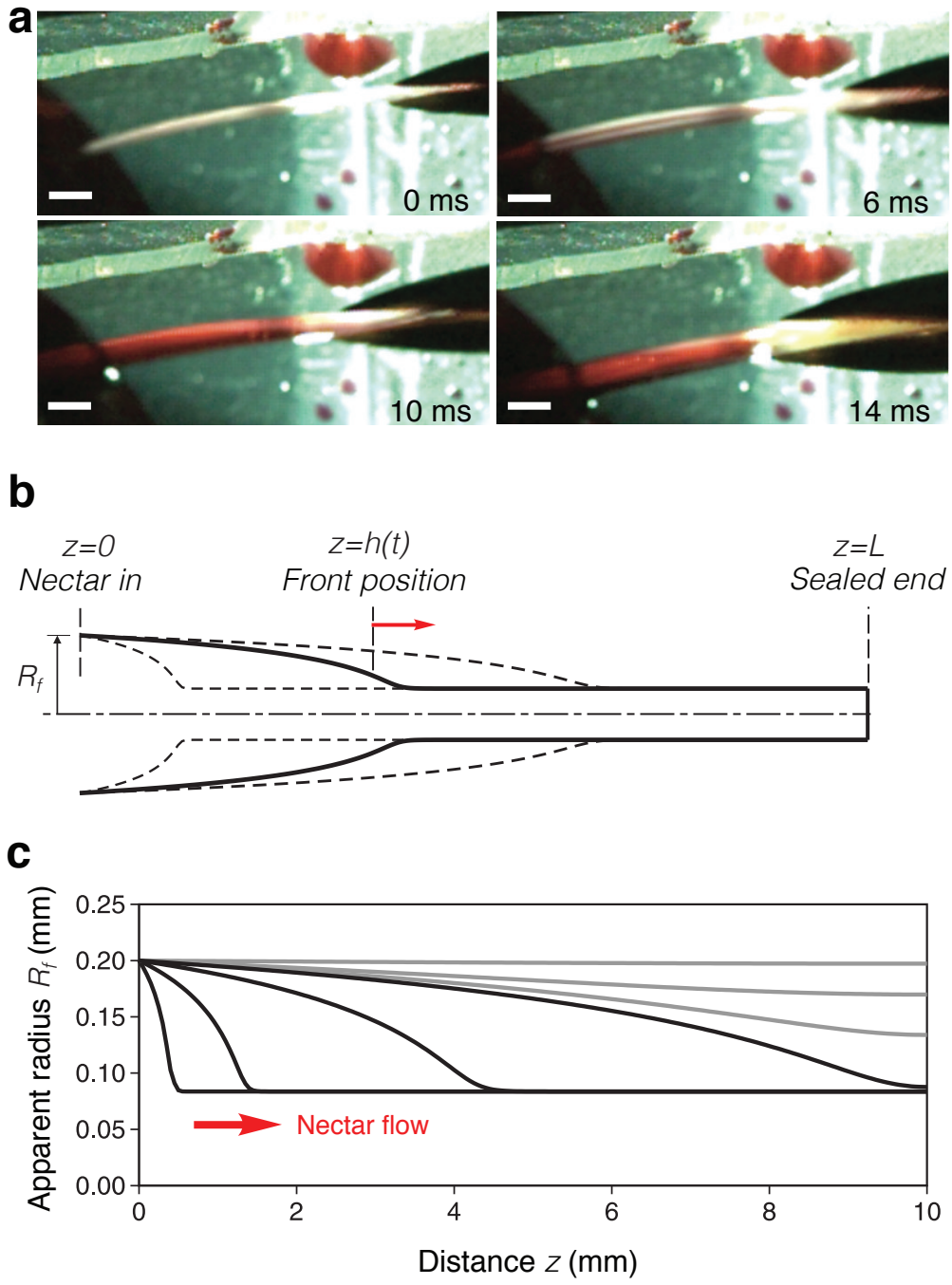



**Figure S3. Modeling the expansive filling mechanism**. **a,** Video frames showing a lateral view of an Amazilia Hummingbird's tongue being protruded and contacting dyed red nectar. The full filling of the grooves is completed in 14 milliseconds (ms). Scale bars (white) = 0.5 mm. **b,** A schematic showing the gradually expanded tube profiles and the front position to be compared with experimental data. During the nectar filling process the groove is wider near the liquid and thinner near the bill tip. **c,** Axi-symmetric modeling results showing the transient profiles of the groove at 0.001, 0.01, 0.1, 0.5, 0.7, 1.0, and 2.0 times of the characteristic time scale $\tau=8\mu L2/(Rf\ 2E)$, with the parameters described in the text. Black lines describe progressive wave-like behavior, and grey lines show the diffusive recovery at the long-time scale.

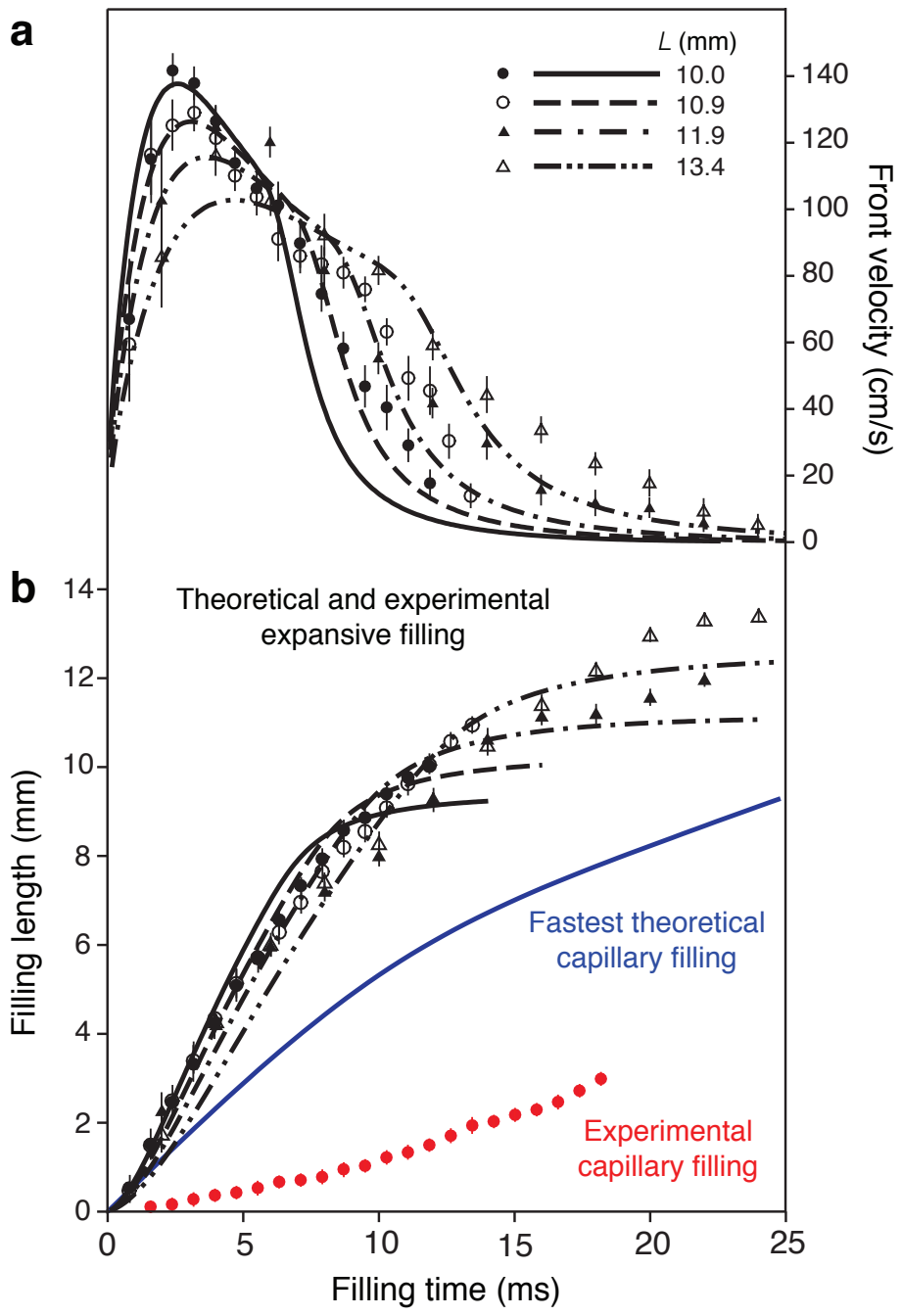



**Figure S4. Theoretical and experimental data for the filling mechanisms. a,** Transient velocity of the traveling liquid front versus time. Data points (black symbols) denote expansive filling data from 4 drinking sequences of free-living birds, exemplifying different filling tube lengths (mm). Lines (black) are predictions from our elastohydrodynamic model. **b,** Transient filling length h versus time at the same tube lengths shown above. The theoretically fastest capillary filling curve (blue) is based on Bosanquet's capillary model [#] under zero contact angle condition (complete wetting). Experimental capillary filling data points (red circles) represent our only observation of capillarity from only one side of the tongue, in a single lick (Video S3). Vertical bars correspond to 95% CIs.

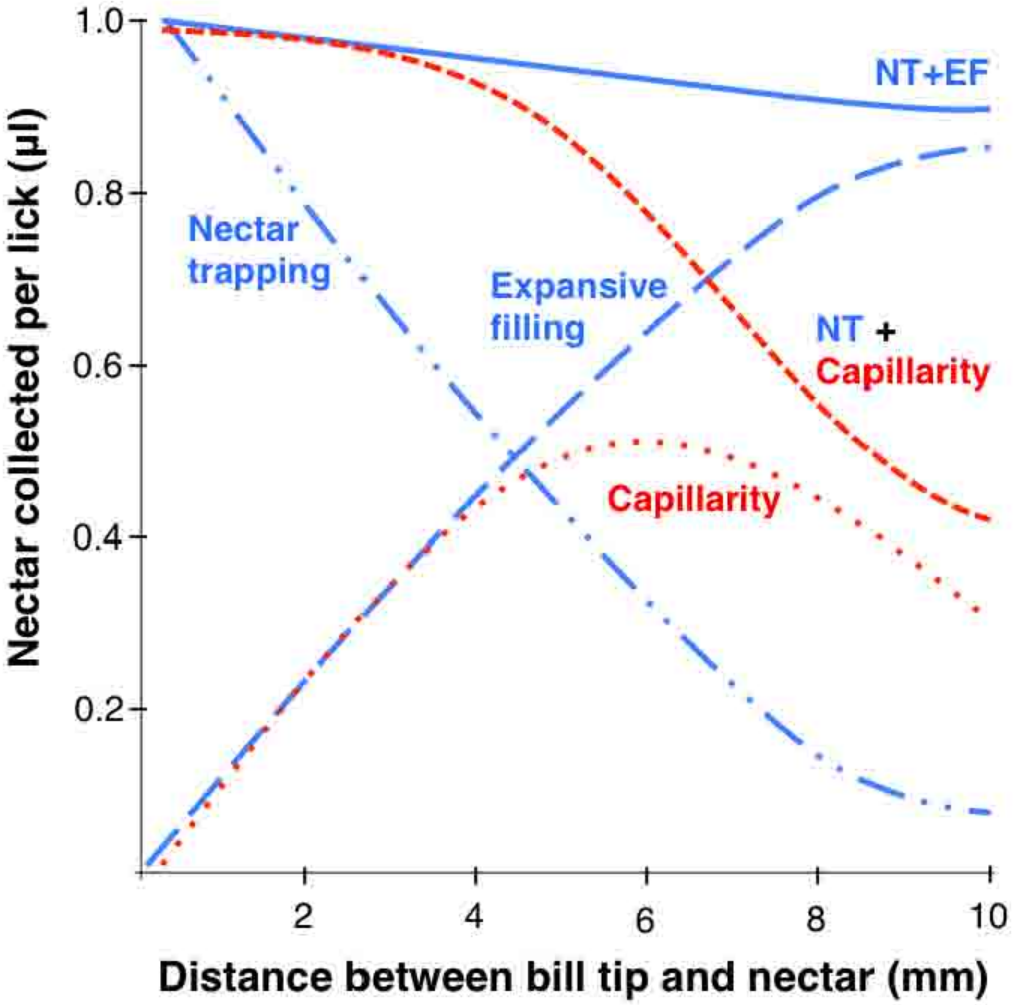



**Figure S5. Predicted tongue nectar collection along a gradient of bill tip to nectar surface distances.** These predictions are made under the assumption (supported by unpublished data at artificial feeders) of constant licking rate (~10Hz) at all distances. In blue (long-dashed lines): Our two proposed mechanisms for tongue loading: Nectar Trapping (NT) and Expansive Filling (EF). Top solid line represents the net result of the combination of those two mechanisms. In red: the predicted filling following capillarity equations (dotted line) and the combination of NT and capillarity (short-dashed line). Note that at small distances between bill and nectar, NT is the mechanism contributing the most to tongue filling. At moderate to large distances between bill and nectar, two alternative processes could occur: EF and capillarity. The combination of NT + EF yields larger amounts of nectar collected per lick compared to the alternative capillarity option. Predictions are based on empirical results of EF and capillarity velocities under experimental conditions (wild birds at artificial feeders, Figs. S4, S6).

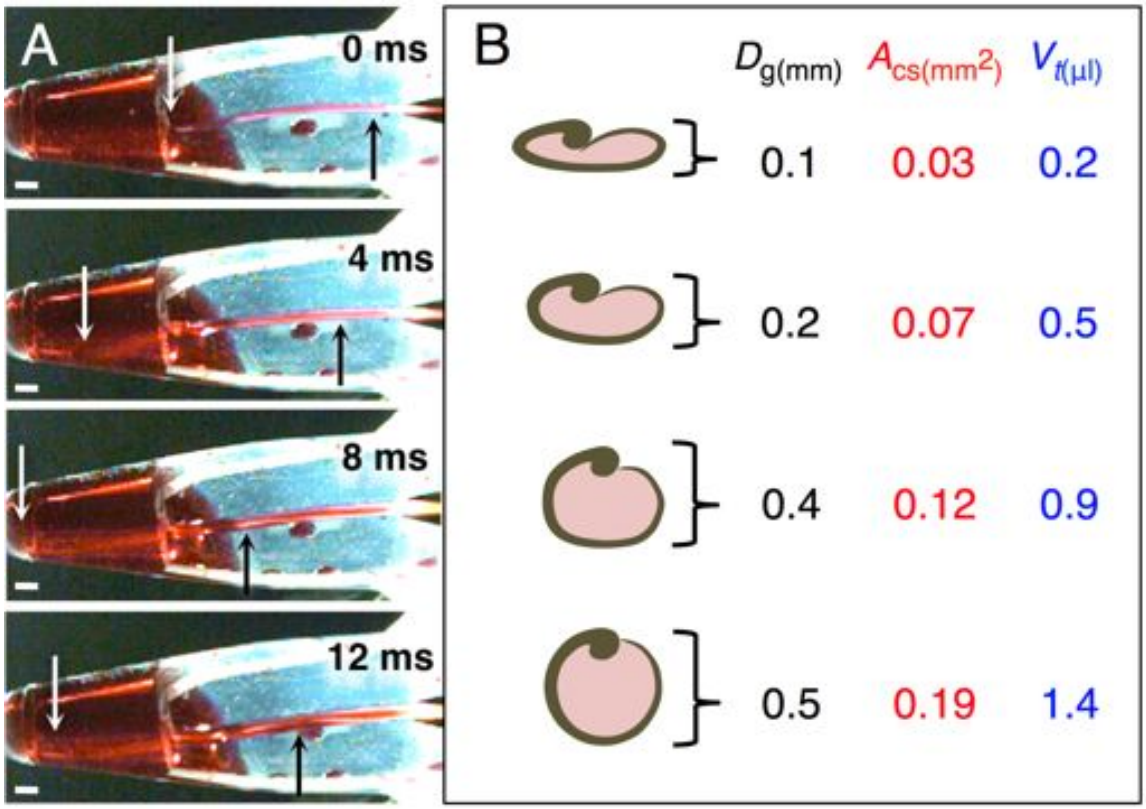



**Figure S6. Volume of nectar collected by the elastic micropump.** (A) Frames from a high-speed video of free-living Amazilia Hummingbird (*Amazilia amazilia*) drinking nectar. Downward (*white*) arrows point at the tongue tip, tracking it through time. Upward (*black*) arrows point at a particular semilandmark, showing the relative position of that point at different times. Note the expansion of the grooves from top to bottom and how the nectar (with red dye to enhance visualization) fills the tongue as the grooves expand (increase in thickness). Scale bars = 0.5 mm. (B) Diagrams of cross sections of a single point in the tongue through time, corresponding to the frames on the left and measurements of the dorsoventral thickness that are a proxy for the minor axis (or diameter) of the nectar column from an elliptical to a circular base cylinder: $D_g$ (*black*); estimated cross-sectional areas, $A_{cs}$ (*red*); and approximate volumes of nectar (using 7 mm of effective groove length) inside the tongue for any given moment matching the frames on the right, $V_t$ (*blue*). Adding up the volume of the two grooves, the tongue is able to take about 1/4 µl per microsecond via expansive filling.

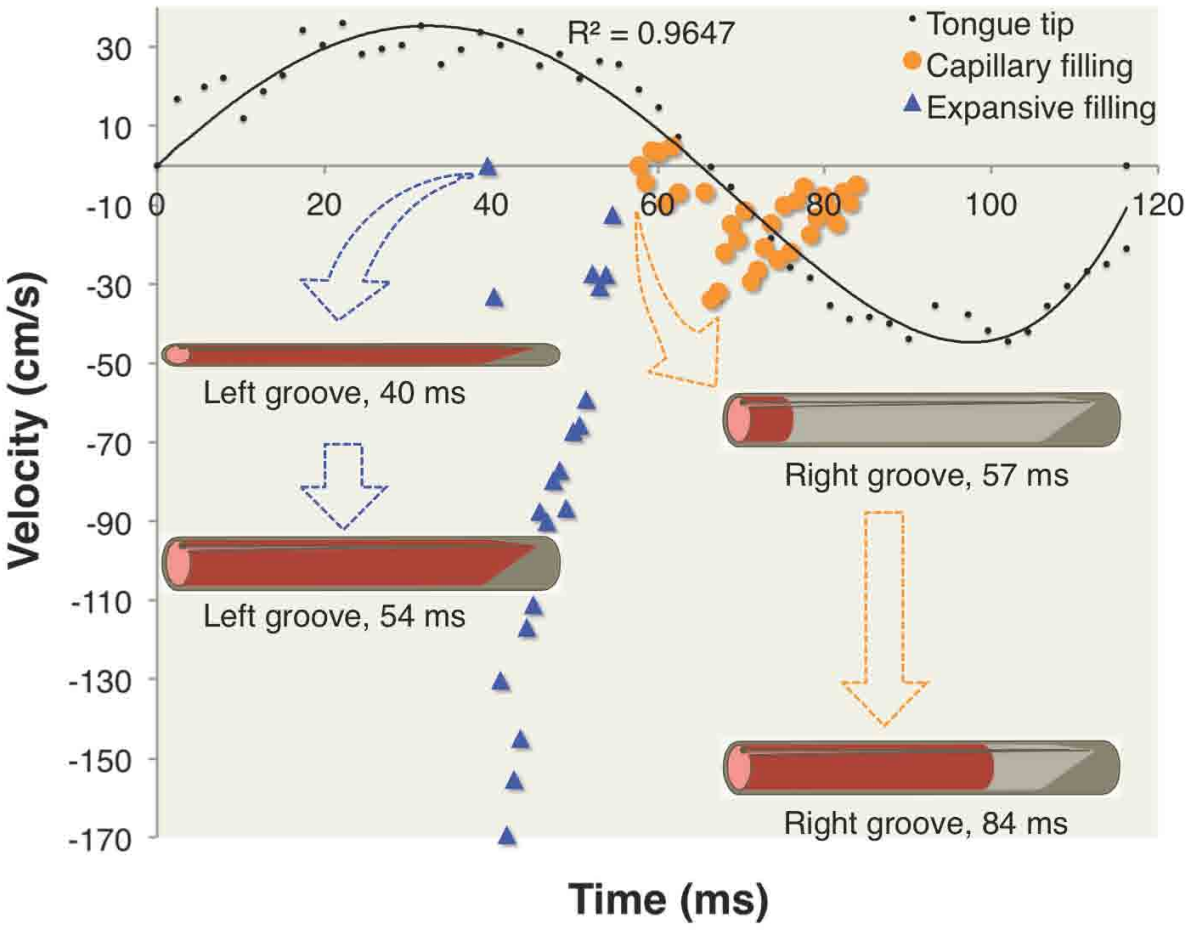



**Figure S7. Parallel capillary and expansive filling velocity profiles.** The time interval (120 ms) corresponds to a single lick cycle. Black dots indicate tongue tip velocity through time; a polynomial trend line describes the reciprocating movement of the tongue, positive values indicate tongue protrusion and negative values tongue retraction. Triangles (*blue*) represent velocities for expansive filling (wave front tracking), and circles (*orange*) correspond to velocities for capillary filling (meniscus tracking). Note that capillary filling is not only slower than expansive filling but also has a longer delay in its start point, probably due to a greater influence of inertial forces on the meniscus movement. By the time capillarity started to move fluid in the right groove (57 ms), the left one had already been filled through expansive filling.

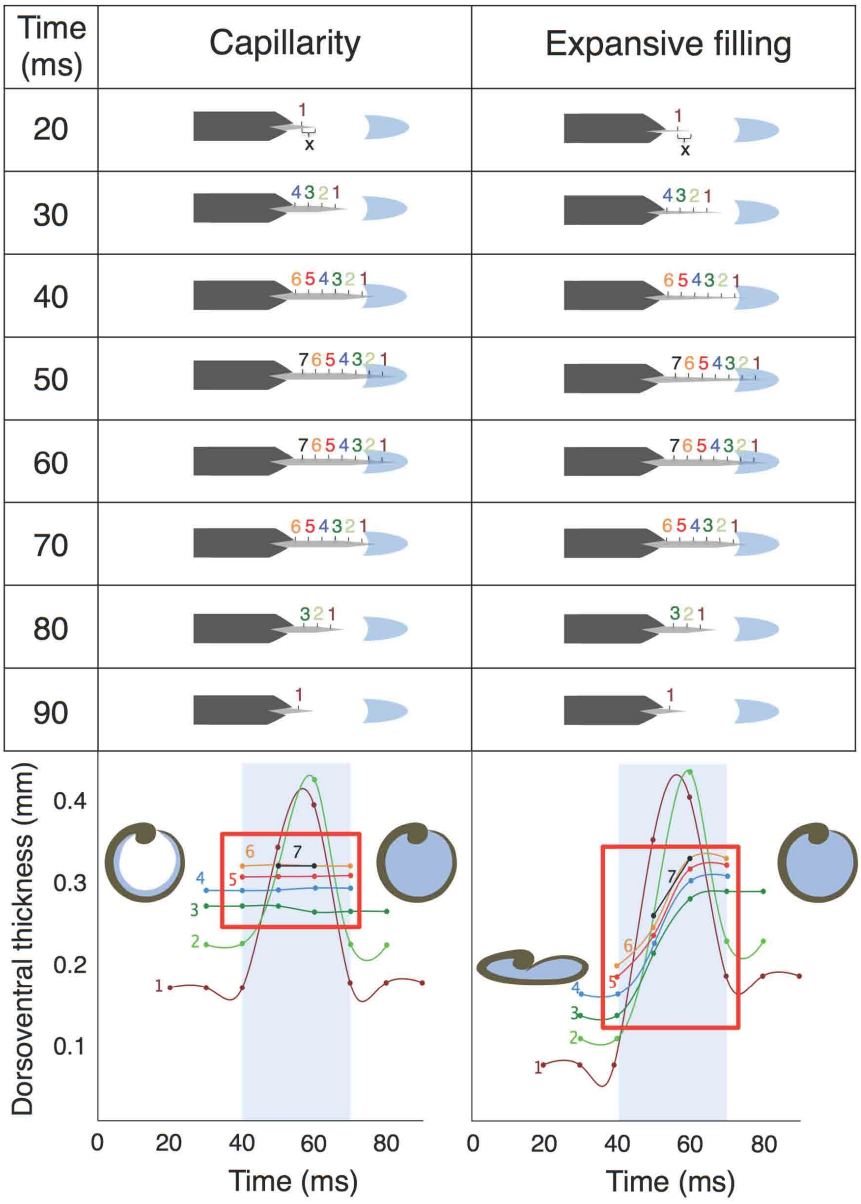



**Figure S8. Measurements to discern between the mutually exclusive possibilities.** *Top:* Placement scheme of semilandmarks to measure thickness along the tongue (tip to base) and through time (licking cycle from top to bottom). Stretched half-moon light blue shades represent the nectar pool. *Bottom:* Theoretical graphs depicting the expected change (or lack of) in dorsoventral thickness for each one of the semilandmarks above. Blue shade denotes the interval in which the tongue is in contact with nectar. Red squares represent the set of points measuring portions of the tongue that never get immersed in the nectar pool. Compare the expected lack of change in dorsoventral thickness under the capillarity hypothesis (*left*) and the increase in thickness under the expansive filling hypothesis (*right*). Cross sections of the tongue, before and after the tongue contacts the nectar, are shown for each graph. Under the capillarity hypothesis the empty tubes do not change thickness but a meniscus moving through the groove fills it with nectar, alternatively, during expansive filling, there are no empty tubes at any moment so a meniscus cannot form (excluding capillarity) and the grooves fill while they expand.

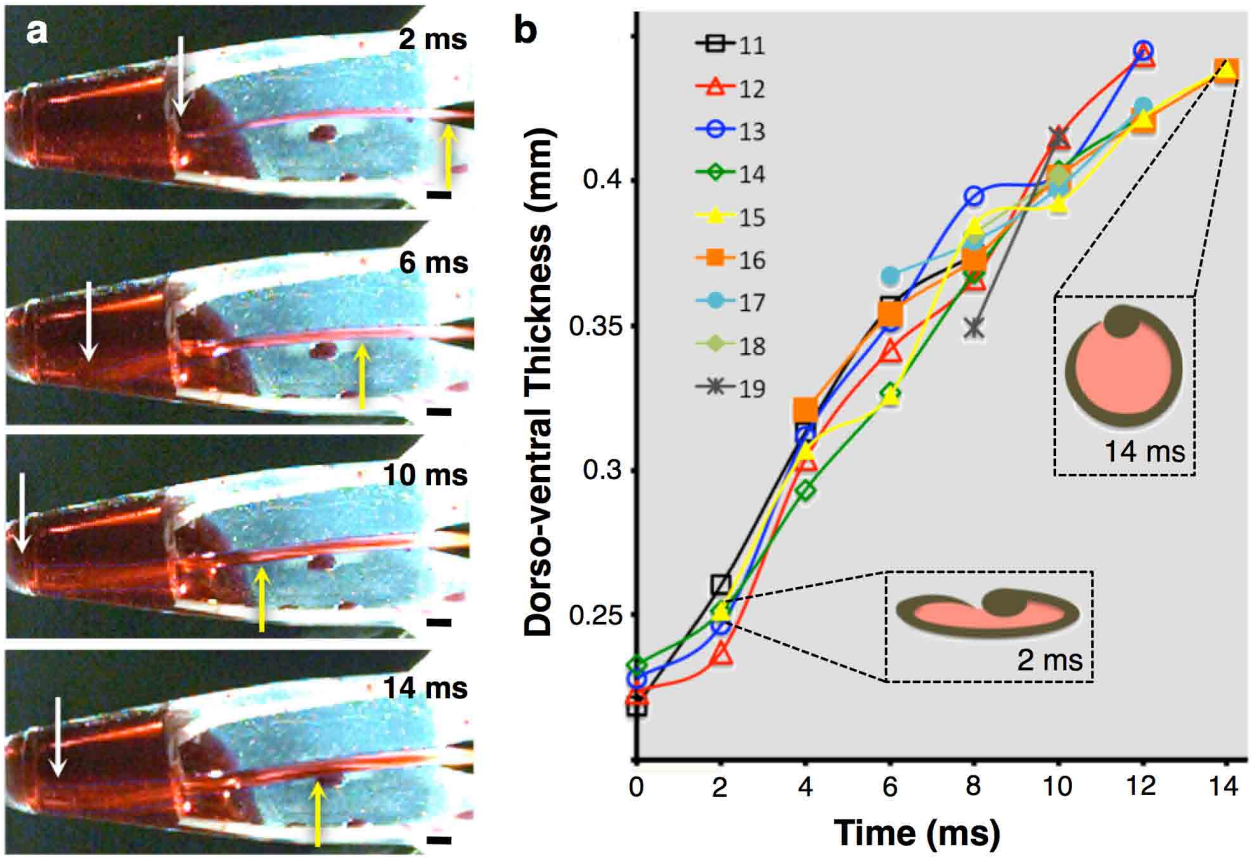
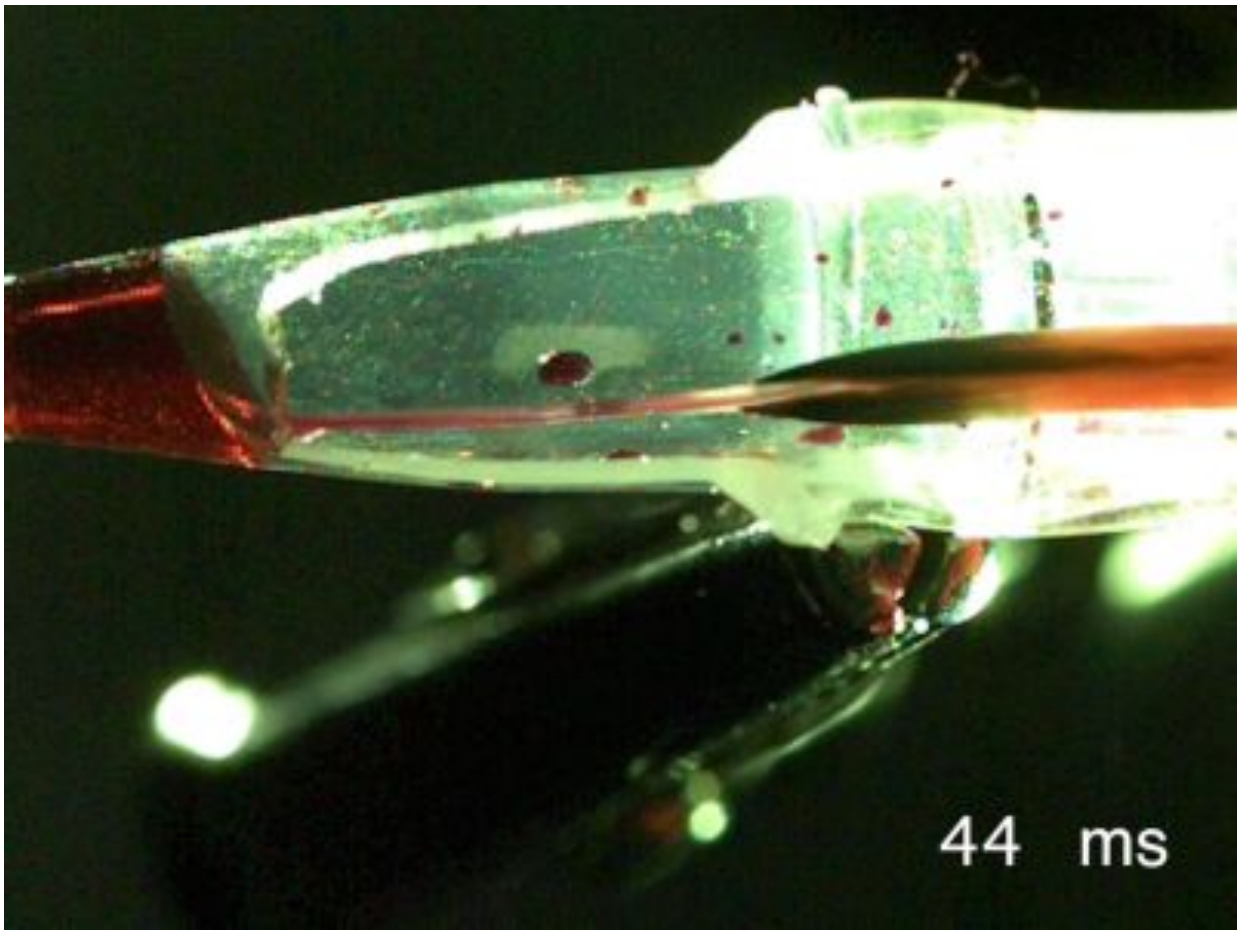
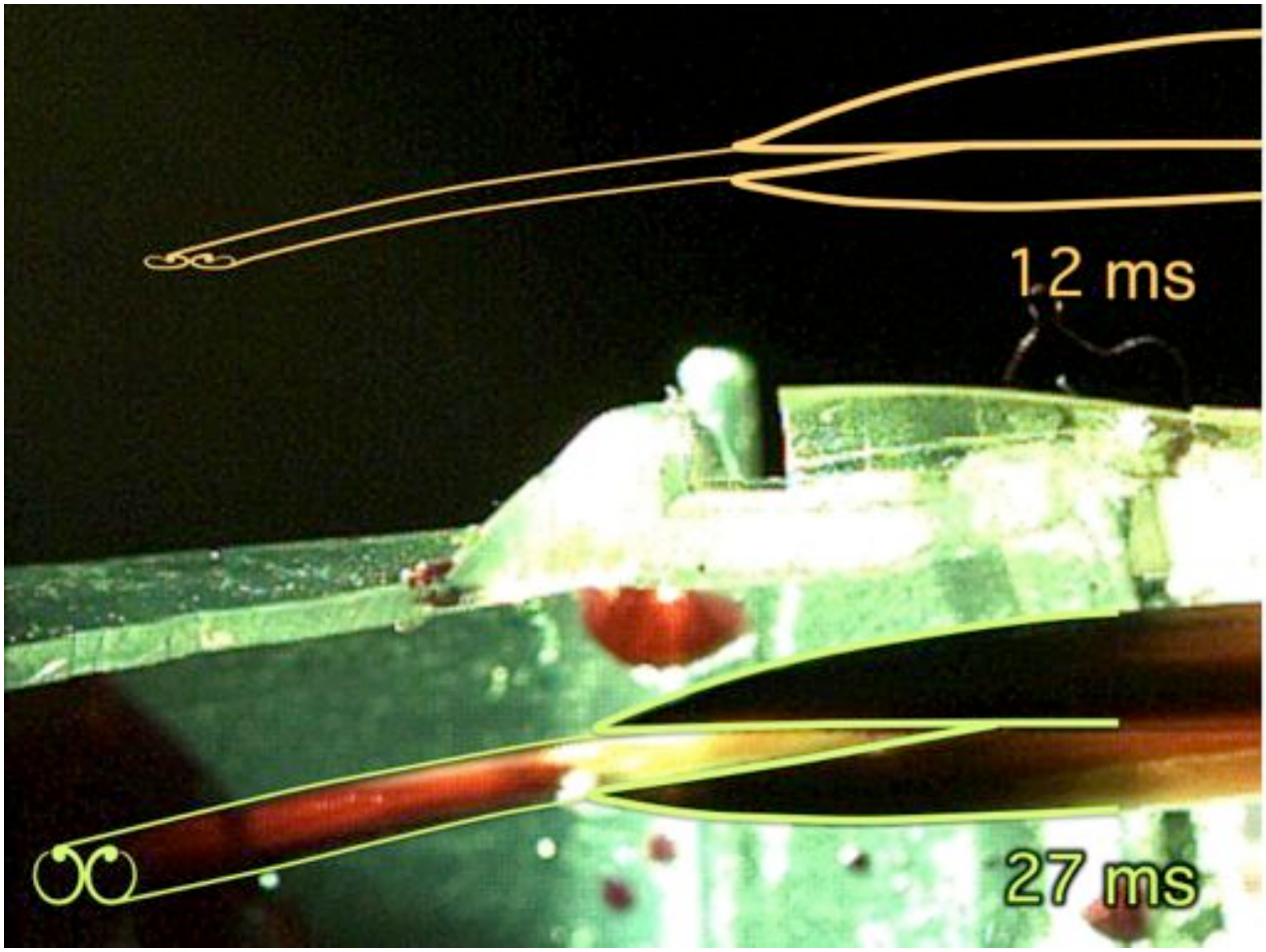



**Figure S9. Semilandmarks to measure expansive filling. a,** High-speed video frames showing a lateral view of the tongue of an Amazilia Hummingbird (*Amazilia amazilia*) drinking nectar. White (downward) arrows show the tongue tip and yellow (upward) arrows track the movement of a single point (semilandmark 15) as the tongue moves through a lick cycle. **b,** Change in thickness of several semilandmarks (11-19) tracked along the tongue through the lick cycle. Note that a single semilandmark can only be tracked from the point in time in which it emerges from the bill tip, and until it submerges into the nectar; hence the different lapses (lengths in the *X-axis*) of the semilandmark traces. Insets are schematics of hypothetical cross sections based in the expansion of the grooves demonstrated by the increase in thickness of the semilandmarks. Scale bar = 0.4 mm.

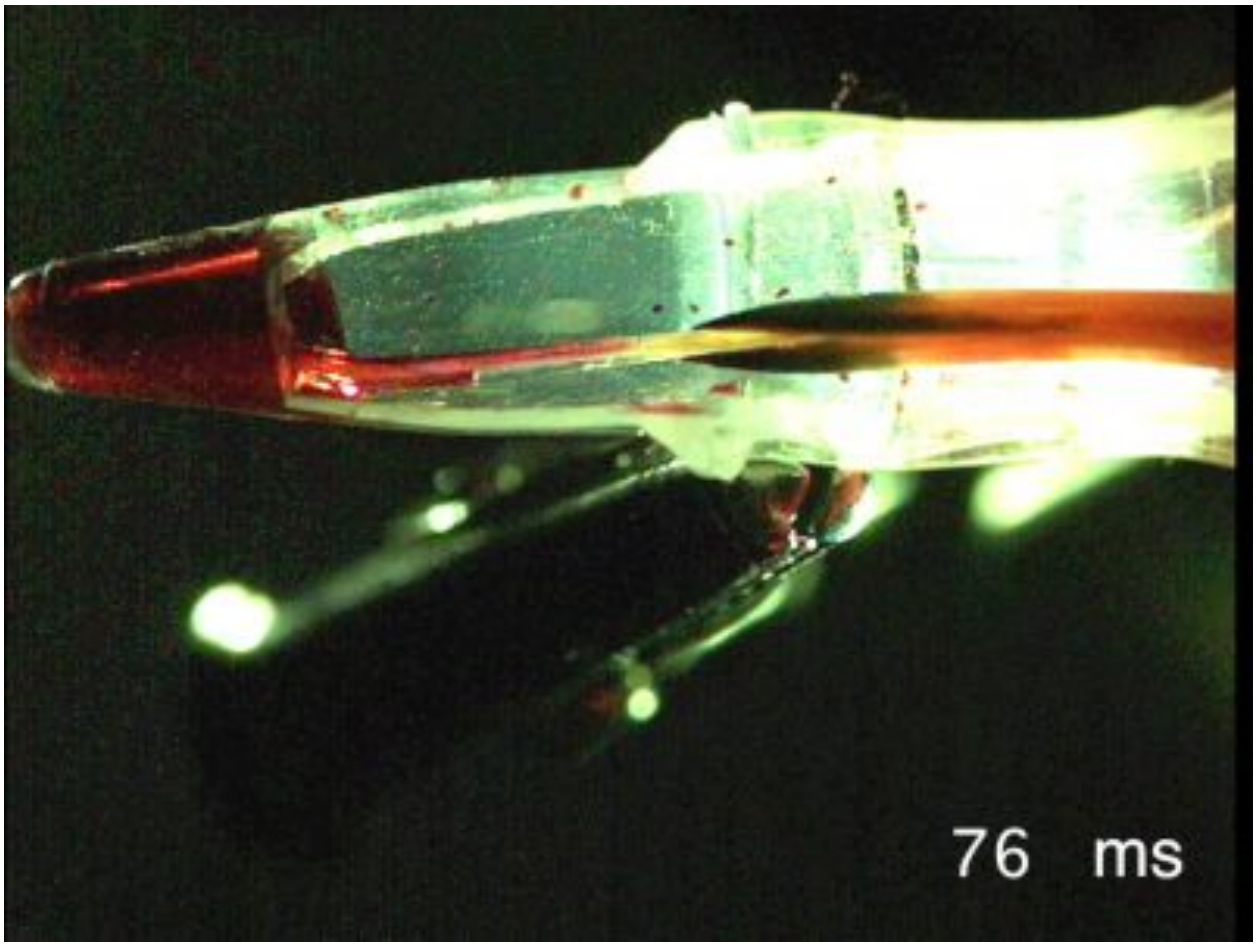
**Supplementary Movies**



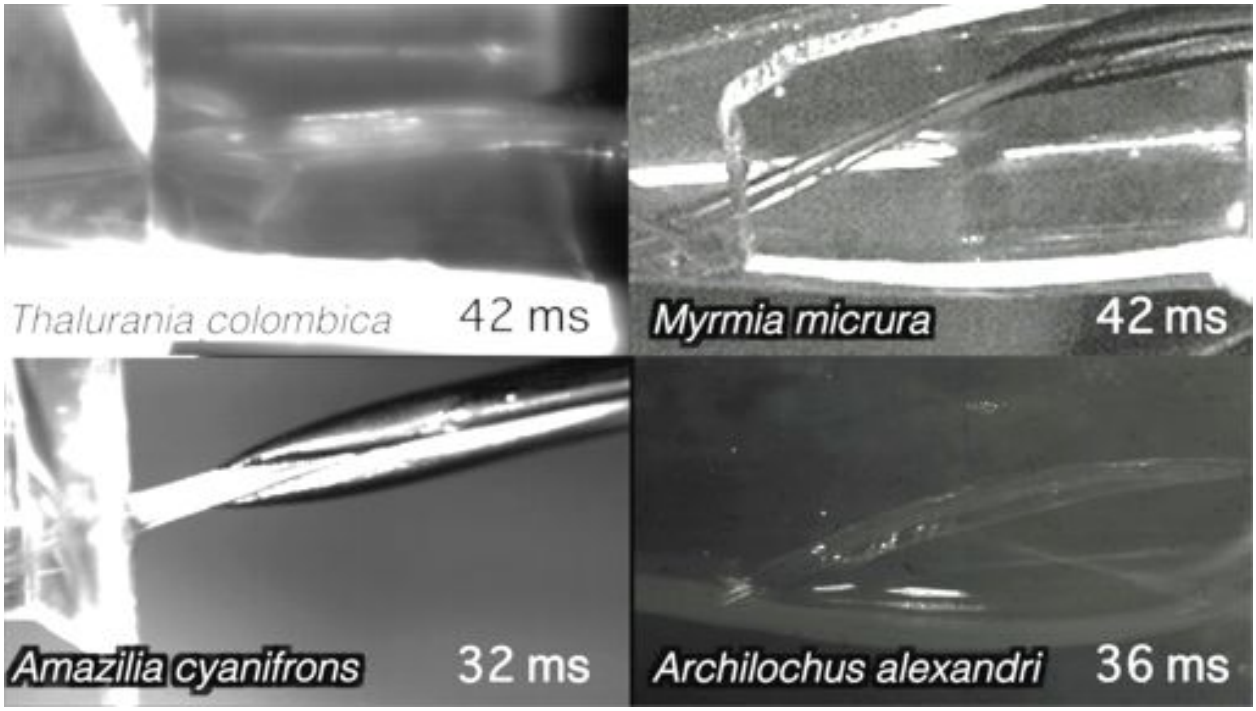

**Video S1. Hummingbird drinking far from the nectar pool.** A slow motion (115 times slower than real time) video in lateral view of an Amazilia Hummingbird (*Amazilia amazilia*) hovering and feeding on artificial nectar. Note the expansion of the tongue grooves as soon as the tips contact the liquid. The footage was taken at 500 frames per second (fps) and the timer is displaying in milliseconds.




**Video S2. Hummingbird drinking far from the nectar pool (close-up).** A slow motion (120 times slower than real time) video of an Amazilia Hummingbird hovering and feeding on artificial nectar. We compare the outlines of the tongue before and after expansion noting the time frame for the expansive filling mechanism (15 ms). The footage was taken at 1000 fps and the timer is displaying in milliseconds.




**Video S3. Expansive filling and capillarity.** A slow motion (135 times slower than real time) video of an Amazilia Hummingbird drinking artificial nectar. In this anomalous lick, one of the tongue grooves sticks to the feeder wall and bends, which terminates its flattened configuration. Compare the expansive filling on the still flattened groove to capillarity in the non-flattened (lower) grove. The footage was taken at 1000 fps and the timer is displaying in milliseconds.




**Video S4. Expansive filling in a variety of hummingbird species.** Slow motion (215-280 times slower than real time) videos of a Crowned Woodnymph (*Thalurania colombica*), a Short-tailed Woodstar (*Myrmia micrura*), an Indigo-capped Hummingbird (*Amazilia cyanifrons*), and a Black-chinned Hummingbird (*Archilochus alexandri*, courtesy of Don Carroll) drinking nectar. Notice the expansive filling of the tongue in all videos. The footage was taken at 400-800 fps and the timers are displaying in milliseconds.

## Supplementary Tables

### Table S1. Expansion of the tongue in 32 hummingbirds of 18 species.

| Species | Ind. # | Dorso-ventral Expansion (mm) | % Filled groove dorso-ventral diameter |
|---|---|---|---|
| *Florisuga mellivora* (0) | 1 | 0.39 ± 0.009 | 60.2 ± 1.59 |
| *Florisuga mellivora* (0) | 2 | 0.38 ± 0.009 | 61.8 ± 0.79 |
| *Phaethornis baroni* (1) | 1 | 0.41 ± 0.017 | 58.6 ± 2.17 |
| *Ramphodon naevius* (1) | 1 | 0.45 ± 0.01 | 61.9 ± 1.01 |
| *Ramphodon naevius* (1) | 2 | 0.44 ± 0.015 | 60.2 ± 1.76 |
| *Colibri coruscans* (2) | 1 | 0.31 ± 0.01 | 53.4 ± 0.43 |
| *Colibri coruscans* (2) | 2 | 0.31 ± 0.022 | 53.3 ± 2.03 |
| *Lophornis chalybeus* (3) | 1 | 0.18 ± 0.009 | 45.3 ± 2.14 |
| *Lophornis chalybeus* (3) | 2 | 0.21 ± 0.012 | 52.4 ± 1.86 |
| *Aglaeactis cupripennis* (4) | 1 | 0.2 ± 0.003 | 46.1 ± 0.76 |
| *Boissonneaua flavescens* (4) | 1 | 0.26 ± 0.007 | 54.3 ± 1.86 |
| *Boissonneaua flavescens* (4) | 2 | 0.26 ± 0.009 | 54.1 ± 1.13 |
| *Clytolaema rubricauda* (4) | 1 | 0.23 ± 0.018 | 47.5 ± 3.63 |
| *Coeligena bonapartei* (4) | 1 | 0.28 ± 0.023 | 53 ± 2.94 |
| *Myrmia micrura* (5) | 1 | 0.19 ± 0.018 | 47.9 ± 4.28 |
| *Myrmia micrura* (5) | 2 | 0.2 ± 0.03 | 48 ± 4.78 |
| *Archilochus colubris* (5) | 1 | 0.23 ± 0.02 | 50.2 ± 3.55 |
| *Archilochus colubris* (5) | 2 | 0.2 ± 0.009 | 44.3 ± 1.39 |
| *Calypte anna* (5) | 1 | 0.24 ± 0.012 | 50.7 ± 1.95 |
| *Calypte anna* (5) | 2 | 0.24 ± 0.013 | 51.8 ± 2.26 |
| *Chalybura buffonii* (6) | 1 | 0.25 ± 0.003 | 49.4 ± 1.35 |
| *Chalybura buffonii* (6) | 2 | 0.27 ± 0.013 | 52.8 ± 1.39 |
| *Thalurania glaucopis* (6) | 1 | 0.3 ± 0.015 | 57.4 ± 1.85 |
| *Thalurania glaucopis* (6) | 2 | 0.3 ± 0.006 | 61.2 ± 0.27 |
| *Eupetomena macroura* (6) | 1 | 0.31 ± 0.003 | 59.2 ± 1.41 |
| *Amazilia tzacatl* (6) | 1 | 0.29 ± 0.012 | 57.5 ± 0.66 |
| *Amazilia tzacatl* (6) | 2 | 0.27 ± 0.026 | 55.2 ± 3.64 |
| *Amazilia amazilia* (6) | 1 | 0.24 ± 0.013 | 54.4 ± 2.24 |
| *Amazilia amazilia* (6) | 2 | 0.24 ± 0.003 | 51.5 ± 1.03 |
| *Amazilia amazilia* (6) | 3 | 0.22 ± 0.027 | 50.5 ± 4.51 |
| *Amazilia cyanifrons* (6) | 1 | 0.19 ± 0.02 | 47.1 ± 4.74 |
| *Amazilia cyanifrons* (6) | 2 | 0.22 ± 0.021 | 51.1 ± 3.92 |

Numbers in parentheses following each species indicate hummingbird main clades: 0=Topazes, 1=Hermits, 2=Mangoes, 3=Coquettes, 4=Brilliants, 5= Bees, 6=Emeralds. Dorso-ventral expansion is the difference between the dorso-ventral diameter of the groove completely filled with nectar (final thickness) and the minor axis of the flattened groove (initial thickness). Such thicknesses were measured at the same semilandmark through time (by tracking the tongue tip) to account for thickness variation along the tongue length. The percentage is calculated as the proportion of the expansion out of the final thickness. For each individual three licks of different foraging bouts were measured. Values in the table are mean ± SEM of these three licks.

# Chapter 4

## Hummingbird bill tips function as tongue wringers

**Abstract**

It is well known that hummingbirds can extract nectar with impressive speed from flowers. Our recent work has shown that the tongue collects nectar in surprising ways. However, despite decades of study on nectar intake rates, the mechanism by which feeding is ultimately achieved – the release of nectar from the tongue so that it can pass into the throat and be ingested– has not been elucidated. Under high magnification (up to 50x) we examined the interior of the bills of 1050 specimens, representing 157 species and 84 genera. The vast majority of the reviewed genera showed a very distinctive set of previously unreported internal structures. We found near the bill tip, in an area of strong lateral compression of internal mandibular width, that the tomia are thinner and sometimes partially inrolled and often hold forward-directed serrations. Aligned with these structures, a prominent pronglike structure projects upward and forward from the internal mandibular keel. Distal to this mandibular projection, another smaller maxillary projection protrudes downwards from the keel of the palate. Four shallow depressions occur at the base of the mandibular projection on the mandibular floor. Of these, two are small depressions located proximally and at the sides of the mandibular projection. A third, slightly larger depression is positioned distally to the first two and directly under the maxillary projection. And the fourth depression, the largest, is found more proximally where the bill becomes thicker, as seen from the side. Variations of this general arrangement occur in different clades of hummingbirds. We hypothesize that this group of structures, integrated into the area of the bill tip where tongue extrusion occurs, helps to enhance the nectar offloading at each lick.

We suggest that this “wringer” or “squeezer device”, in conjunction with bill, tongue and gular area movements, helps to move nectar towards the throat. Taking into account bill and tongue morphology, we propose a new model for nectar transport in hummingbirds, including a suction component rejected by most previous authors.

*Keywords*: Beaks, functional morphology, birds, feeding mechanics, tongues.

## Introduction

Although nectarivory is commonly viewed as a specialized way of life in birds, repeated independent evolutions of the nectarivorous lifestyle provide evidence of the wide range of variation in the degree of reliance on nectar across the nectarivore clades, *i.e.* there are various degrees of specialization for nectarivory in birds (Stiles 1981, Paton and Collins 1989). In the same way, plants employing animal pollinators have a wide range of options, from insects to several kinds of vertebrates; and birds stand out as the main vertebrate pollinators (Fleming and Muchhala 2008). At the vertex in which these two continua converge, several cases of plant-bird coevolution have appeared independently, and on several continents (Stiles 1981). Various studies have noted strong and repetitive patterns in the bill morphology of avian nectarivores (see review in Paton and Collins 1989), and it has been assumed that a similar feeding mechanism underlies these convergent morphologies (Collins 2008, Köhler *et al.* 2010).

However, despite decades of study of morphological variation in bill size and shape, there is a surprising lack of detailed examination of the morphology of the interior of the bill, where nectar handling actually occurs. Focusing on hummingbirds (the most specialized nectarivorous vertebrates), we describe here previously unreported structures that we hypothesize are adaptations for nectar feeding. We propose a functional hypothesis for their placement and structure that provides testable predictions for future studies, and that can be applied to similar (convergent) traits found in other nectarivores. This hypothesis could facilitate the design of experiments to test other previously proposed hypotheses (*e.g.* Scharnke 1931, Böker 1937, Kingsolver and Daniel 1983, Heyneman 1983, Cheke and Mann 2009), thereby shedding light on the functional constraints on the evolution of bill morphology in these birds.

The bills of hummingbirds have long been regarded as highly specialized instruments for probing the tubular corollas of flowers (see reviews in Faegri and van der Pijl 1979, Stiles 1981). The role of the bill in nectar feeding has seemed clear: it provides a rigid sheath that permits the instrument for nectar extraction, the tongue, to traverse the corolla tube and enter the nectar chamber (Stiles 1981, 1985). Numerous studies have described the correspondence between the lengths and curvatures of bill and corolla and its relation to the rate and efficiency of nectar uptake (*e.g.* Wolf *et al.* 1972, 1976; Temeles *et al.* 2009). Even small differences in the bill-corolla "fit" may have a major impact on flower choice by the hummingbirds (Stiles 1981) and thus affect resource partitioning in hummingbird-flower communities (*e.g.* Rodríguez-Flores and Stiles 2005, Gutiérrez-Zamora 2008).

The morphology of the hummingbird tongue has also received detailed study (Scharnke 1931, Weymouth *et al.* 1964, Hainsworth 1973), and on the basis of its anatomy Kingsolver and Daniel (1983) developed a widely accepted biophysical model to explain how nectar is collected via capillarity. This model suggested that nectar was removed from the flower by capillary action of the rolled-up tongue tips with each lick of the tongue (Hainsworth 1973, Roberts 1996, Collins 2008, Köhler *et al.* 2010, Kim *et al.* 2012).

Our work has demonstrated that the capillary model is inaccurate, and we documented intake mechanisms that take advantage of the tongue groove walls' elastic properties (Rico-Guevara and Rubega 2011, Rico-Guevara 2014 Chap. 3). In spite of these advances, it is still not clear how the nectar is removed from the tongue (which, after all, functions by collecting nectar on its grooves) and passed to the pharynx for ingestion. Scharnke (1931) and others (see review in Böker 1937) suggested that the nectar uptake is initiated via capillary forces, but later is completed by a vacuum created by the tongue retraction into the oral cavity and swallowing process. Ewald and Williams (1982) reported compression of the tongue at the bill tip during protrusion, and apparently coordinated movements of the throat with opening and closing of the bill tip with each lick of the tongue. Heyneman (1983) suggested that a suction component might be involved. Although high-speed videography supports the dorso-ventral compression of the tongue while it is being extruded (Rico-Guevara and Rubega 2011, Rico-Guevara 2014 Chap. 3), the details of how the morphology of the beak interacts with this compression to liberate nectar from the tongue, while retaining it inside the bill, have not been elucidated.

It is generally overlooked by all but anatomists that the edges of hummingbird beaks are not smooth. In a previous study of the minute serrations on the cutting edge of the bills of hummingbirds (Stiles and Rico-Guevara unpub. manusc.), we distinguished three classes of tomia: Class A with numerous tall serrations over 15% or more of the tomia; class B with fewer and lower, forward-directed serrations concentrated on the distal rhamphotheca just proximal to the bill tip; and class C with rudimentary or no serrations. Class B serrations were of particular interest in that they occurred in the great majority of genera examined, and had been overlooked in the previous most complete study on hummingbird serrations (Ornelas 1994) because they occur at a point where the tomia are rolled inward in museum specimens, rendering them inconspicuous or invisible from side view. This is precisely the point at which the tongue is extruded during nectar uptake, and we hypothesized that such serrations might play a role in removal of nectar from the tongue with each lick.

That the story might be still more complex was suggested to us when we discovered hitherto undescribed structures on the inner (maxillar and mandibular) surfaces of the rhamphotheca that might interact with the serrations and the medially deflected (rolled inwards) tomia. We therefore decided to reexamine the bills of as many species of hummingbirds as possible at higher magnifications, to determine the occurrence of these structures. We report here the distribution and variation among hummingbirds of these structures. The discovery of this structural complexity inside hummingbirds' bills, plus these birds' capabilities of bill bending (Yanega and Rubega 2004, Smith *et al.* 2011, Rico-Guevara 2014 Chap. 5), enable us to propose a new theoretical model of nectar uptake and transport from the tongue grooves, through the bill, to the pharynx.

This model necessitates a revision of the biophysical mechanisms cited by Böker (1937), and a reevaluation of the intake-rate equations proposed on the basis of a capillarity-based transport model (Kingsolver and Daniel 1983, Kim *et al.* 2012). We provide new hypotheses about the nectar extraction and transport processes, and use them to generate predictions that are testable by performance experiments.

**Methods**

*Morphological survey of museum specimens*

We examined the bills of hummingbird specimens in the following museums: Instituto de Ciencias Naturales, Universidad Nacional de Colombia (ICN); Museo de Zoología "Alfonso Herrera" and the Colección Nacional de Aves del Instituto de Biología, Universidad Nacional Autónoma de México (UNAM); Colección Ornitológica Phelps, Caracas, Venezuela (COP); Museu de Zoologia, Universidade de São Paulo (MZUSP); National Museum of Natural History, Smithsonian Institution, Washington D.C. (USNM); the American Museum of Natural History, New York, NY (AMNH); the Museo de Zoología, Universidad de Costa Rica (UCR); the Vertebrate Collection at the Yale Peabody Museum, Yale University, New Haven, CT, and the Vertebrate Research Collection, University of Connecticut, Storrs, CT.

We observed the interior of the bills at magnifications of 10-50x using Wild-Heerbrugge dissecting microscopes or a Leica GZ6 stereomicroscope. Samples comprised 6-15 adult specimens (typically 3 per sex) for most species, depending on availability of specimens with the bill tip open and enough remaining flexibility that bills could be opened without damaging the specimens.

Because it was necessary to have bills opened more widely to see the internal structures than for observing tomial serrations, we were unable to use many older specimens unless they had been "improperly" prepared with bills slightly open, which limited to some extent the present survey. In all, we examined 1050 specimens representing 157 species and 84 genera (*ca.* 48% of the species and 82% of currently recognized genera of hummingbirds, *cf.* Schuchmann 1999).

We analyzed our results at the level of hummingbird genera rather than species because our survey was much more complete for genera than for species, and because we found that intrageneric variation in structures was relatively limited (see below). After preliminary observations, we described the internal structures in detail. We used that description to conduct a pilot survey (1 individual per each of 40 genera, opportunistically chosen) to assess the range of variation of the internal bill structures, and of the degree of inrolling of the distal tomia just proximal to the bill tip.

*Phylogenetic signal tests*

Using the results of the pilot survey, we developed classifications for the character states of the structures we found inside of the bills, and for the degree of inrolling of the tomia. We defined character states (Fig. S1) for the different bill structures in each genus (Appendix 1) and then used Spearman rank correlations to test for co-variations among the categories defined for each one of the bill traits. We performed G-test for goodness-of-fit to test if the bill structures surveyed here vary according to Stiles and Rico-Guevara's (unpub. manusc.) classification of hummingbird bills' tomia (see Supplementary methods).

Finally, we used a modification (including *Ramphodon*, *Sappho*, *Cynanthus* and *Goldmania,* McGuire *et al.* 2014, J. McGuire, pers. comm.) of the topology reported by McGuire *et al.* (2007), available in TreeBASE (study accession number SI 825, matrix accession number M3354), to determine whether the character states of the bill traits reported here showed any relation to the hummingbird phylogeny. Our resulting tree is concordant with the most complete time-calibrated phylogenetic tree for hummingbirds (McGuire *et al.* 2014). We plotted the traits on the topology to examine trait evolution qualitatively, and tested for phylogenetic signal of discrete traits (in Mesquite v. 2.75, Maddison and Maddison 2014).

*MicroCT and High-Speed Videography*

To examine the three-dimensional arrangement of the structures inside the bill, we used the Xradia MicroXCT scanner, of the High-Resolution X-ray Computed Tomography Facility at The University of Texas. This scanner provided 5-micron resolution of osmium stained tissues (highlighting the keratin of the structures inside the bill), effectively covering the smaller size ranges (<1 cm) of the structures involved in this study (see staining method in Rico-Guevara 2014 Chapt. 1). We obtained scans for three salvaged specimens, a Ruby-throated Hummingbird (*Archilochus colubris*), an Anna's Hummingbird (*Calypte anna*), and a Short-tailed Woodstar (*Myrmia micrura)*.

In order to visualize the functional interactions among the bill tips and the tongue, we filmed free-living hummingbirds feeding on artificial nectar (18.6% sucrose concentration). We used a high-speed camera (Phantom Miro ex4) with a special high-magnification macro lens (MP-E 65mm f/2.8 1-5x Macro Photo - Canon USA, Inc.).

We worked in Colombia and Ecuador at two different elevations (0 and 2400 m.a.s.l.) and opportunistically filmed Indigo-capped Hummingbirds (*Amazilia cyanifrons*), Amazilia Hummingbirds (*Amazilia amazilia*), and Short-tailed Woodstars (*Myrmia micrura)*. All filming activities were reviewed and authorized by the Institutional Animal Care and Use Committee at the University of Connecticut; Exemption Number E09-010.

**Results**

*Description of the squeezer device structures*

All the structures described in this paper are restricted to the most distal portion of the bill (Fig. 1a). In most of the genera this area is easily seen in lateral view as an apparent slight dorso-ventral thickening (especially evident in straight bills) of the entire bill just proximal to the tip (Fig. 1a). Starting close to the bill base, the mandible fits inside the maxilla along almost the entire length of the bill (*cf.* Fig. 127b, Böker 1937). The apparent thickening near the tip is partially due to the decoupling of maxilla and mandible (resulting in a seeming increase in bill depth) and partially due to a depression on the mandibular floor ("proximal basin" in Fig. 1d), which translates into a downward external curvature of the mandibular profile (ventrum). The inner surfaces of both maxillary and mandibular rhamphotheca possess medial keels in nearly all species. In the mandibular floor, from base to tip this keel is interrupted by the aforementioned depression but reappears distally to it (Fig. 1d). Projecting anteriorly from the mandibular keel and dorsally at an angle of *ca.* 30º from it, just posterior to the point at which the tomia start to roll inwards in most species, is a fine-tipped prong that in most cases is not visible from the outside of the bill in side view (Fig. 1c-e).

In some species (see Appendix 1), projecting anteriorly and ventrally from the maxillary keel, slightly anterior to the mandibular prong, is a smaller prong of similar shape (Fig. 1b,c). Most species showed a pronounced mandibular prong, but in many the maxillary prong was much reduced or lacking.

In most species with a pronounced mandibular prong, two small depressions (smaller than the more proximal depression described above) were located just behind and on either side of its base in the floor of the mandibular rhamphotheca; in some cases a hole in the base of each of these depressions, connecting to internal ducts running posteriorly, was noted (Fig. 1f). A fourth depression was located medially in the mandibular floor just posterior to the bill tip, and especially when the maxillary prong presented a high character state, this fourth basin was located directly beneath it, and distal to the base of the mandibular prong ("distal basin" in Fig. 1d). This depression is intermediate in size compared to the two smaller ones and the most proximal large one.

All the structures described above appear in the distal portion of the bill in the area in which the maxillary and mandibular tomia are sufficiently thin that in living birds this region of the tomia is flexible at the contact with the tongue (Movie S1). In museum specimens, because of drying, this section of the tomia appears partially inrolled (curled inwards).

We classified prongs, basins, and tomial curvature in categorical classifications of character states in order to make comparisons and study patterns across the family (see Supplementary methods, Fig. S1).

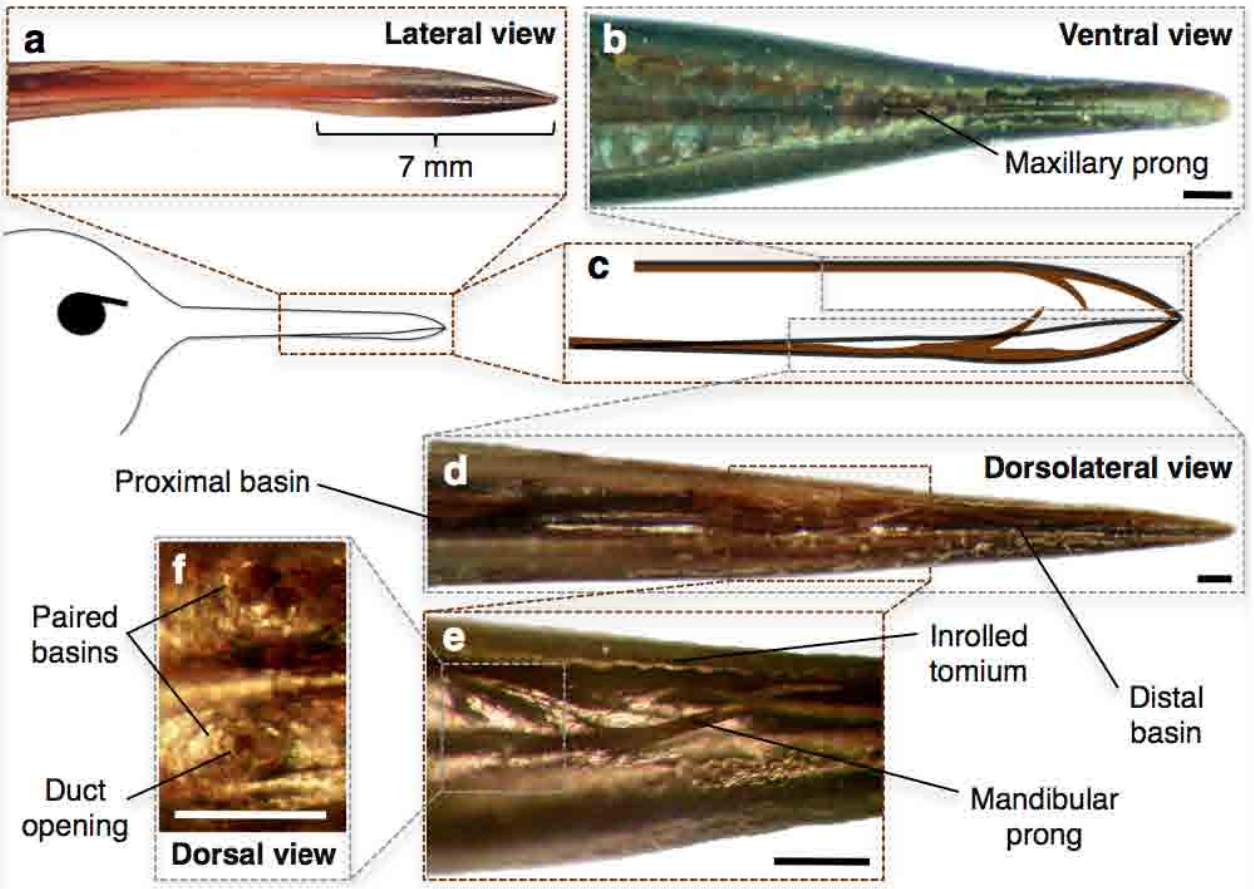



**Figure 1. Description of the internal bill structures that construct a) squeezer device in hummingbird bill tips. a)** Photograph of a museum specimen of a Broad-billed Hummingbird (*Cynanthus latirostris*) showing the last portion of the bill, which appears thickened dorso-ventrally. **b)** Ventral view of the tip of the maxilla (maxillary rhamphotheca) in which the degree of inrolling of the serrated tomia is noticeable; thin black line indicates maxillary prong. **c)** Sketch of the distal portion of the bill in which the maxillary and mandibular prongs and proximal and distal depressions are depicted. **d)** Ventral view of the tip of the mandible (mandibular rhamphotheca) in which the proximal and distal basins are visible. **e)** Close-up of the mandibular prong and the inrolled tomia. **f)** Dorsal view close-up of the paired basins with duct openings at their bottoms, located at the base of the mandibular prong. All unlabeled scale bars are 0.5 mm.

*Presence and state of prongs, basins and inrolled tomia*

Two-thirds of the genera examined showed distinct maxillary and mandibular prongs (categories 3 and 4); an additional 20% showed a distinct mandibular prong but only a rudimentary maxillary prong (category 2).

A further 10% showed a mandibular prong but none on the maxilla, thus the presence of at least one prong appears nearly universal among the hummingbirds (96 % of all genera reviewed, Table 1).

**Table 1. Numbers of genera in each major hummingbird clade showing different character states of prongs and basins on the inside of the bill.**

| **Clade** | 0: Topazes | 1: Hermits | 2: Mangoes | 3: Coquettes | 4: Brilliants | 5: Giant | 6: Mt. gems | 7: Bees | 8: Emeralds |
|---|---|---|---|---|---|---|---|---|---|
| **Prong type** | **Highest character state of prongs** | | | | | | | | |
| **0** | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| **0-1,1** | 0 | 1 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| **1-2,2** | 1 | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 0 |
| **2-3,3** | 1 | 3 | 2 | 3 | 1 | 0 | 0 | 5 | 1 |
| **3-4,4** | 0 | 0 | 3 | 9 | 12 | 1 | 5 | 5 | 22 |
| **Basin type** | **Highest character state of basins** | | | | | | | | |
| **0** | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| **0-1,1** | 0 | 1 | 4 | 1 | 0 | 0 | 0 | 0 | 0 |
| **1-2,2** | 2 | 0 | 2 | 4 | 4 | 0 | 1 | 0 | 5 |
| **2-3,3** | 0 | 3 | 5 | 8 | 9 | 1 | 2 | 8 | 14 |
| **3-4,4** | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 2 | 4 |
| **Total genera** | **2** | **5** | **11** | **14** | **13** | **1** | **5** | **10** | **23** |

Only three genera never showed prongs (or they were very rudimentary): *Eutoxeres, Androdon* and *Heliothryx*. All have distinctive, atypical bills. The bill of *Eutoxeres* is very strongly decurved, such that when the base of bill is horizontal, the bill tip points downward at an angle of nearly 90º.

In *Eutoxeres* maxillary overlap of the mandibles is pronounced, except for the distal 6 mm. In that distal area, both maxillar and mandibular tomia become thinner and in many specimens appear strongly inrolled, facing medially and in many cases touching and even overlapping each other. In the mandibular floor, approaching the inrolled area, the medial keel is thicker and taller, only in some specimens discontinous (but never projecting upward, *i.e.* prongless, Fig. S5). The bill of *Androdon* is long, straight and it does not show the lateral thickening evident in most of the genera. In the vast majority of hummingbirds the mandibular rami fuse in the distal half of the bill, just posterior to the dorso-ventral thickening; however in this genus the point at which the rami join is much closer to the bill base (about half way along its 4 mm long bill, Fig. S6). *Androdon* also shows the most highly serrated tomia (class A) of all hummingbirds (Fig. S6); the keels are enlarged from near the base to the tip but never show projections.

In *Heliothryx* the rami fuse relatively closer to the bill base compared to other hummingbirds with bills of similar length (about 5 mm from the base in its 15 mm bill, Fig. S7), and the distal half of the bill is strongly compressed laterally and tapers to a sharp point, as is also the case in *Schistes* (Fig. S8). An enlarged gonys (formed by proximad fusion of the rami, creating a longer symphysis) is also present in *Threnetes ruckeri* (Zusi 2013), a species known to rob nectar (frequent piercing of *Calathea spp.*, Stiles 1980). Given that *Heliothryx* and *Schistes* are also nectar robbers (*cf.* Schuchmann 1999), it is plausible that the enlarged gonys is an adaptation for nectar robbing, perhaps reinforcing the tip for piercing (pers. obs.). *Heliothryx* specimens showed an enlarged mandibular ridge starting close to the bill base and continuing through the middle region but disappearing near the bill tip, again never forming prongs.

Similarly, some 70% of all genera showed four depressions on the mandibular floor (categories 3 and 4, Table 1) and an additional 23% showed three; only 7% showed a single broad but shallow depression (category 1, Appendix 1). Only *Eutoxeres* lacked any trace of a basin: the distal palatal surfaces of its rhamphotheca are entirely smooth.

*Phylogenetic signal in the presence of the squeezer device structures*

The distribution of prongs and basins among the hummingbirds is influenced by phylogenetic relationships (Table 2, Supplementary character state trees). All members of the more derived clades 4-8 (brilliants, giant, mountain-gems, woodstars and bees, emeralds) in the phylogeny of McGuire *et al.* (2007, 2009, 2014) have prongs of category 2-4 and basins of category 1-3; the vast majority of genera in these clades also show class B tomia (Appendix 1). The mangos (clade 2), all of which show class A tomia, exhibit all states of prongs and basins, as is also the case with the hermits (clade 1); although in hermits there is a trend for low character states of the squeezer device structures.

**Table 2. Summary of phylogenetic signal tests.** Steps of parsimonious reconstructions (see text for topology explanation) are shown in the second column. Terminal taxa were reshuffled 10000 times and a threshold of (0.05) on the steps distribution was fixed as a confidence boundary (third column). Characters that have fewer steps than the confidence threshold are characterized by significant phylogenetic signal.

| Character | Tree steps (parsimony) | Percentile boundary (0.05) | Phylogenetic signal |
|---|---|---|---|
| **Tomial class** | 13 | 20 | **Significant** |
| **Tomial curvature Maxilla Females** | 10 | 10 | Marginal |
| **Tomial curvature Maxilla Males** | 16 | 14 | Not significant |
| **Tomial curvature Mandible Females** | 21 | 18 | Not significant |
| **Tomial curvature Mandible Males** | 21 | 21 | Marginal |
| **Projections** | 16 | 18 | **Significant** |
| **Depressions** | 23 | 23 | Marginal |

The two genera in the basal clade (0, topazes) were incompletely (category 2, *Florisuga*) to partially (category 3, *Topaza*) pronged and presented category 2 basins (Table 1, Appendix 1). Given the otherwise universal presence of prongs, their absence in *Eutoxeres, Androdon* and *Heliothryx* almost certainly represents secondary losses of these structures; as is the case of the lack of depressions (basinless) in *Eutoxeres*.

*MicroCT and High-Speed Videography*

Exploring the three-dimensional organization of the internal bill structures, and the bill as a whole in general, in conjunction with close-up (5x) high-speed (1260 fps) observations of hummingbirds drinking artificial nectar, was an informative first step towards linking morphology and function. Using this visualization technique it is easier to graphically portray the relative positions of the structures that we found in our morphological survey (*e.g.* Fig. 2), and the high-speed videos of the bill tips provide key functional insights (*e.g.* Fig. 3, Movie S1).

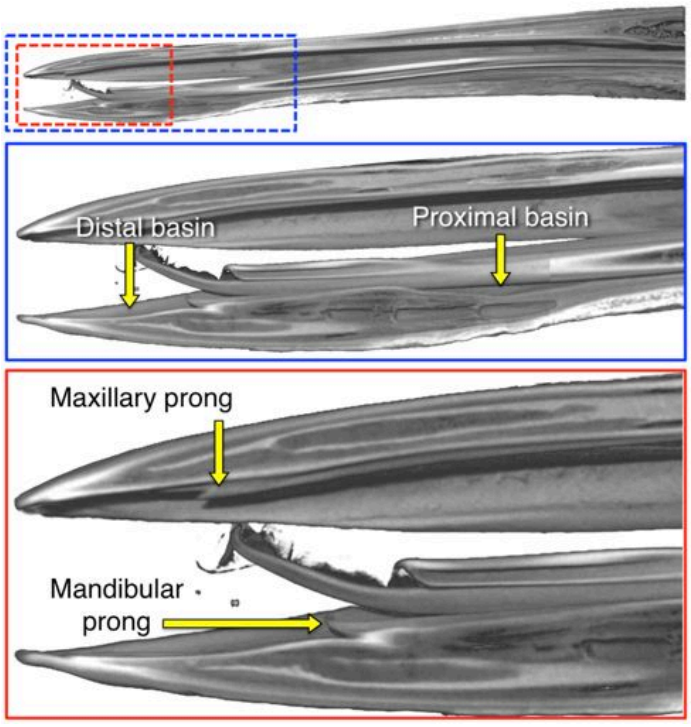



**Figure 2. MicroCT rendering (lateral view – longitudinal section) of the bill of a Short-tailed Woodstar (*Myrmia micrura*).** On top the general overview of the bill with the blue (outer) and red (inner) rectangles indicating the location of the close up views, middle and bottom panels respectively. In the middle a close up showing the distal portion of the bill in which a dorso-ventral thickening is evident, as well as the proximal and distal basins. At the bottom a further zoomed in view indicating the maxillary and mandibular prongs. One of the paired basins is only partially viewed at the base of the mandibular prong. In all views, one curled tongue groove tip is visible between the maxillary and mandibular tips.

We found that maxillary and mandibular prongs are staggered and located just before the point in which the tongue is in direct contact with the tomia during its protrusion (Figs. 2 and 3). The proximal basin is located at the cranio-rostral (proximal to distal) start of the region in which the bill thickens dorso-ventrally (*e.g.* Fig. 1a), this thickening is partly due to the decrease in proximity between maxilla and mandible, therefore lack of tomia overlapping, and partly due to the dorso-ventral thickening of the mandibular rhamphotheca itself (Fig. 2).

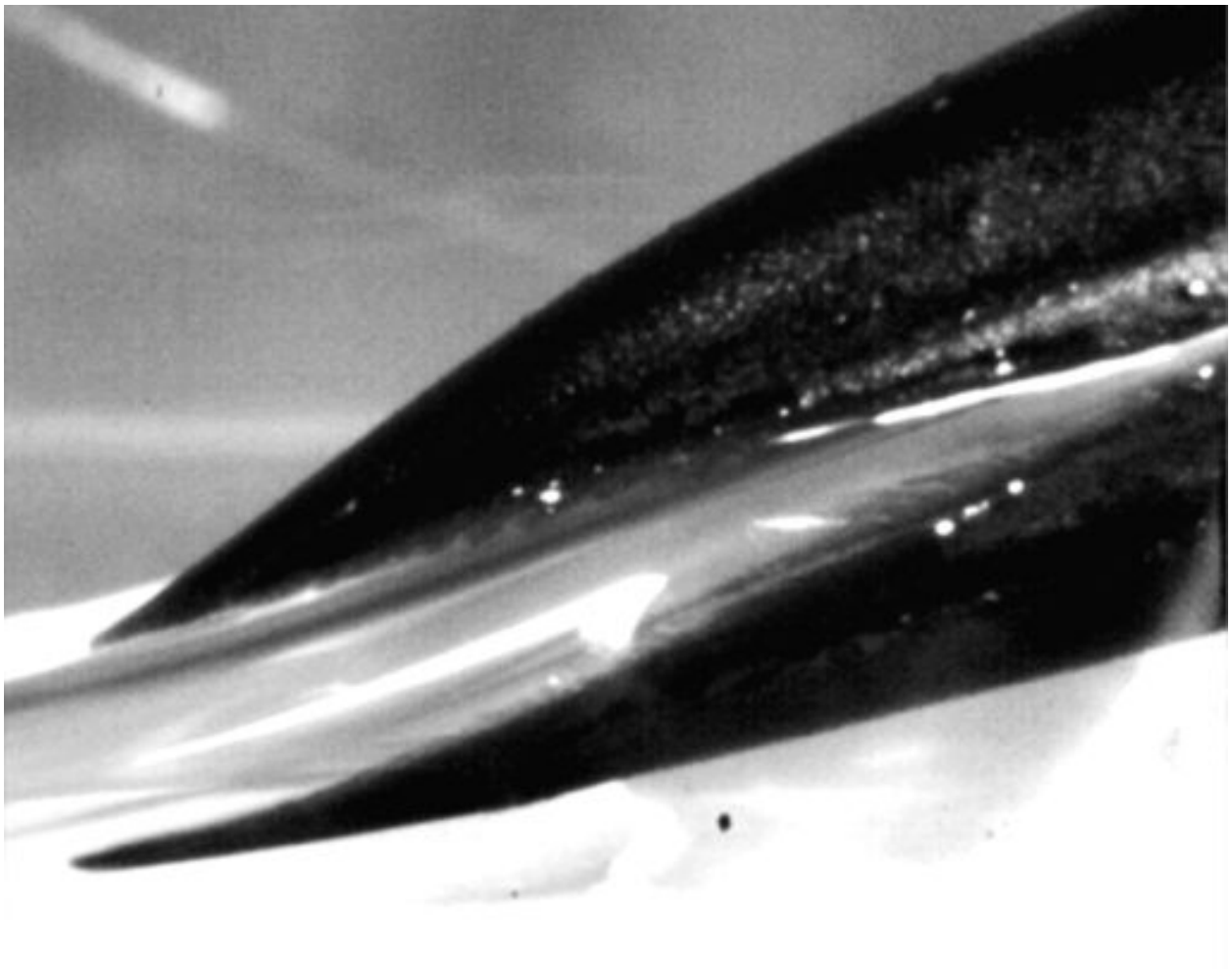


**Figure 3. Still picture taken from a high-speed video of a Short-tailed Woodstar (*Myrmia micrura)* drinking artificial nectar.** Serrations are conspicuous along both the mandibular and maxillary tomia, but larger in the former and more distal in the latter. It is important to point out that the serrations are extensions of the flexible tomia, and move pivoting on their base, molding to the tongue, and always keeping a close contact between the surfaces (*e.g.* Movie S1)

In the dorso-ventrally thickened region the mandibular rami fuse (Fig. 4) and the ventral rhamphotheca forms a downward pointing keel, the gonys (*cf.* Fig. 49 in Baldwin *et al.* 1931, but see Proctor and Lynch, p. 65). In some birds, the gonys appears as a dorso-ventrally thickened region (*e.g.* gulls) probably because of structural reinforcement for the fusion of the rami (pers. obs.), but in hummingbirds the mandibular rhamphotheca also thickens internally in this region (Figs. 2 and 5).

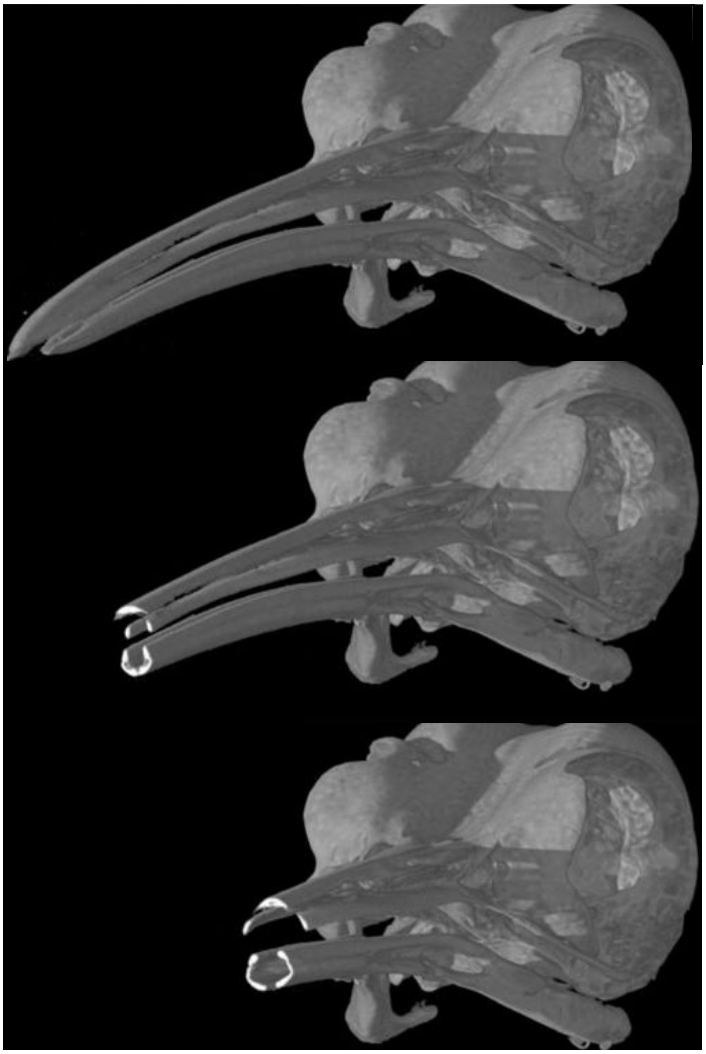


**Figure 4. MicroCT rendering (rostro-cranial coronal cross sectioning) of the bill of a Ruby-throated Hummingbird (*Archilochus colubris*).** Only the bony components of the skull are shown. Note how the mandibular rami, cross-sectionally concave, form a dorso-ventrally flattened mandible cranially (bottom). Rostrally however, the rami become less concave and form a laterally compressed mandible at the symphysis. A similar transition occurs in the maxilla between the dorsal and ventral bars (bone names *sensu* Zusi 2013).

We found that the mandibular tomia thin cranio-rostrally before the maxillary tomia (Fig. 5), in other words, near the mandibular prong the mandibular tomia are thinner than the maxillary tomia, and the latter thin more distally around the maxillary prong (Figs. 5 and 6).

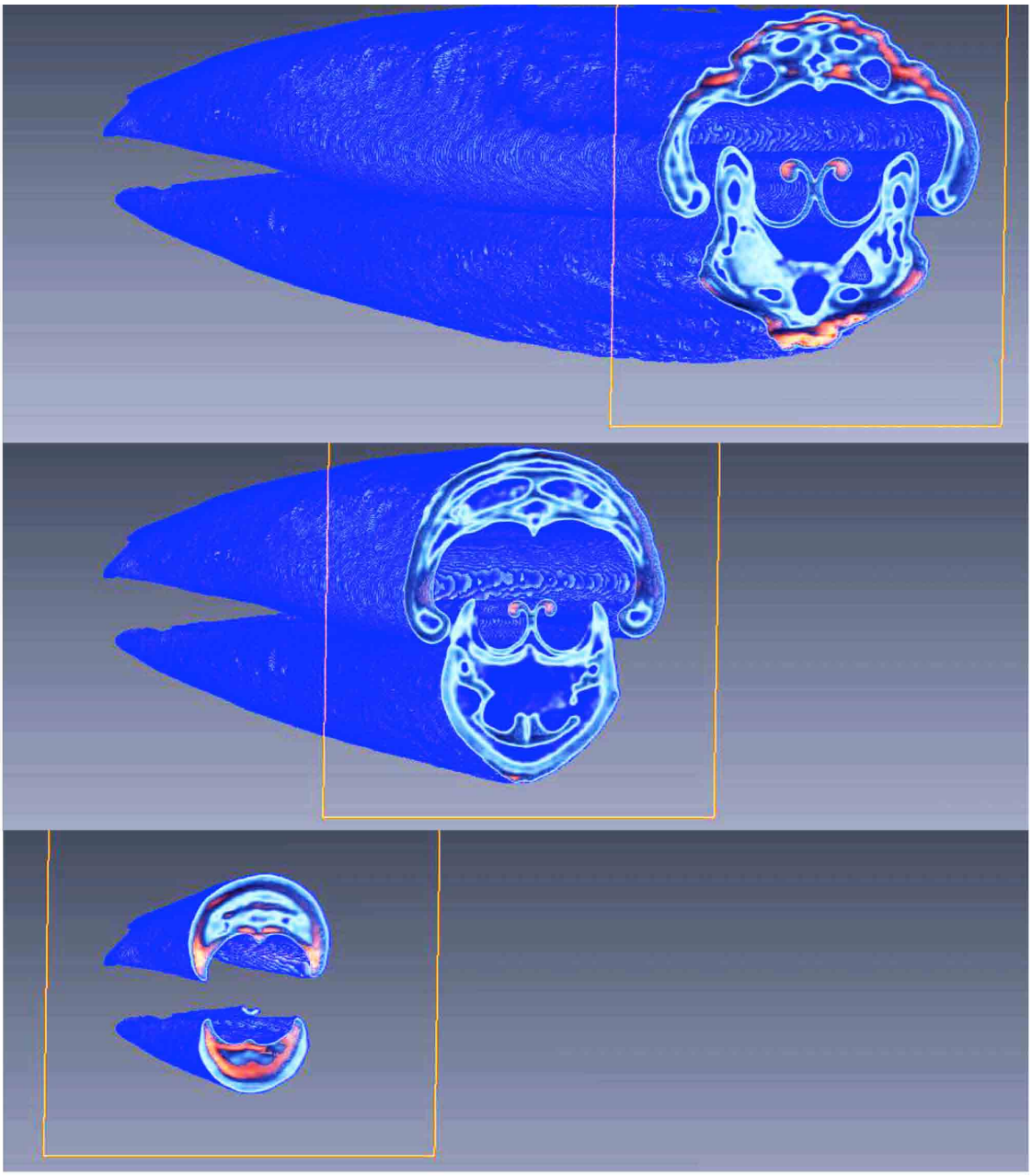


**Figure 5. MicroCT rendering (cranio-rostral coronal cross sectioning) of the bill of a Ruby-throated Hummingbird (*Archilochus colubris*).** On top the cross section is located at the proximal depression and a space below the tongue is noticeable. In the middle, this cross section near the mandibular prong shows how the space below the tongue has disappeared and the match between tongue and internal mandibular shape is much tighter. At the bottom the section shows the concave shapes at the bill tip that match the tongue tip shape (two parallel tube like grooves).

Because the mandible fits inside the maxilla along almost the entire length of the bill (*cf.* Fig. 127b, Böker 1937), the mandibular walls are clearly in close contact with the tongue (except from the top side). Thus, it is not surprising that structures enhancing the extrusion process are bigger and more proximally located (*i.e.* prong), unique (*i.e.* depressions), and more extended (*i.e.* flexible tomia) in the mandible when compared to the maxilla. We observed that the internal space for the tongue inside the bill starts to form a funnel distally after the proximal depression (Fig. 2). The internal bill space that is up to that point dorso-ventrally flattened becomes laterally flattened distally (Fig. 4).

The mandibular internal "tongue space" is further reduced near the bill tip, and the mandibular tomia and prong match the tongue shape like pieces of a puzzle (Fig. 5). At this location the mandibular serrated tomia are thin and flexible and are the last point of contact with the ventral sides of the tongue (Fig. 3). Slightly more distally, the maxillary tomia become thinner, allowing another close match between the tongue shape and the maxillary prong in the middle of the two grooves (Fig. 6). The serrated and flexible maxillary tomia are the last point of contact with the dorsal sides of the tongue during protrusion (Fig. 3, Movie S1). We found salivary ducts openings (*cf.* Scharnke 1931, Weymouth *et al.* 1964, Fig. 6) at the base of the mandibular prong; saliva near the bill tip could help with tongue lubrication, or even antibacterial coating (*cf.* Marcotte and Lavoie 1998). In addition, hummingbirds sometimes use their tongues to pick up dry particles of calcium-rich compounds (*e.g.* Graves 2007, Estades *et al.* 2008, Hickman *et al.* 2012, Zusi 2013) and they may use saliva to dilute and collect those particles.

We found that the bill structures we describe here are made entirely of keratin, and there are no signs of them in the underlying bone, which ends about 1 mm behind the rhamphothecal bill tips (Fig. S3). This result is in agreement to what Zusi (2013) found for *Androdon*, that its enlarged tomial serrations and distal hook are not reflected in the underlying bone.

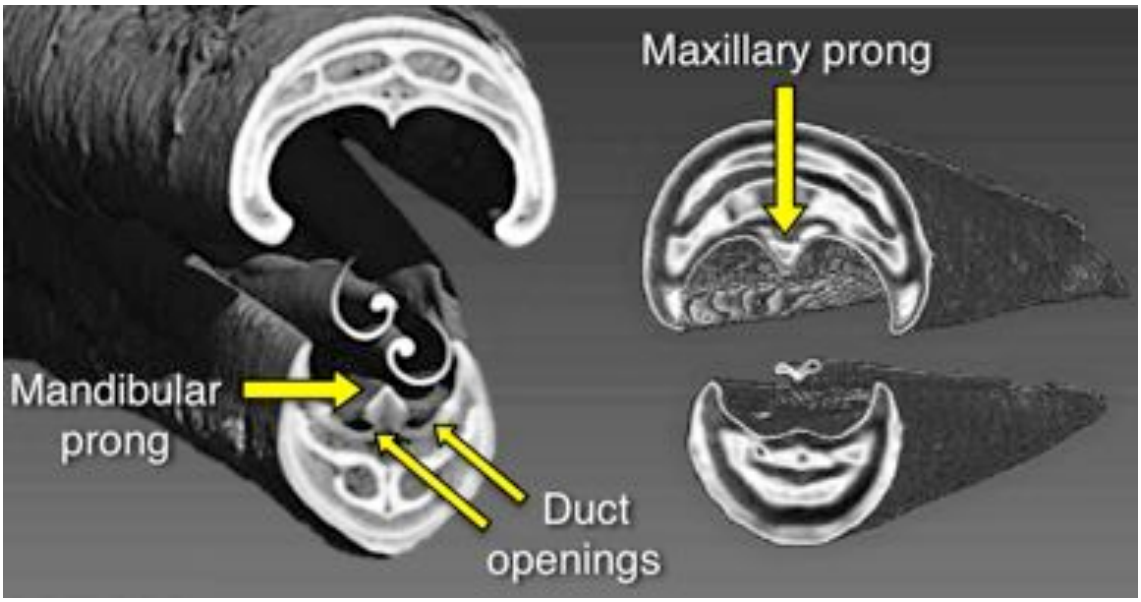



**Figure 6. MicroCT rendering (rostro-cranial [left] and cranio-rostral coronal [right] cross sectioning) of the bill of a Ruby-throated Hummingbird (*Archilochus colubris*).** The cross section on the left is located at the mandibular prong. The thin mandibular tomia are visible as well as the paired basins at the bottom of the mandibular prong with the duct openings at their bottom. The cross section on the right is located at the maxillary prong and the change in thickness (left *vs.* right) along the maxillary tomia is manifest.

**Discussion**

Our results document the existence of a previously unknown set of structures in the hummingbird bill, and demonstrate that these structures are essentially ubiquitous in hummingbirds; we found only 3 cases out of 157 species examined in which there was no sign of prongs (or they were rudimentary).

The small size, and interior position under the tongue helps explain how these prongs have gone undiscovered through over two centuries of anatomical studies of hummingbirds (several species described in the 18th century, *cf.* Schuchmann 1999). The universal appearance of prongs in hummingbird bills indicates that they must serve some important function. There is a strong association of prongs and basins with inrolled tomia; in both sexes these character states are associated with tomia exhibiting class B serrations (Tables 3-5). This association/correlation is especially strong in genera in the more derived clades of hummingbirds (see Supplementary character state trees), which suggests that prongs, basins, inrolled tomia and class B serrations are functionally related. We propose that this suite of structures enhances the removal of nectar from the tongue, and its release into the bill. We suggest that suction (but applied through the bill instead of through the tongue) is used, after release of nectar from the tongue, for moving nectar to the gullet. Our model is detailed below.

*Hypothesis for the functioning of bill structures: the squeezer device*

When hummingbirds are extracting nectar they slightly separate maxilla and mandible at the bill tip with coordinated movements, keeping a larger gap during tongue retraction (full nectar load), and a smaller gap during tongue protrusion. Ewald and Williams (1982) showed that the tongue is fully compressed dorsoventrally (by the bill tips) and laterally (by the sides of the bill) during protrusion. The inrolled tomia and class B serrations of the squeezer device are located in this area of lateral compression. In the first paper of this series (Stiles and Rico-Guevara unpub. manusc.), we proposed that these forward-directed serrations operate during tongue extrusion; here we explain in more detail the possible functioning of each piece of the puzzle.

Forward serrations and flexible tomia:

Since the lamellae on each of the tongue tips assume a conical form as a means of retaining the nectar collected inside flowers (Rico-Guevara and Rubega 2011), these two cones must be re-opened in order to release the nectar inside the bill. Upon tongue extrusion, forward serrations "comb" the surface of the tongue tips, breaking the adhesion between the tongue lamellae and nectar, thus releasing it onto the bill basins. Serrated tomia provide the traction needed to bend each lamella backwards, releasing the nectar that is held by the fimbriated tongue tip. The serrations should be deflected inwards when they contact the tongue in order to offer the best traction possible without damaging the tongue itself, hence their forward orientation.

By the same token, we hypothesize that flexible tomia function to orient the serrations to keep them in contact with the tongue long enough to release the nectar inside the bill, and to serve as wipers that empty the tongue during protrusion (Movie S1). This hypothesis may also explain why serrations are lower (preventing damage) but more widely distributed (more contact with the tongue) on the mandible, and conversely larger (separating the lamellae) in the maxilla. The lesser degree of bill bending (smaller opening) during protrusion may permit the tomia to inroll more strongly during tongue extrusion, producing greater lateral compression, however rhynchokinesis in hummingbirds is still controversial (see below).

Mandibular and maxillary prongs:

In all cases the mandibular prong was larger and more proximal in location than the maxillary projection. We hypothesize that prongs serve to separate the tongue tips and orient them in the right position (avoiding twists) to be extruded past the tomia and their serrations.

Mandibular basins:

We hypothesize that the mandibular depressions function as a holding area, to collect the nectar while it is squeezed from the tongue, prior to transport up the bill. Mandibular depressions thus function as transitory nectar reservoirs, allowing sufficient amounts of fluid to accumulate in order to permit a suction mechanism to operate while the tongue is being extruded. A relevant characteristic of the paired depressions (Fig. 1f) is that they have duct openings at the bottom that seem to connect to paired salivary glands at the bill base (*cf.* Scharnke 1931, Weymouth *et al.* 1964). The functional significance (possibly lubrication of the squeezer device) of such glands and duct openings near the bill tip needs further study.

*Hypothesis for nectar transport from the bill tip to the gullet*

More than seven decades ago, several hypotheses were conceived (varying slightly from one another) suggesting that capillarity fills the distal portion of the tongue, and that upon tongue protrusion the interaction between the tongue and palate creates a pumping mechanism that moves the nectar towards the bill base (review in Böker 1937). On the basis of our morphological study, we suggest that the squeezer device, in conjunction with general bill and throat movements during nectar feeding, could shed light into this intra-oral transport mechanism. Kingsolver and Daniel (1983) argued that since hummingbirds' tongues consist of open grooves which cannot sustain a pressure differential (*cf.* Weymouth *et al.* 1964, Hainsworth 1973), suction feeding cannot occur and proposed that movement of nectar results from the action of capillarity alone. However, Ewald and Williams (1982) noticed a bulge below the bill as the tongue was being extended that suggested that suction might occur.

Here we propose a new idea about how hummingbirds may transport nectar up their bills by focusing our model on the squeezing mechanism, and taking into account the potential importance of bill base bending capabilities recently demonstrated in hummingbirds (Yanega and Rubega 2004).

In the bill's middle region, the maxilla and mandible fit tightly together (Fig. 127b, Böker 1937). The outside surfaces of the mandibular tomia possess long furrows, into which the maxillary tomia fits, and in the maxilla two palatal ridges match with the inner surfaces of the fitted mandibular tomia, thus doubly sealing the bill from the tip to its base (pers. obs.). We suggest that the increased bill compression by the inrolled tomia at the bill tip, in conjunction with the tightly sealed middle portion of the bill, functions to create a sealed passage that facilitates nectar transport from the mandibular depressions to the bill base. In long-billed species it is noticeable that the mandibular tomia from the base to the inrolled area are less melanized (and presumably more flexible) than in shorter-billed birds, thus increasing the effectiveness of the seal. A pressure drop could potentially be created at the bill base by intramandibular flexion separating the rami laterally (Yanega and Rubega 2004), and by expansion of the throat itself through hyoid depression, as observed by Ewald and Williams (1982).

Following this line of reasoning, the transport mechanism from the bill tip to the throat would have two main components. First, the squeezer device would generate a "nectar pressure wave" inside bill, moving the fluid accumulated in the mandibular basins back by the compression of the liquid inside.

Since the nectar is being retained inside the bill, at every tongue stroke, its accumulation near the tip will push nectar remaining in the middle region towards the bill base. The second component would be a suction mechanism driven by movements of the remarkably flexible mandibular base. Close observation of the bill's base during foraging bouts revealed a slight separation between jaws (without creating separation of the edges); the maxilla and mandible separate only near the base, keeping the middle region tightly closed (Rico-Guevara *et al.* unpub. manusc.). This could be performed only by a highly flexible structure with modified movements, since in rigid bills even a small aperture near the base would cause a wide gap in the middle region. It is likely that the bending mechanism described by Yanega and Rubega (2004) and Smith *et al.* (2011), used during aerial prey catching, is involved in this movement.

A long tightly closed middle region and a mobile mandibular base could make possible a suction mechanism. The complex musculature at the base of the mandible and in the throat region described by Weymouth *et al.* (1964) and Zusi and Bentz (1984) could operate such a mechanism, permitting expansion and contraction of the bill base and throat. When sufficient fluid fills the nectar reservoirs, a vacuum generated by the separation of the mandibular rami could suck the liquid thorough the sealed middle region to the throat to be swallowed. In this conception of nectar transport, the unfitness of the tongue grooves to support a pressure differential is immaterial, since the pressure differential is being applied across the closed interior of the whole beak, and the nectar flowing from areas of high to low pressure has already been liberated from the tongue grooves.

We note that keeping the bill middle portion tightly closed would require a mechanism of distal rhynchokinesis to control the opening and closing of the bill tips in coordination with tongue movements. In a comparative morphological survey Zusi (1984) did not find osteological evidence supporting this kind of bill bending. However, close inspection of high-speed film footage of feeding (Rico-Guevara 2011, Zusi 2013) confirmed that distal bill bending definitely occurs. The precise mechanism will probably be elucidated only by using high-speed videography under controlled conditions in conjunction with high magnification instruments, and performing flexural rigidity (Field *et al.* 2011) and finite element analyses (Soons *et al.* 2010, 2012) to determine differential stress magnitudes for different bill shapes and material properties.

*Variation in the squeezer device among the Trochilidae*

Each one of the bill's internal structures discussed above presents variation among the different genera of the family. The entire suite of structures comprising the squeezer device presented high character states (large prongs, deep basins, curled tomia, *cf.* Fig. S1) among the genera of the most derived clades of the phylogeny of McGuire *et al.* (2008, 2014), most of which have class B serrations. Several genera in various clades have unserrated (class C) tomia, but the degree of tomial inrolling (hence tomial flexibility) does not differ appreciably from that shown by the majority of genera with class B serrations (Appendix 1). Moreover, the occurrence of class B serrations may vary widely among individuals of any given species in these genera; we will show elsewhere that such variation likely results from such serrations being produced by wear of the tomia during tongue movements. Hence, given flexible tomia and high character states of prongs and basins, the lack of serrations in genera with class C tomia in these clades might not affect greatly the operation of the mechanisms we propose.

The genus with class C tomia that is most exceptional in this regard is *Eutoxeres*, which lacks prongs and basins altogether. In this genus the extreme curvature of the bill implies that when the bird is perching with the head in normal (level) position, the distal part of the bill points downward, which would challenge both the squeezing and distal rhynchokinesis mechanisms. However, the distal tomia are moderately inrolled (category 2) and this genus has the widest commissure relative to its body mass of any hummingbird (unpubl. data); thus, it might have an exceptionally powerful suction mechanism. Moreover, when feeding from many very curved flowers (*e.g.* many *Heliconia*), *E. aquila* perches below the flower and actually tilts its head backwards (unpubl. data), such that gravity may facilitate nectar transport within the bill. Thus, other aspects of its bill morphology and behavior could compensate for the apparent lack of high character states of the structures in its squeezer device. Clearly the mechanism of nectar ingestion in this genus merits further study.

The genera with class A tomia (high serrations) are especially problematic in this respect. These genera are exceptional in that most of them present sexual dimorphism in the orientation of the serrations (backwards in males, perpendicular or forward-directed in females, *cf.* Stiles and Rico-Guevara unpub. manusc.). Moreover, especially in genera in which males have backward-directed serrations, their distal maxillary tomia are stiffer (less inrolled) and the bill tip itself is more rigid and pointed (unpub. data). We have suggested that such sharp bill tips might function in male-male combat or courtship (Rico-Guevara and Araya-Salas *in rev.*, Stiles and Rico-Guevara unpub. manusc.), and modifications of the bill tip could impose trade-offs on the efficiency of the squeezing mechanism.

Females of most of these same genera have more inrolled tomia, such that their squeezer device may be less affected. It is noteworthy that there is much less sexual dimorphism in tomial inrolling on the mandible: males of these genera have more inrolled, and usually less serrated, mandibular tomia, which might compensate for the limitations imposed by highly serrated and stiffened maxillary tomia. However, the components of the squeezer device are present in especially low character states (*cf.* Fig. S1) in *Heliothryx* and *Androdon*, and nectar ingestion in these genera also would repay further study. Tongue morphology in these genera also remains unexamined. A detailed study of feeding performance in species with pronounced bill dimorphism (*e.g. Ramphodon naevius* or *Colibri coruscans*) would also be interesting to determine whether such dimorphism might affect (and to what extent) the efficiency of nectar ingestion.

*General implications for hummingbird foraging theory*

The serrations and the new internal structures found are at precisely the point at which the bifid tongue is extruded from the slightly open bill tip. We therefore suggest that these structures serve, in effect, as tongue-strainers or squeezers that help to release nectar from the tongue, grooves and brush-tip as the tongue is extruded for each successive lick. This arrangement assures that nectar taken up in one lick is not lost in the next. The prongs separate the bifid tongue tips, which after nectar collection are joined by liquid adhesion, and the depressions collect the nectar so that it reaches a sufficiently large quantity to be moved by a vacuum operating in the bill base.

Foraging time at flowers is often long due to the time required to take the nectar (Wolf *et al.* 1972); given the extreme energetic constraints under which hummingbirds operate, efficiency in transport of nectar in the bill plays an important role in minimizing foraging time.

This study demonstrates that hummingbird bills are more complex than we had ever thought. The hidden structures described here are important clues to advance our understanding of hummingbird feeding mechanisms. Despite hundred years of study, we still have much to learn regarding the function of hummingbird bill their functional significance.

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## Literature cited


BALDWIN, S. P., OBERHOLSER, H. C., & WORLEY, L. G. 1931. Measurements of birds (No. 17). Cleveland Museum of Natural History.

BLEIWEISS, R., J. A. W. KIRSCH AND J. C. MATHEUS. 1997. DNA hybridization evidence for the principal lineages of hummingbirds (Aves, Trochilidae). Mol. Biol. Evol. 14:325-343.

BÖKER, H. 1937. Einführung in die vergleichende biologische Anatomie der Wirbeltiere. Gustav Fischer, Jena. Vol. 1, xii, 228 p.

CHEKE, R. A. AND MANN, C. 2008. *Family Nectariniidae (Sunbirds)*. In: del Hoyo, Josep, Elliot, Andrew and Christie, David A., (eds.) Handbook of the birds of the world: Penduline-tits to Shrikes. Handbook of the Birds of the World, Volume 13. Lynx Edicions, Barcelona, Spain, pp. 196-243.

COLLINS, B. G. 2008. Nectar intake and foraging efficiency: responses of honeyeaters and hummingbirds to variations in floral environments. The Auk,125(3), 574-587.

ESTADES, C. F., VUKASOVIC, M. A., & TOMASEVIC, J. A. 2008. Giant Hummingbirds (*Patagona gigas*) ingest calcium-rich minerals. The Wilson Journal of Ornithology, 120(3):651-653.

EWALD, P. W. AND W. A. WILLIAMS. 1982. Function of the bill and tongue in nectar uptake by hummingbirds. Auk 99:573-576.

FAEGRI, K., & VAN DER PIJL, L. 1979. The principles of pollination ecology. Pergamon.

FIELD, D. J., LIN, S. C., BEN-ZVI, M., GOLDBOGEN, J. A., & SHADWICK, R. E. 2011. Convergent evolution driven by similar feeding mechanics in balaenopterid whales and pelicans. The Anatomical Record 294(8):1273-1282.

FLEMING, T. H., & MUCHHALA, N. 2008. Nectar‐feeding bird and bat niches in two worlds: pantropical comparisons of vertebrate pollination systems. Journal of Biogeography 35(5):764-780.

GASS, C. L. AND W. M. ROBERTS. 1992. The problem of temporal scale in optimization: three contrasting views of hummingbird visits to flowers. Amer. Nat. 140:839-853.

GRAVES, G. R. (2007). Jamaican hummingbirds ingest calcareous grit. *Journal of Caribbean Ornithology*, *20*, 56-57.

GUTIÉRREZ-ZAMORA, A. (2008). Las interacciones ecológicas y estructura de una comunidad altoandina de colibríes y flores en la cordillera oriental de Colombia. *Ornitol. Colomb*, *7*, 17-42.

HAINSWORTH, F. R. 1973. On the tongue of a hummingbird: its role in the rate and energetics of feeding. Comp. Biochem. Physiol. 46A:64-78.

HAINSWORTH, F. R. 1977. Foraging efficiency and parental care in *Colibri coruscans.* Condor 79:69-75.

HEYNEMAN, A. J. 1983. Optimal sugar concentrations of floral nectars: dependence on sugar intake efficiency and foraging costs. Oecologia 60:198-213.

HICKMAN, B. R., HARRIS, J. B. C., & JUIÑA, M. E. 2012. Apparent soil ingestion by female Esmeraldas woodstars (*Chaetocercus berlepschi*) in western Ecuador. Ornitologia Neotropical 23:335-340.

JARDINE, W. 1834. The naturalist's library, vol. 1: humming-birds. W. H. Lizars, Edinburgh.

KIM, W., PEAUDECERF, F., BALDWIN, M. W., & BUSH, J. W. 2012. The hummingbird's tongue: a self-assembling capillary syphon. Proceedings of the Royal Society B: Biological Sciences 279(1749):4990-4996.

KINGSOLVER, J. G. AND T. L. DANIEL. 1983. Mechanical determinants of nectar feeding strategy in hummingbirds: energetics, tongue morphology and licking. Oecologia 60:214-226.

KÖHLER, A., LESEIGNEUR, C. D., VERBURGT, L., & NICOLSON, S. W. 2010. Dilute bird nectars: viscosity constrains food intake by licking in a sunbird. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 299(4):R1068-R1074.

MADDISON, W. P. AND D. R. MADDISON. 2014 MESQUITE: a modular system for evolutionary analysis. Version 2.75 See http://mesquiteproject.org

MARCOTTE, H., & LAVOIE, M. C. 1998. Oral microbial ecology and the role of salivary immunoglobulin A. Microbiology and molecular biology reviews 62(1):71-109.

MCGUIRE, J. A., C. C. WITT, D. L. ALTSHULER AND J. V. REMSEN, JR. 2007. Phylogenetic systematics and biogeography of hummingbirds: Bayesian and maximum likelihood ananysis of partitioned data and the selection of an optimum partitioning strategy. Syst. Biol. 56:837-856.

MCGUIRE, J. A., C. C. WITT, J. V. REMSEN, JR., R. DUDLEY AND D. L. ALTSHULER. 2009. A higher-level taxonomy for hummingbirds. J. Ornithol. 150:155-165.

MCGUIRE, J. A., WITT, C. C., REMSEN JR, J. V., CORL, A., RABOSKY, D. L., ALTSHULER, D. L., & DUDLEY, R. (2014). Molecular phylogenetics and the diversification of hummingbirds. Current Biology 24(8):910-916.

MIELKE, P. W. JR., AND K. J. BERRY. 2001. Permutation methods: a distance function approach. Springer Series in Statistics. 344 pp.

MOYER, B. R., PETERSON, A. T., AND D. H. CLAYTON. 2002. Influence of bill shape on ectoparasite load in Western Scrub-Jays. Condor 104:675-678.

ORNELAS, J. F. 1994. Serrate tomia: an adaptation for nectar-robbing in hummingbirds? Auk 111:703-710.

PATON, D. C., & COLLINS, B. G. 1989. Bills and tongues of nectar-feeding birds: A review of morphology, function and performance, with intercontinental comparisons. Australian Journal of Ecology 14(4):473-506.

PROCTOR, N. S., & LYNCH, P. J. 1993. Manual of Ornithology: Avian Structure & Function. 1993. Yale University Press.

RICO-GUEVARA, A. 2005. Relaciones entre morfología y forrajeo de artrópodos en colibríes del bosque altoandino. Unpublished thesis, Universidad Nacional de Colombia, Bogotá.

RICO-GUEVARA, A. 2008. Morfología y forrajeo para buscar artrópodos por colibríes altoandinos. Ornitol. Colombiana 7:43-58.

RICO-GUEVARA, A. 2014. Morphology and Function of the Drinking Apparatus in Hummingbirds. Doctoral dissertation. University of Connecticut.

RICO-GUEVARA, A. AND M. RUBEGA. 2011. The hummingbird tongue is a fluid trap, not a capillary tube. Proc. Natnl. Acad. Sci. USA 108(23):9356-9360.

RICO-GUEVARA, A. AND M. ARAYA-SALAS. 2014. Bills as daggers? A test for sexually dimorphic weapons in a lekking hummingbird. Behavioral Ecology *In review*.

RIDGWAY, R. 1891. The humming birds. Annual report, U.S. Natnl. Mus. 1890: 253-283.

RIDGWAY, R. 1911. The birds of North and Middle America. Bull. U.S. Natnl. Mus. 50, part V.

ROBERTS, M. W. (1996). Hummingbirds' nectar concentration preferences at low volume: the importance of time scale. Animal Behaviour, 52(2), 361-370.

RODRÍGUEZ-FLORES, C. I., & STILES, F. G. (2005). Ánalisis ecomorfológico de una comunidad de colibríes ermitaños (Trochilidae, Phaethorninae) y sus flores en la amazonía colombiana. Ornitología Colombiana, 3, 7-27.

SALVIN, O. 1892. Catalogue of the Picariae in the collection of the British Museum: Upupae and Trochili. Longmans & Co.and British Museum (Natural History), London.

SCHARNKE, H. 1931. Beiträge zur Morphologie und Entwicklungsgeschichte der Zunge der Trochilidae, Meliphagidae und Picidae. Journal of Ornithology 79(4):425-491.

SCHUCHMANN, K.-L. 1995. Taxonomy and biology of the tooth-billed hummingbird (*Androdon aequatorialis*). Mitt. Zool. Mus. Berlin 71:109-113.

SCHUCHMANN, K.-L. 1999. Family Trochilidae (hummingbirds). Pp. 468-680 *in*: J. Del Hoyo, J. Sargatal and A. Elliott (eds.). Handbook of birds of the world, vol. 5. Lynx Edicions, Barcelona.

SKUTCH, A. F. 1954. Life histories of Central American birds, vol. 1. Pacific Coast Avifauna, no. 31.

SMITH, M. L., G. M. YANEGA AND A. RUINA. 2011. Elastic instability model of rapid beak closure in hummingbirds. J. Theor. Biol. 282:41-51.

SOONS, J., HERREL, A., GENBRUGGE, A., AERTS, P., PODOS, J., ADRIAENS, D., ... & DIRCKX, J. 2010. Mechanical stress, fracture risk and beak evolution in Darwin's ground finches (*Geospiza*). Philosophical Transactions of the Royal Society B: Biological Sciences, 365:1093-1098.

SOONS, J., HERREL, A., AERTS, P., & DIRCKX, J. 2012. Determination and validation of the elastic moduli of small and complex biological samples: bone and keratin in bird beaks. Journal of The Royal Society Interface 9:1381-1388.

STILES, F. G. 1980. The annual cycle in a tropical wet forest hummingbird community. Ibis 122:322-343.

STILES, F. G. 1981. Geographical aspects of bird-flower coevolution, with particular reference to Central America. Annals of the Missouri Botanical Garden 68(2):323-351.

STILES, F. G. 1985. Seasonal patterns and coevolution in the hummingbird-flower community of a Costa Rican subtropical forest. Ornithological Monographs 36: 757-787.

STILES, F. G. 1995. Behavioral, ecological and morphological correlates of foraging for arthropods by the hummingbirds of a tropical wet forest. Condor 97:853-878.

STILES, F. G. AND A. F. SKUTCH. 1989. A guide to the birds of Costa Rica. Cornell University Press, Ithaca, NY.

TEMELES, E. J., KOULOURIS, C. R., SANDER, S. E., & KRESS, W. J. 2009. Effect of flower shape and size on foraging performance and trade-offs in a tropical hummingbird. Ecology 90(5):1147-1161.

WEYMOUTH R.D., LASIEWSKI, R.C., BERGER, A. J. 1964. The tongue apparatus in hummingbirds. Acta Anatomica 58: 252-270.

WOLF, L. L., HAINSWORTH, F. R., & STILES, F. G. 1972. Energetics of foraging: rate and efficiency of nectar extraction by hummingbirds. Science 176(4041):1351-1352.

WOLF, L. L., F. G. STILES AND F. R. HAINSWORTH. 1976. The ecological organization of a tropical highland hummingbird community. J. Animal Ecol. 32:349-379.

YANEGA, G. M. AND M. A. RUBEGA. 2004. Feeding mechanisms: hummingbird jaw bends to aid insect capture. Nature 428:615.

ZUSI, R. L. 1984. A functional and evolutionary analysis of rhynchokinesis in birds. Smithsonian Institution Press.

ZUSI, R. L. 2013. Introduction to the Skeleton of Hummingbirds (Aves: Apodiformes, Trochilidae) in Functional and Phylogenetic Contexts. Ornithological Monographs, 77:1-94.

ZUSI, R. L., & BENTZ, G. D. 1984. Myology of the purple-throated carib (*Eulampis jugularis*) and other hummingbirds (Aves: Trochilidae). Smithsonian Institution Press.

**Supplementary Methods and Figures**

*Intergeneric variation in internal bill structures*

Prongs: We found that maxillary prongs are less widespread than mandibular prongs, and never found in the absence of mandibular prongs. We combined maxillary and mandibular prongs in a single categorical classification system that characterizes the state of both structures (Fig. S1a). Our categories are:

0 (prongless) = prongs absent above and below, both maxillary and mandibular keels smooth, without projections;

1 (prong below, but not above) = maxillary keel smooth, mandibular keel with a small (projecting up to halfway from the keel to the tomium, or less) but distinct prong;

2 (incompletely pronged) = maxillary keel pronounced, sometimes with a rudimentary prong, mandibular prong larger (extending more than half the distance to the tomium) but not projecting above the tomium in side view;

3 (partially pronged) = a distinct but small maxillary prong (Fig. 1b) projecting up to halfway from the keel to the tomium, the mandibular prong larger and reaching or barely exceeding the level of the tomium and visible from the side; and

4 (fully pronged) = maxillary prong larger, extending more than half the distance to the tomium, mandibular prong large (Fig. 1e) and distinctly projecting above the tomium in side view. We found no cases in which the maxillary prong was sufficiently large that it was visible in side view.

Basins: We combined the presence and size of the mandibular basins in a single categorical classification scheme (Fig. S1b). Our categories are:

0 (basinless) = no obvious depressions;

1 (proximal basin only) = a single broad, shallow medial basin, located in the area near the tip where the bill appears thicker in side view (Fig. 1a, d, proximal basin), posterior to the mandibular prong and ending posterior to where the mandibular tomia get thinner and start to inroll;

2 (proximal and paired basins) = three basins present, the proximal basin, plus two smaller, shallower ones anterior to it, and just posterior to the base of the mandibular prong; in some specimens a duct opening at the bottom of each of the two shallow depressions was noticeable (paired basins, Fig. 1f);

3 (four small basins) = four depressions, including a fourth shallow depression distal to the mandibular prong (distal basin, Fig. 1e), usually present when a maxillary prong was present, and located below it, and generally close to the distal margin of the inrolled area of the tomia; and

4 (four large basins) = four large depressions present on the mandibular floor, the distal basin deeper and extending far anterior to the mandibular prong. We never found distinctive depressions in the surface of the maxillary palate.

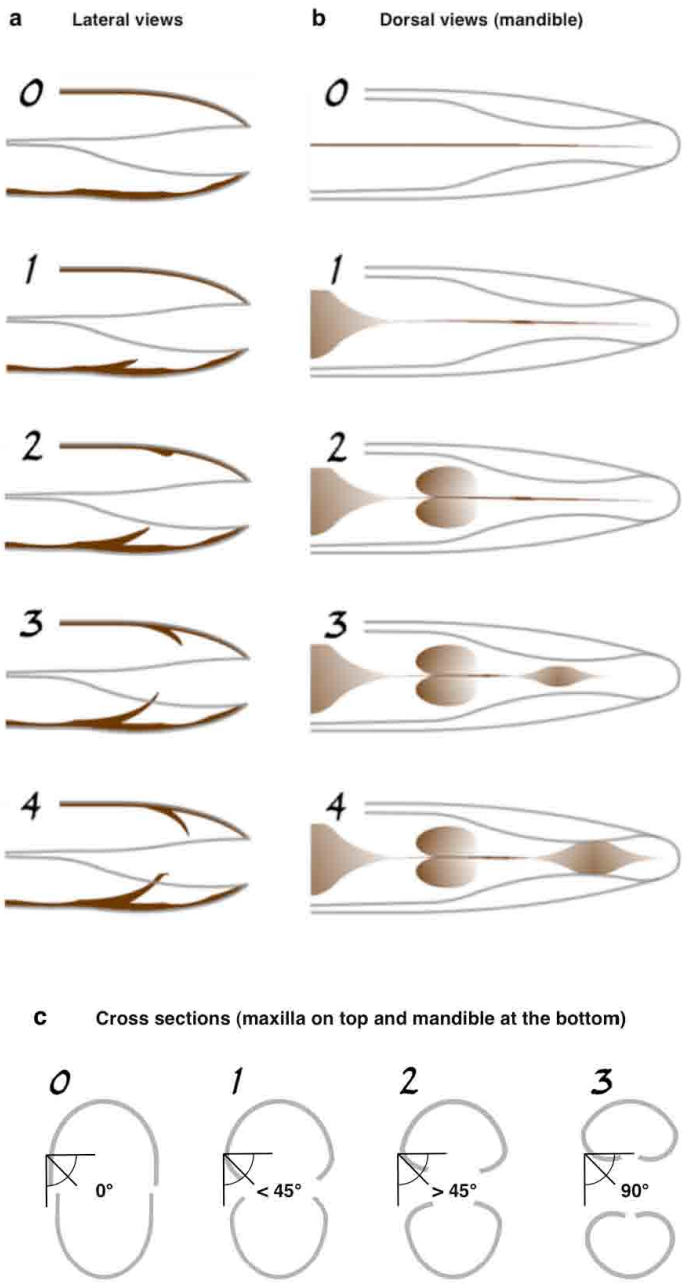



**Figure S1. Classification system for the character states of the squeezer device structures. a)** Schematics of lateral views of hummingbird bill tips representative of each one of the prong categories defined in the Supp. Methods text. **b)** Schematics of dorsal views of hummingbird mandible tips representative of each one of the basin categories defined in the Supp. Methods text. **c)** Cross-sectional schematics of hummingbird bill tips representative of each one of tomial curvature categories defined in the Supp. Methods text.

Tomial curvature: We categorically classified the degree of deflection (inrolling) of the tomia just proximal to the bill tip (the area where class B serrations occur, *cf.* Stiles and Rico-Guevara unpub. manusc.) as follows (Fig. S1c):

0 = tomia not inrolled: when present, tomial serrations projecting perpendicularly from the palatal surface of the maxilla (pointing straight down) or mandible (pointing straight up);

1 = slightly inrolled: serrations projecting less than 45º medially (inwards) from the perpendicular to the palatal surface;

2 = moderately inrolled: serrations projecting between 45º and 90º inwards from the perpendicular to the palatal surface and difficult to see from the side; and

3 = tomia strongly inrolled: the serrations projecting inwards 90º or more from the perpendicular and invisible from the side. The degree of inrolling may be an indicator of the flexibility of a given tomium: stiff tomia (in living birds) will show little or no inrolling (when they become museum specimens) while flexible tomia may show slight to strong inrolling.

Sometimes within a genus, we observed categorical overlapping, for instance: Class 2 means that all individuals of the genus observed had tomia inrolled at least 45º; class 2-3 indicates that no individual seen had less than 45º of inrolling and some individuals or species had tomia inrolled 90º or more. Class 3 includes those genera in which all individuals of all species observed had tomia inrolled 90º or more (Appendix 1).

We considered only adult individuals (*i.e.*, without corrugations on the maxillary rhamphotheca and not showing obvious juvenile plumage) in all of these classifications to avoid biases reflecting ontogenetic changes. In juveniles (specimens with heavily corrugated maxillary rhamphotheca near the bill base) the tomia were always strongly inrolled, even in species in which adults show no inrolling (class 0). The ontogeny of these structures is outside the scope of this study, so we excluded juveniles from the analysis.

In many genera, the state of these structures varied somewhat among species and even among individuals within species. However, such variation was usually limited; hence, in Appendix 1 we have included such variation as transitions between categories *e.g.*, 2-3 where some species or individuals were classified in category 2 and others in category 3. For purposes of comparisons, we used the higher state-category for each genus (*e.g.*, category 3 for the example above).

We also excluded adult specimens that were "molting" the internal structures; in our previous study we had found a correlation of a white (not yet melanized) replacement line of the tomia and the molt of the hummingbirds studied. In the present study we found that some specimens had white translucent small prongs (Fig. S2a) or white basins (Fig. S2b), which were conspicuously different from the character states typical of the genus. We hypothesize that these internal structures are replaced annually with the molt of the rhamphotheca.

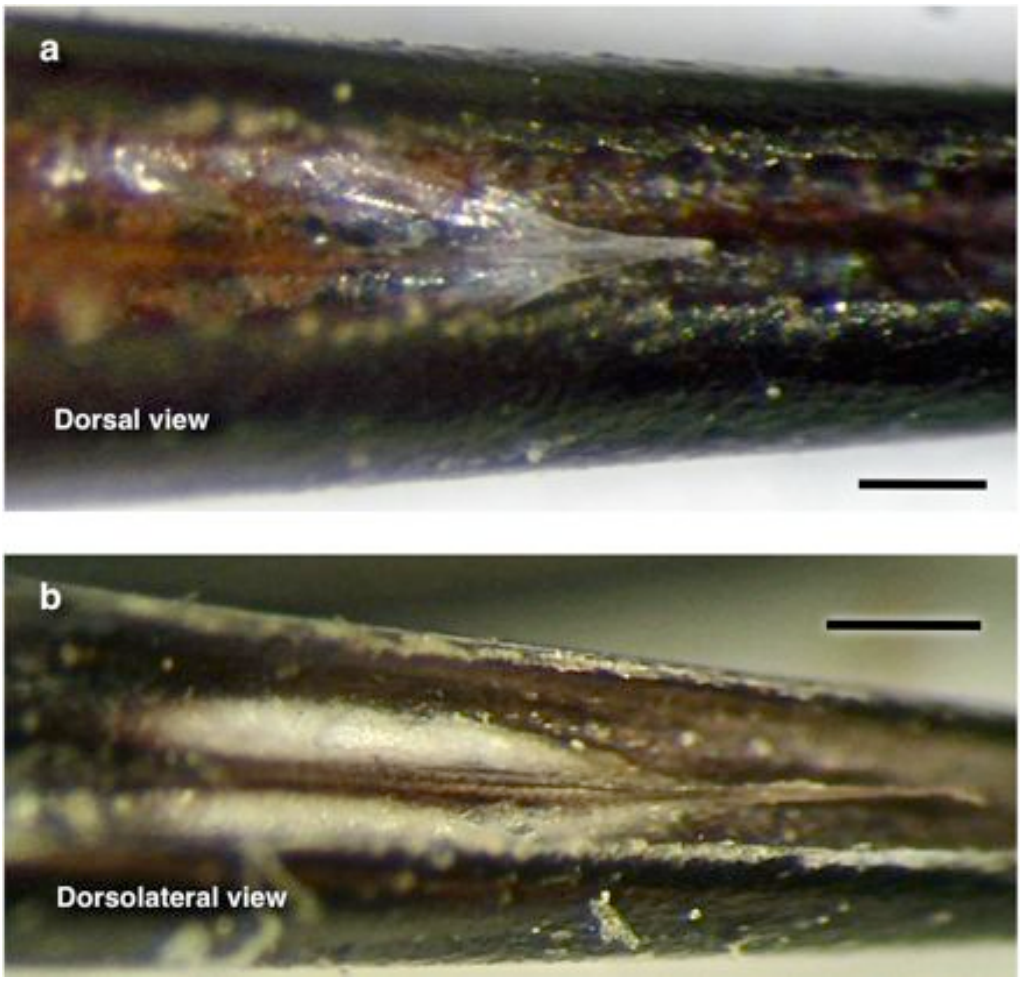



**Figure S2. Replacement of the squeezer device structures.** (a) This considerable smaller projection (in comparison to conspecifics) of a green violetear (*Colibri thalassinus*) suggest that the specimen was collected when the projection was in growing, since this is an adult individual and we have observed that the projection appears even in juveniles, we consider that this is an instance of replacement of the structure. This inference is supported by the fact that the structure is unmelanized whilst fully grown structures are melanized. (b) Different case of inferred replacement in a specimen of a glowing puffleg (*Eriocnemis vestita*). In this case the "replacement zone" is now in the area of the paired basins. Unlabeled scale bars are 0.5 mm.

Many of the same trends were evident in the distribution of strongly inrolled distal tomia: 94% of the species with class B tomia showed strongly inrolled tomia, as also did 77% of those with class C tomia. By contrast, 90% of species with class A tomia showed at most weakly inrolled tomia (Table S1), a highly significant difference ($G = 39.8$, $p<0.001$, $df = 1$, combining tomial classes B and C and inrolling categories 0 and 1 vs. 2 and 3). The same tendency occurred in mandibular tomia but was less marked ($G = 11.8$, $p<0.001$, $df = 1$).

We also found that sexual dimorphism in maxillary tomial inrolling differed according to tomial class: little or none in genera with classes B or C tomia but in a majority of those with class A tomia, inrolling was most pronounced in females (Table S2), again a highly significant difference ($G = 54.27$, $p<0.001$, $df = 1$, combining tomial classes B and C and adding the two cases of males with more strongly inrolled tomia to the "no dimorphism" category). It is noteworthy that in all genera of tomial class A in which females had more strongly inrolled tomia, there was also dimorphism in tomial serrations: males had backward-directed serrations whereas females had serrations directed perpendicularly or forwards. In such genera, the tomia of males appeared stiffer and the bills more sharply pointed as well (Rico-Guevara and Araya-Salas in rev.). These are also the genera in which the inrolling of the mandibular tomia is more pronounced than that of the maxillary tomia inrolling, especially in males (Appendix 1).

The size and incidence of prongs is strongly correlated with that of basins (Spearman rank correlation $r_s = 0.606$ with correction for tied ranks, $p << 0.001$): genera with prongs of high character states usually had basins of high character states as well (Table S3). There is also a relationship between the character states of prongs and basins and the maxillary tomial class (Appendix 1; see Stiles and Rico-Guevara unpub. manusc.). Virtually all (96%) of the 50 genera with class B tomia have both prongs and basins of medium to high character states (category 2 or higher for both prongs and basins). The only exceptions were *Oxypogon*, with only a rudimentary mandibular prong but basins of medium character states) and *Discosura* with the reverse condition of prongs of high character states but only a single shallow basin. By contrast, a considerable proportion (29%) of the genera with strongly serrated, class A tomia showed prongs, basins, or both, with low character states as did a few of the genera with unserrated, class C tomia (Table S4).

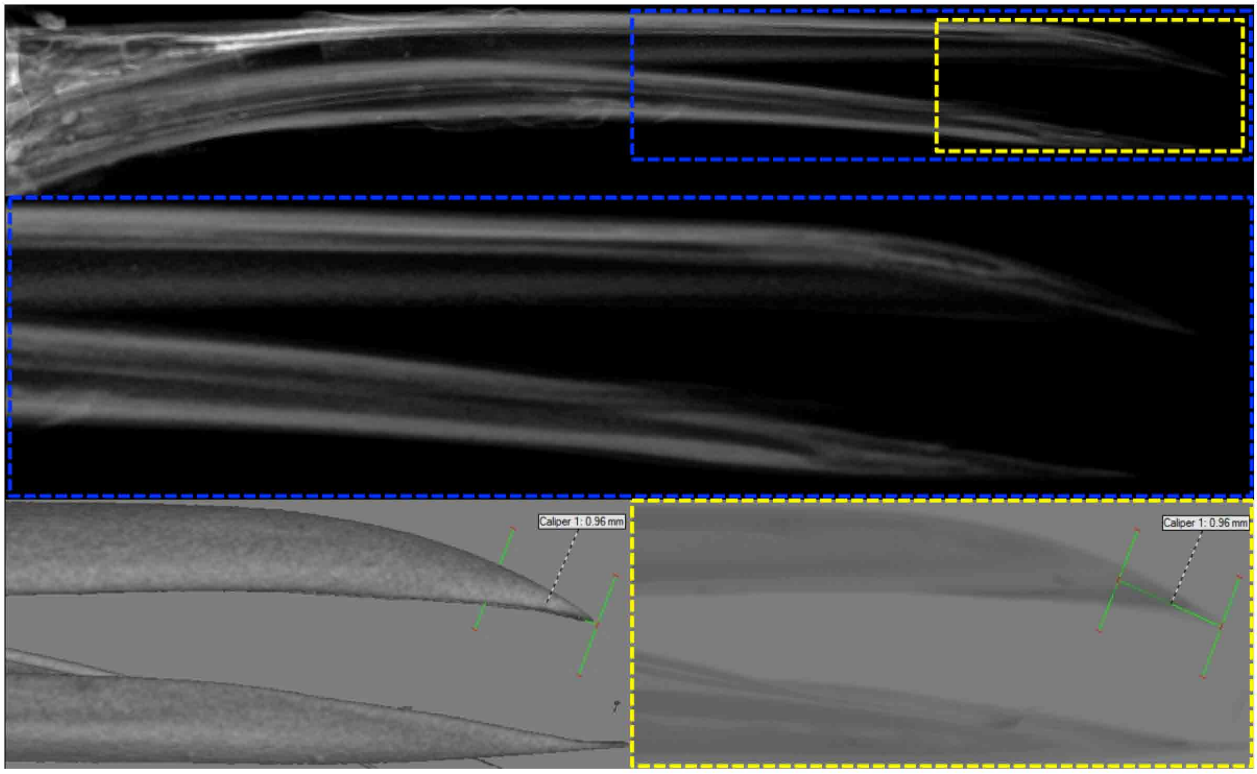



**Figure S3. MicroCT renderings (lateral view and longitudinal sections) of the bill of an Anna's Hummingbird (*Calypte anna*).** The upper image shows a general X-ray overview. The middle image is an X-ray view in which the different terminations of bone -maxilla and mandible- and maxillary and mandibular rhamphothecae are visible. The bottom right image shows a measurement of the difference between bone and rhamphotheca at the maxilla (~ 1 mm), and the bottom left image shows a three-dimensional rendering showing the same difference.

Given that we used an *a priori* classification of tomial classes (A, B, and C) to interpret and compare our results (Stiles and Rico-Guevara unpub. manusc.), we corroborate here the classification. We performed a nonmetric multidimensional scaling (PC-ORD version 5) as an ordination method to obtain a graphical representation of the relationships inside our nonnormal and discontinuous dataset (Fig. S4). The nonmetric multidimensional scaling graph (Axes 1 and 2 explained 86% of the variance) shows consistency among groups; averages of females of the only three species classified as class A overlap with species of class B, and two averages of females of class C species overlap with class B.

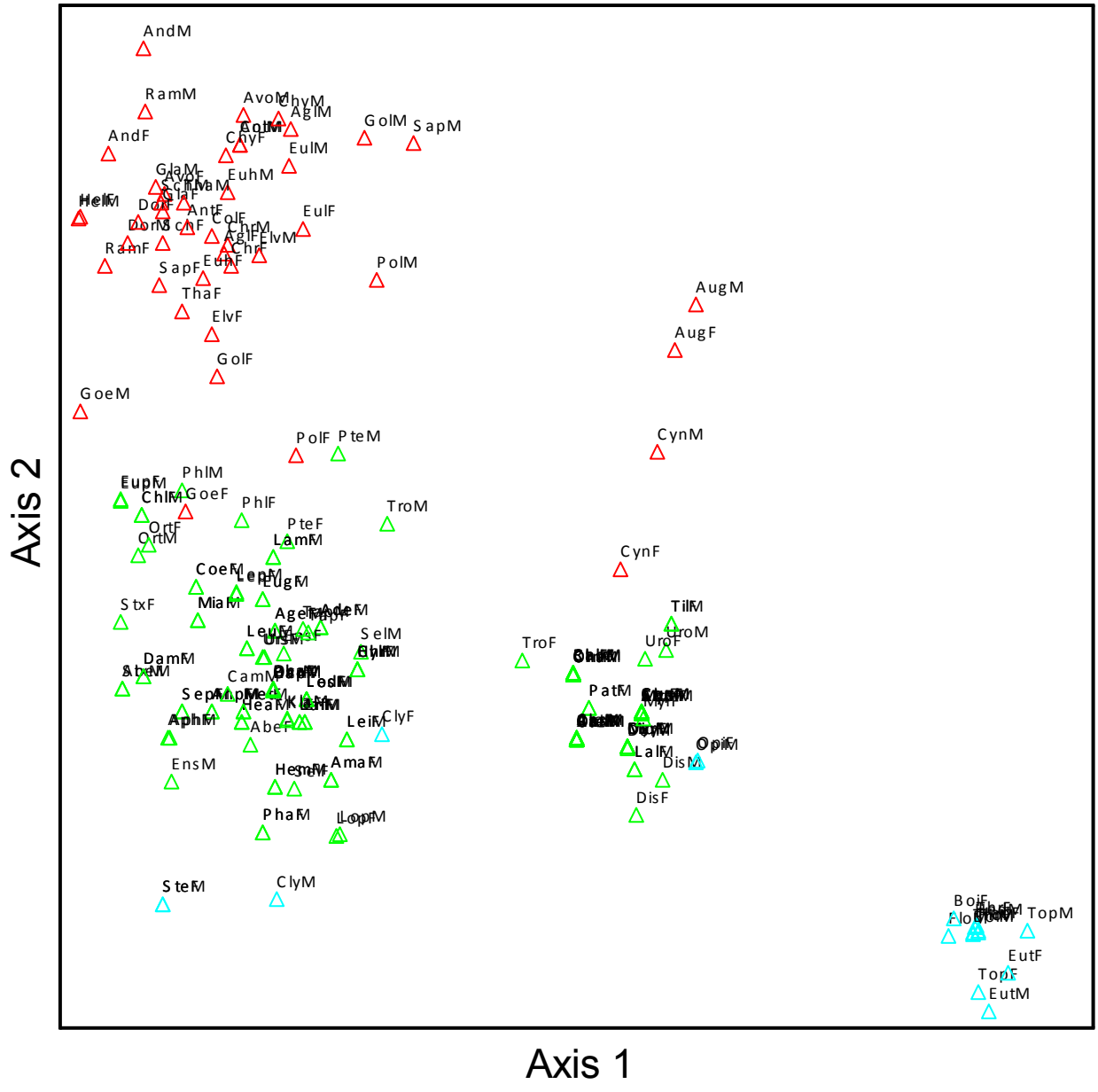



**Figure S4. Nonmetric multidimensional scaling graph.** The analysis was performed using Sørensen distance. Triangles represent averages discriminated by species and sex. The group at the top left (red) corresponds to tomial class A (large, sexually dimorphic [backwardly directed in males] serrations), the larger group at the bottom left (green) corresponds to the tomial class B (small forwardly directed serrations), and the smaller group at the bottom right (blue) corresponds to the tomial class C (no distinct serrations).

In order to test the hypothesis of no difference among the groups we defined, we used a multi-response permutation procedure (PC-ORD version 5); we obtained clear differences among groups ($T= -73.72$, $A= 0.28$, $p<0.001$). Together the nonmetric multidimensional scaling and the multi-response permutation procedure support the use of tomial classes to interpret and compare our results.

***Supplementary figures of hummingbird genera with bills presenting extreme morphology and unusual squeezer structures***

**Figure S5. Bill tip of a White-tipped Sicklebill (*Eutoxeres aquila*) specimen.** This photograph features a 4-mm section of the wringer area. Note the mostly smooth, inrolled, tomia with few small serrations, the small prong, and the developed basins.

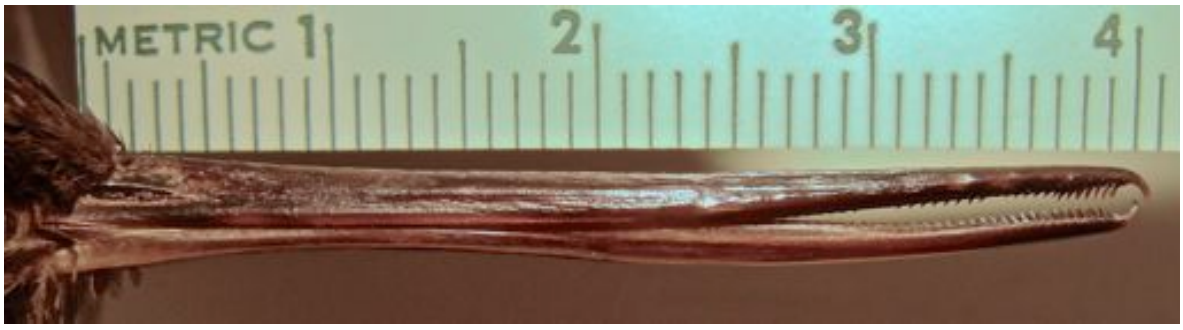



**Figure S6. Bill of a Tooth-billed Hummingbird (*Androdon aequatorialis*).** Note the extreme degree of serration development, and the downward curvature of the mandible (which usually matches the wringer) starting about 20 mm from the tip, a positioning unusual when compared to other species with less "bizarre" bills.

**Figure S7. Bill of a Purple-crowned Fairy (*Heliothryx barroti*).** Compressed laterally near the tip, this species' bill shows an unusual shape for the family. Similarly to *Androdon*, the downward mandibular curvature starts farther away from the tip than usual.

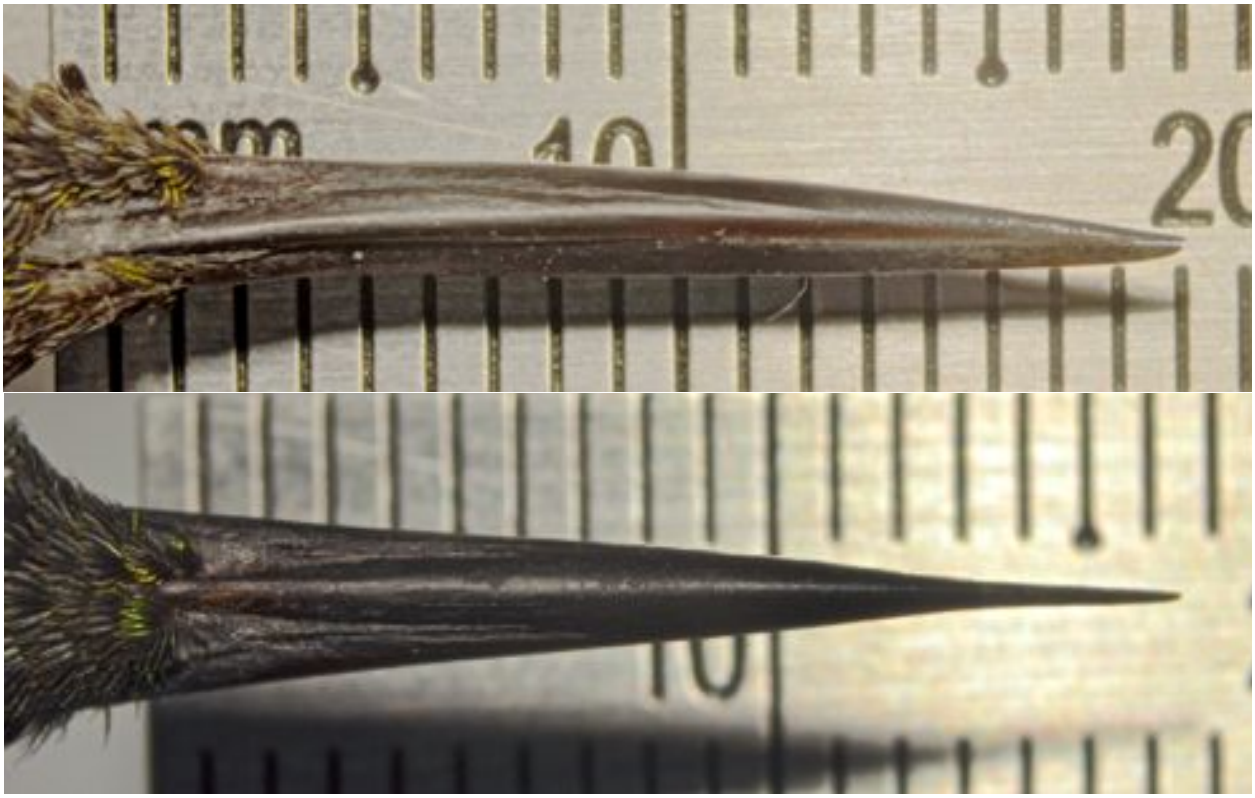
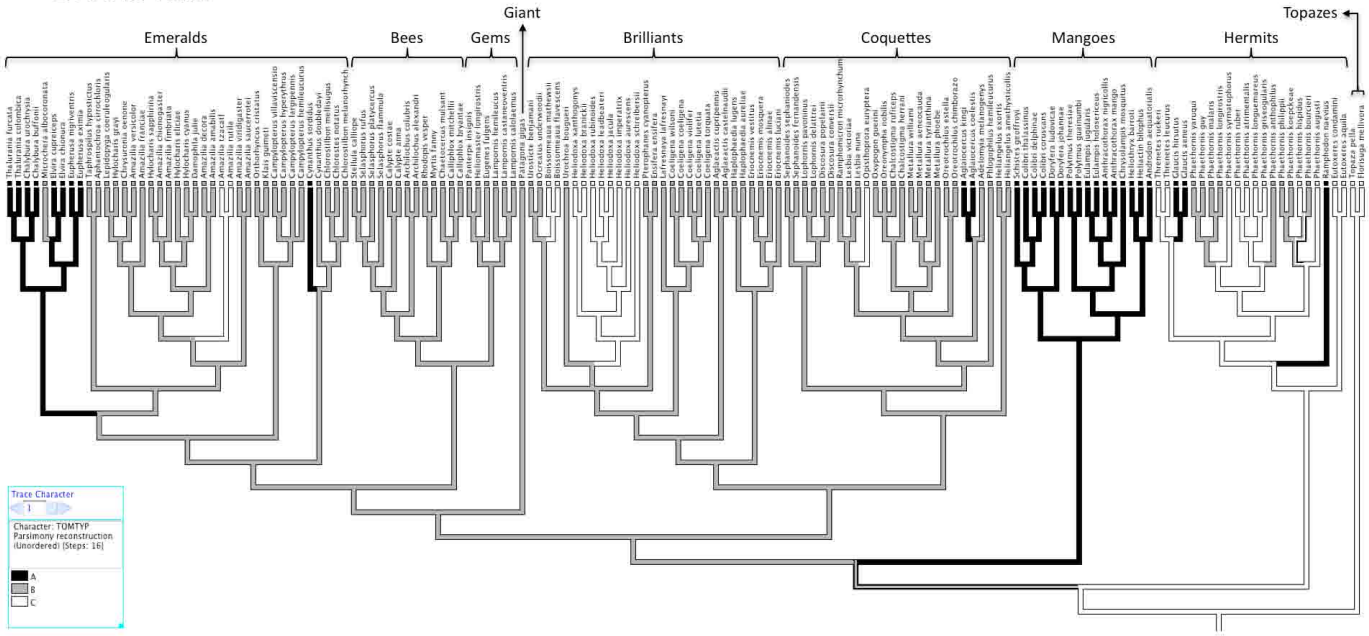


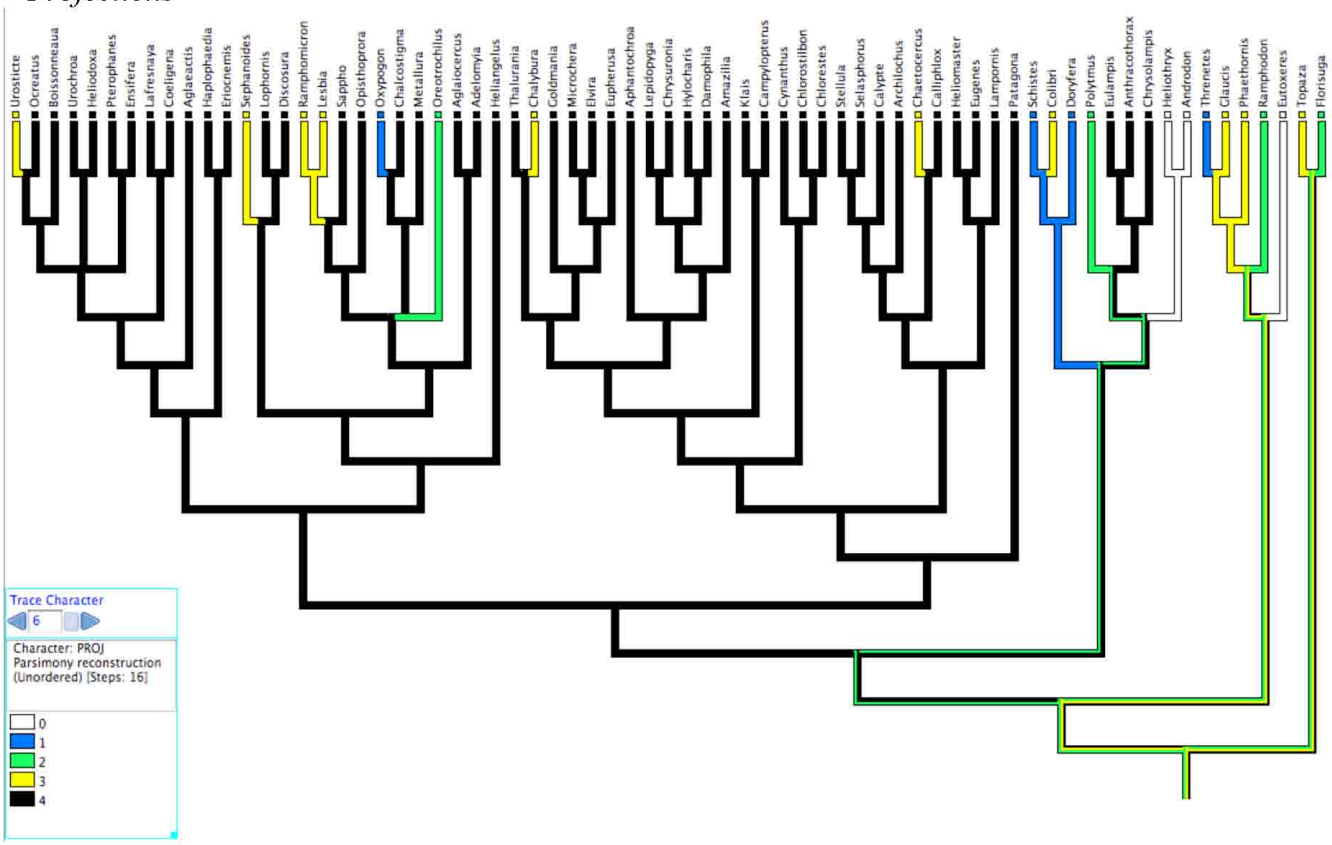
**Figure S8. Wedge-billed Hummingbird (*Schistes geoffroyi*).** Similar to *Heliothryx,* the bill of this species is compressed near the bill tip. In this case, there is not a clear downward curvature of the mandible, problably because starts to close to the bill base.

## Supplementary character states trees

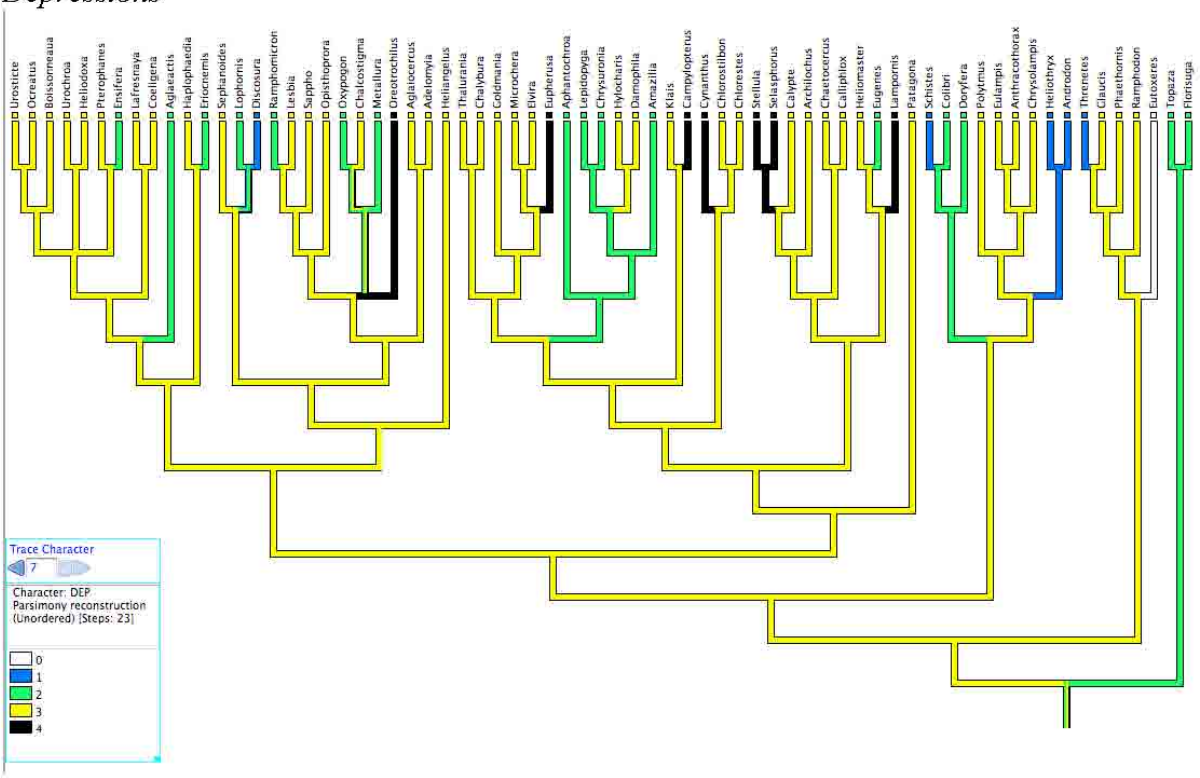
*Tomial class*



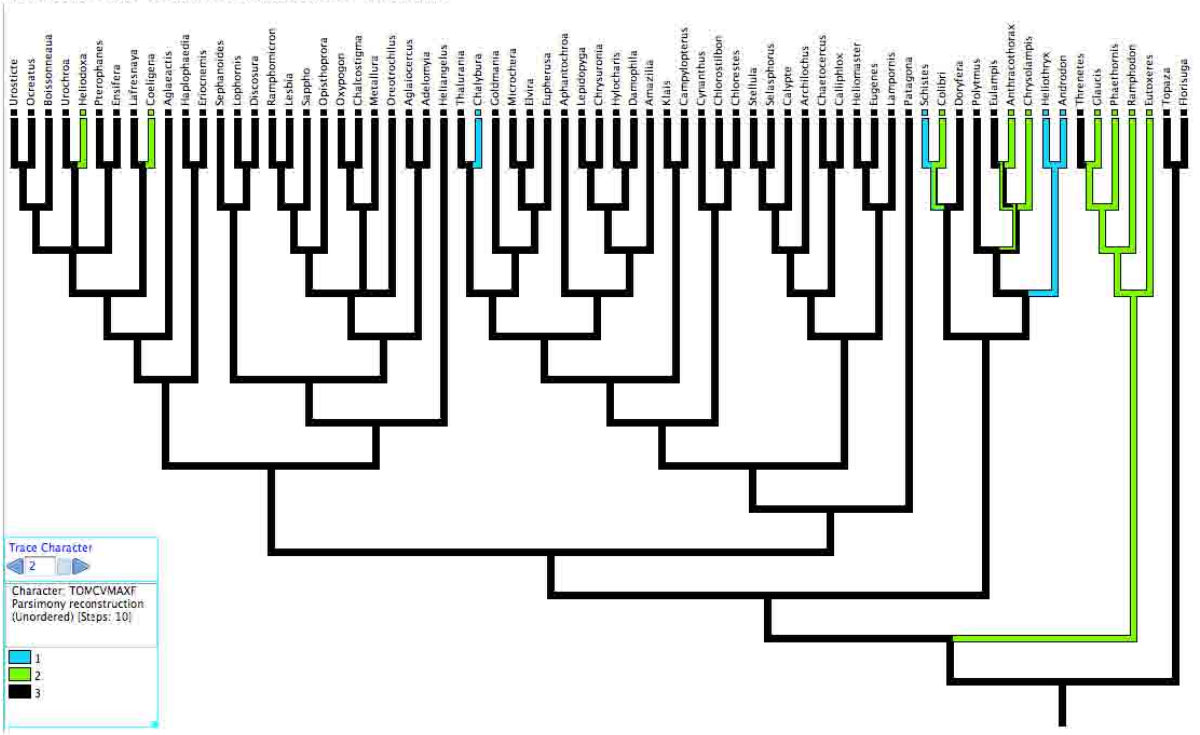

*Projections*



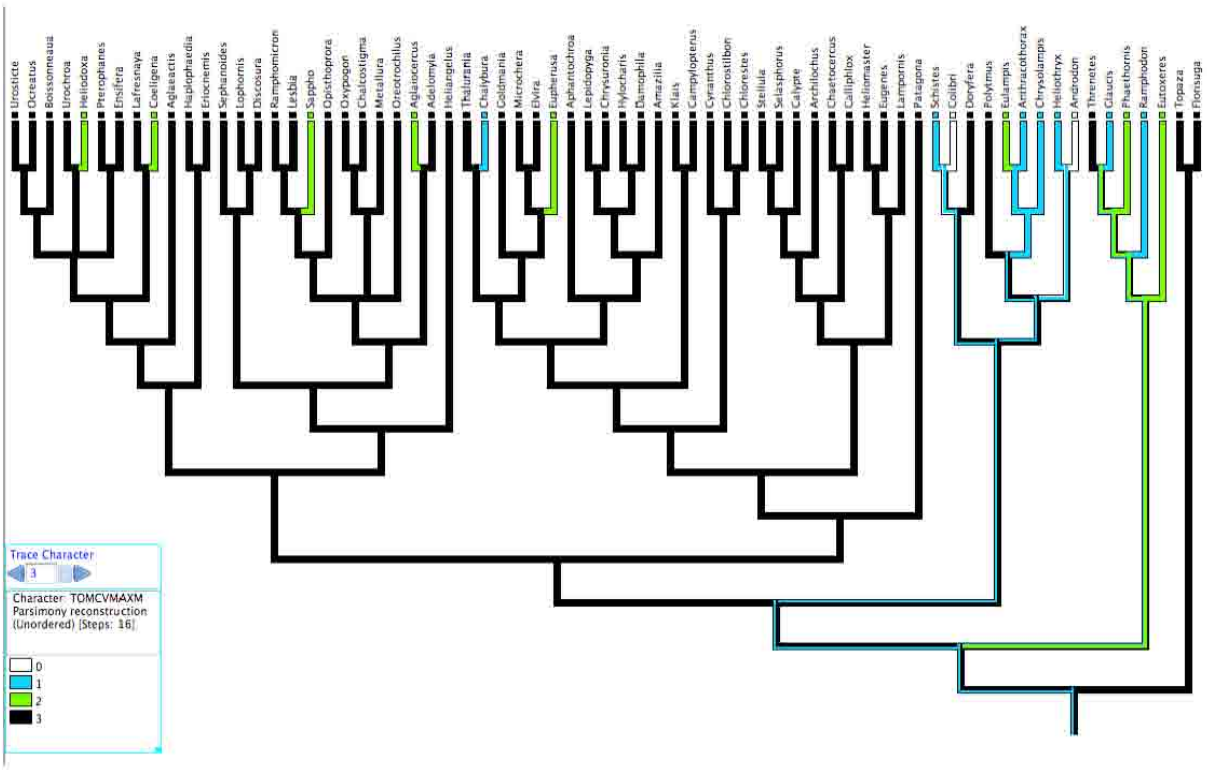
*Depressions*



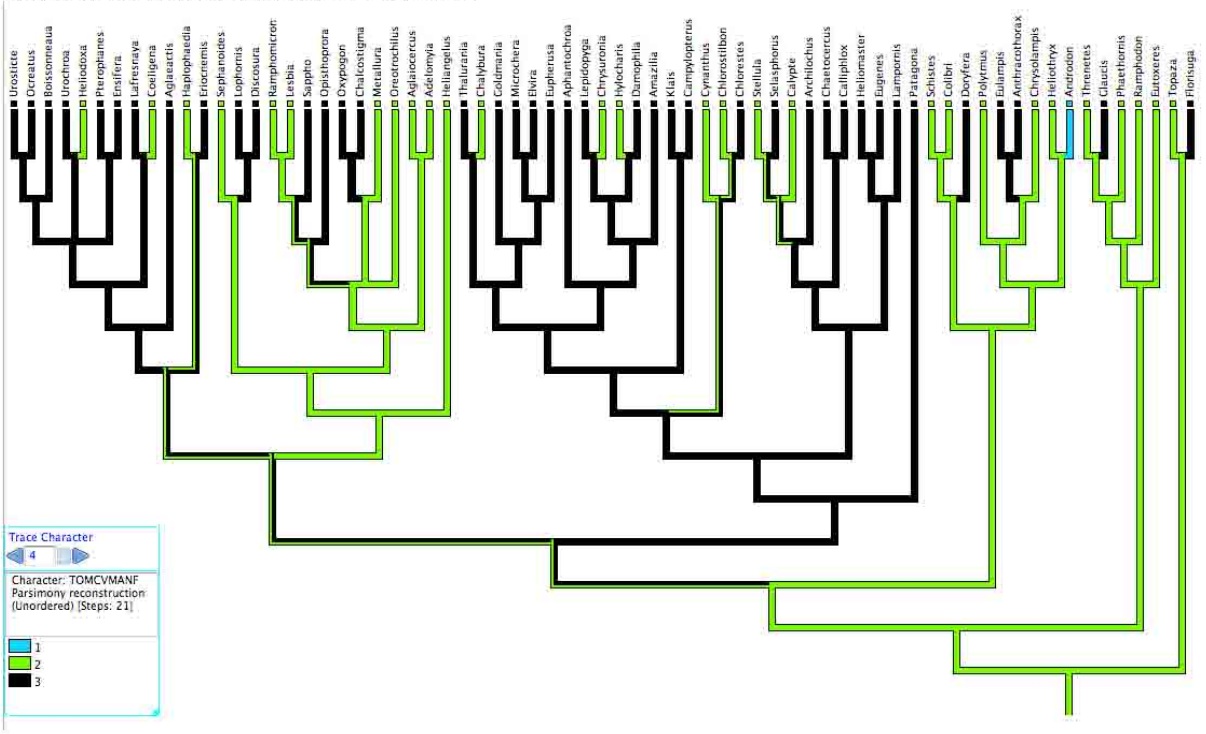

*Tomial curvature Maxilla Females*



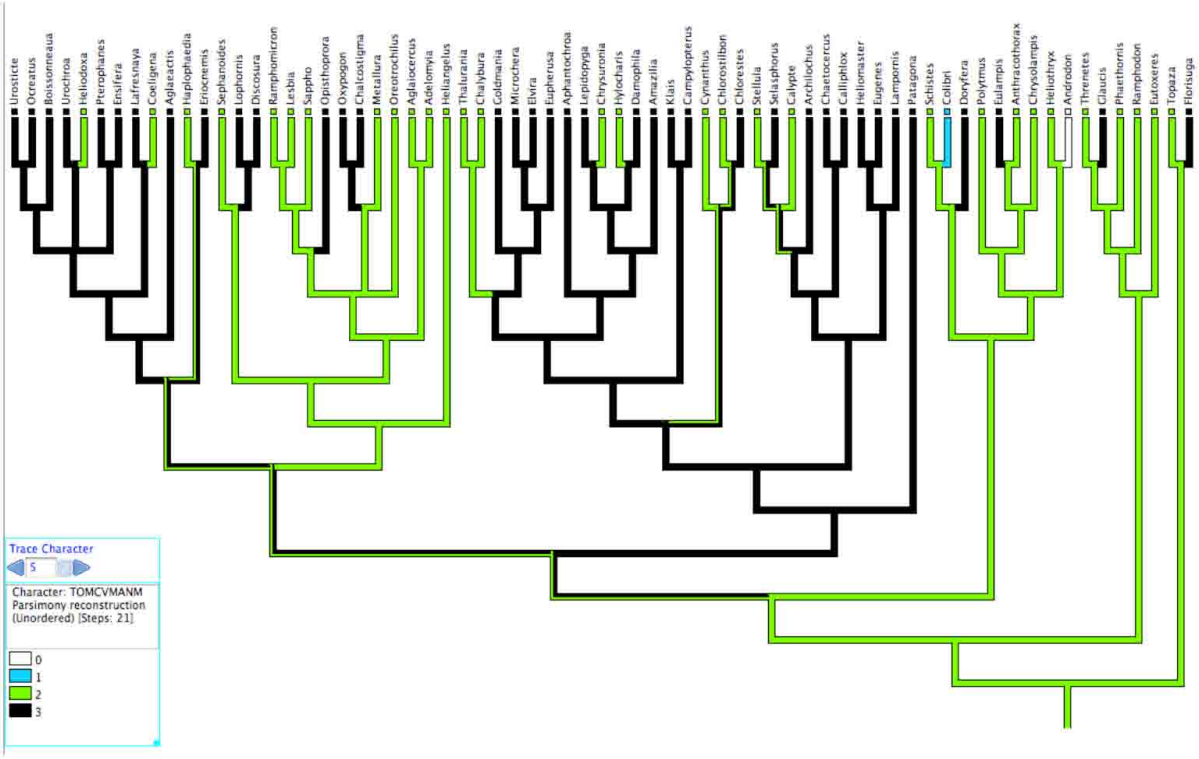
*Tomial curvature Maxilla Males*



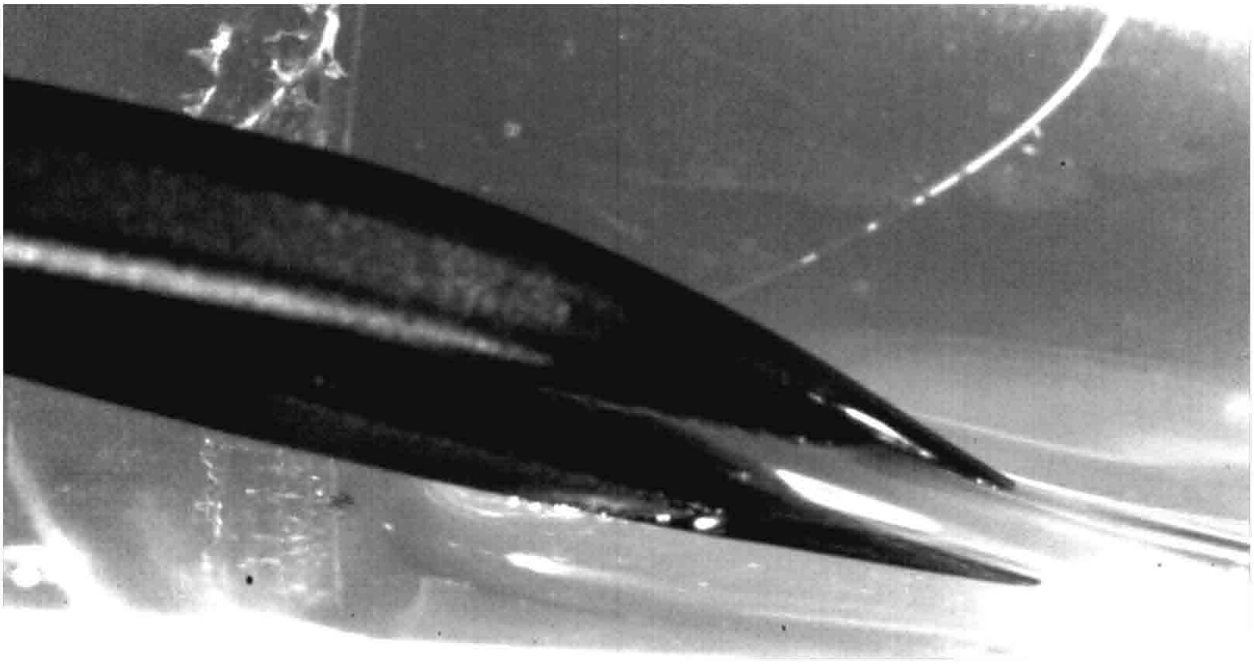

*Tomial curvature Mandible Females*



*Tomial curvature Mandible Males*




## Supplementary Movies



**Movie S1. High-speed video of a Short-tailed Woodstar (*Myrmia micrura)* drinking artificial nectar.** Hummingbirds compress their tongues with their bill tips while extrude them. Flexibilty of the serrated tomia is noticeable.

**Supplementary Tables**

**Table S1. Degree of inrolling of the distal tomia in relation to tomial class in 84 genera of hummingbirds (*cf.* Appendix 1).**

| | Categories of tomial inrolling in males/females | | | | |
|---|---|---|---|---|---|
| | **0-1/0-3** | **1-3/1-3** | **2-3/2-3** | **3/3** | **Totals** |
| **Tomial class** | **Maxilla** | | | | |
| **A, A-B** | 9 | 0 | 2 | 0 | **21** |
| **B-A, B, B-C** | 0 | 3 | 19 | 28 | **50** |
| **C-B, C** | 0 | 3 | 9 | 1 | **13** |
| **Totals** | **9** | **16** | **30** | **29** | **84** |

| **Tomial class** | **Mandible** | | | | |
|---|---|---|---|---|---|
| **A, A-B** | 2 | 10 | 8 | 1 | **21** |
| **B-A, B, B-C** | 0 | 7 | 39 | 4 | **50** |
| **C-B, C** | 0 | 3 | 10 | 0 | **13** |
| **Totals** | **2** | **20** | **57** | **5** | **84** |

**Table S2. Degree of inrolling of the distal tomia in males *vs.* females in 84 genera of hummingbirds.**

| | Tomial inrolling in males *vs.* females | | | |
|---|---|---|---|---|
| | ♂ > ♀ | ♂ ≈ ♀ | ♂ < ♀ | |
| **Tomial class** | **Maxilla** | | | **Totals** |
| **A, A-B** | 0 | 5 | 16 | **21** |
| **B-A, B, B-C** | 2 | 48 | 0 | **50** |
| **C-B, C** | 0 | 13 | 0 | **13** |
| **Totals** | **2** | **66** | **16** | **84** |

| **Tomial class** | **Mandible** | | | |
|---|---|---|---|---|
| **A, A-B** | 0 | 14 | 7 | **21** |
| **B-A, B, B-C** | 0 | 50 | 0 | **50** |
| **C-B, C** | 0 | 13 | 0 | **13** |
| **Totals** | **0** | **77** | **7** | **84** |

**Table S3. Character states of prongs relative to those of basins on the inside of the bills in 84 genera of hummingbirds.**

| Character states of basins | Character states of prongs: 0 | 0-1, 1 | 1-2,2 | 2-3,3 | 3-4,4 | Totals |
|---|---|---|---|---|---|---|
| **0** | 1 | 0 | 0 | 0 | 0 | **1** |
| **0-1, 1** | 2 | 2 | 1 | 0 | 1 | **6** |
| **1-2, 2** | 0 | 2 | 1 | 3 | 12 | **13** |
| **2-3, 3** | 0 | 0 | 1 | 13 | 36 | **50** |
| **3-4, 4** | 0 | 0 | 1 | 0 | 8 | **9** |
| **Totals** | **3** | **4** | **4** | **16** | **57** | **84** |

**Table S4. Character states of prongs and basins in relation to the class of maxillary tomium in 84 genera of hummingbirds[1].**

| | Character states of prongs | | | | | |
|---|---|---|---|---|---|---|
| **Tomial class** | **0** | **0-1, 1** | **1-2, 2** | **2-3, 3** | **3-4, 4** | **Totals** |
| **A, A-B** | 2 | 2 | 2 | 5 | 10 | **21** |
| **B-A, B, B-C** | 0 | 1 | 0 | 9 | 40 | **50** |
| **C-B, C** | 1 | 1 | 2 | 2 | 7 | **13** |
| **Totals** | **3** | **4** | **4** | **16** | **57** | **84** |

| | Character states of basins | | | | | |
|---|---|---|---|---|---|---|
| **Tomial class** | **0** | **0-1, 1** | **1-2, 2** | **2-3, 3** | **3-4, 4** | **Totals** |
| **A, A-B** | 0 | 4 | 2 | 13 | 2 | **21** |
| **B-A, B, B-C** | 0 | 1 | 11 | 31 | 7 | **50** |
| **C-B, C** | 1 | 1 | 5 | 6 | 0 | **13** |
| **Totals** | **1** | **6** | **18** | **50** | **9** | **84** |

[1] = For genera in which different species have different tomial classes, both of these are given, with the prevalent class of the genus given first: thus, A-B = most species of a genus with tomial class A, a minority with class B. The same convention is used for categories of prongs and basins.

## Appendix 1. A survey of bill structures in hummingbirds (Trochilidae).

| Genus | # *spp.* | Tomial Curvature ♂/♀[1] Maxila | Mandible | Internal Structures Projections[2] | Depressions[3] | Tomial class[4] |
|---|---|---|---|---|---|---|
| **Clade 0: "Topazes"** | | | | | | |
| *Topaza* | 1 | 2-3/2-3 | 2/2 | 3 | 1-2 | C |
| *Florisuga* | 2 | 1-3/1-3 | 2-3/2-3 | 2 | 1-2 | C |
| **Clade 1: "Hermits"** | | | | | | |
| *Eutoxeres* | 2 | 2/2 | 2/2 | 0-1 | 0-1 | C |
| *Threnetes* | 1 | 2-3/2-3 | 1-2/1-2 | 1 | 1 | C |
| *Glaucis* | 2 | 0-1/1-2 | 3/3 | 2-3 | 3 | A |
| *Ramphodon* | 2 | 0-1/1-2 | 2/2 | 2 | 3 | A |
| *Phaethornis* | 10 | 1-2/1-2 | 1-2/1-2 | 2-3 | 2-3 | B |
| **Clade 2: "Mangoes"** | | | | | | |
| *Avocettula* | 1 | 0-1/1-3 | 1-2/2-3 | 3 | 3 | A |
| *Eulampis* | 1 | 1-2/2-3 | 2-3/2-3 | 3-4 | 3 | A |
| *Augastes* | 1 | 0-1/1-2 | 2-3/2-3 | 2 | 2 | A |
| *Doryfera* | 2 | 2-3/3 | 2-3/2-3 | 1 | 2 | A |
| *Colibri* | 3 | 0/2 | 0-1/1-2 | 2-3 | 2 | A |
| *Schistes* | 1 | 1/1 | 1-2/1-2 | 1 | 1 | A |
| *Androdon* | 1 | 0/1 | 0/1 | 0 | 0-1 | A |
| *Heliothryx* | 2 | 0-1/0-1 | 2/2 | 0 | 0-1 | A |
| *Anthracothorax* | 3 | 0-1/1-2 | 1-2/2-3 | 3-4 | 2-3 | A |
| *Chrysolampis* | 1 | 1/2 | 2/2 | 3-4 | 1-3 | A |
| *Polytmus* | 2 | 2-3/2-3 | 1-2/1-2 | 2 | 3 | A |
| **Clade 3: "Coquettes and High Andeans"** | | | | | | |
| *Lophornis* | 4 | 2-3/2-3 | 2-3/2-3 | 3-4 | 2 | B |
| *Discosura* | 2 | 2-3/2-3 | 3/3 | 3-4 | 1 | B |
| *Oreotrochilus* | 1 | 3/3 | 2/2 | 2 | 3-4 | B |
| *Heliangelus* | 2 | 2-3/2-3 | 1-2/1-2 | 4 | 2-3 | B |
| *Adelomyia* | 1 | 3/3 | 1-2/1-2 | 4 | 3 | B |
| *Aglaiocercus* | 2 | 1-2/2-3 | 1-2/1-2 | 4 | 2-3 | A |
| *Sappho* | 1 | 1-2/2-3 | 1-2/2-3 | 3-4 | 3 | A |
| *Chalcostigma* | 2 | 3/3 | 2-3/2-3 | 3-4 | 2-3 | B |
| *Oxypogon* | 1 | 3/3 | 2-3/2-3 | 1 | 2 | B |
| *Lesbia* | 2 | 2-3/2-3 | 2/2 | 3 | 2-3 | B |
| *Ramphomicron* | 1 | 3/3 | 2-3/2-3 | 3 | 1-2 | B |
| *Sephanoides* | 1 | 3/3 | 2/2 | 2-3 | 2-3 | B |
| *Metallura* | 3 | 2-3/2-3 | 2/2 | 4 | 2 | B |
| *Opisthoprora* | 1 | 2-3/2-3 | 2-3/2-3 | 3-4 | 3 | C |

| Genus | # *spp.* | Tomial Curvature ♂/♀[1] | | Internal Structures | | Tomial class[4] |
|---|---|---|---|---|---|---|
| | | Maxila | Mandible | Projections[2] | Depressions[3] | |
| **Clade 4: "Brilliants"** | | | | | | |
| *Eriocnemis* | 4 | 2-3/2-3 | 2-3/2-3 | 3-4 | 2 | B |
| *Haplophaedia* | 2 | 2-3/3 | 1-2/1-2 | 4 | 3 | B |
| *Aglaeactis* | 1 | 3/3 | 2-3/2-3 | 4 | 2 | B |
| *Lafresnaya* | 1 | 3/3 | 3/3 | 4 | 3 | B |
| *Coeligena* | 5 | 1-2/1-2 | 2/2 | 4 | 2-3 | B |
| *Heliodoxa* | 4 | 1-2/1-2 | 1-2/1-2 | 4 | 2-3 | C |
| *Sternoclyta* | 1 | 2-3/2-3 | 2/2 | 4 | 2 | C |
| *Boissonneaua* | 2 | 2-3/2-3 | 2-3/2-3 | 4 | 3 | C |
| *Pterophanes* | 1 | 3/3 | 2-3/2-3 | 4 | 3 | B |
| *Ensifera* | 1 | 2-3/2-3 | 2-3/2-3 | 4 | 2 | B |
| *Urochroa* | 1 | 3/3 | 2-3/2-3 | 4 | 3 | B |
| *Ocreatus* | 1 | 3/3 | 2-3/2-3 | 4 | 2-3 | B |
| *Urosticte* | 1 | 2-3/2-3 | 1-3/1-3 | 3 | 3 | B |
| **Clade 5: "Giant"** | | | | | | |
| *Patagona* | 1 | 3/3 | 3/3 | 4 | 3 | B |
| **Clade 6: "Mountain Gems"** | | | | | | |
| *Eugenes* | 2 | 2-3/2-3 | 2-3/2-3 | 3-4 | 3-4 | B |
| *Hylonympha* | 1 | 2/2 | 2-3/2-3 | 4 | 2 | C |
| *Lampornis* | 3 | 3/3 | 2-3/2-3 | 3-4 | 3-4 | B |
| *Lamprolaima* | 1 | 3/3 | 2-3/2-3 | 3-4 | 2-3 | B |
| *Heliomaster* | 2 | 2-3/2-3 | 1-3/1-3 | 4 | 2-3 | B |
| **Clade 7: "Woodstars and Bees"** | | | | | | |
| *Tilmatura* | 1 | 3/3 | 2-3/2-3 | 3 | 3 | B |
| *Doricha* | 1 | 3/3 | 2/2 | 3 | 2-3 | B |
| *Calliphlox* | 3 | 3/3 | 2-3/2-3 | 3-4 | 2-3 | B |
| *Calothorax* | 1 | 3/3 | 2/2 | 2-3 | 2-3 | B |
| *Chaetocercus* | 3 | 2-3/2-3 | 2-3/2-3 | 3 | 2-3 | B |
| *Atthis* | 1 | 3/3 | 2-3/2-3 | 3 | 3 | B |
| *Archilochus* | 2 | 2-3/2-3 | 2-3/2-3 | 3-4 | 3 | B |
| *Calypte* | 2 | 2-3/2-3 | 2/2 | 3-4 | 2-3 | B |
| *Stellula* | 1 | 2-3/2-3 | 2/2 | 3-4 | 3-4 | B |
| *Selasphorus* | 3 | 3/3 | 2-3 | 3-4 | 3-4 | B |

| Genus | # *spp.* | Tomial Curvature ♂/♀[1] | | Internal Structures | | Tomial class[4] |
|---|---|---|---|---|---|---|
| | | Maxila | Mandible | Projections[2] | Depressions[3] | |
| **Clade 8: "Emeralds"** | | | | | | |
| *Abeillia* | 1 | 3/3 | 2-3/2-3 | 3-4 | 2-3 | B |
| *Cynanthus* | 2 | 1-3/3 | 1-2/1-2 | 3-4 | 3-4 | A |
| *Klais* | 1 | 2-3/2-3 | 2-3/2-3 | 3-4 | 2-3 | B |
| *Chlorostilbon* | 5 | 2-3/2-3 | 1-2/1-2 | 4 | 3 | B |
| *Chlorestes* | 1 | 3/3 | 2-3/2-3 | 4 | 3 | B |
| *Eupetomena* | 1 | 2-3/2-3 | 1-2/1-2 | 4 | 1-2 | B |
| *Aphantochroa* | 1 | 2-3/2-3 | 3/3 | 4 | 2 | B |
| *Phaeochroa* | 1 | 3/3 | 2-3/2-3 | 3-4 | 3 | B |
| *Campylopterus* | 3 | 3/3 | 2-3/2-3 | 4 | 3-4 | B |
| *Clytolaema* | 1 | 2-3/2-3 | 2-3/2-3 | 4 | 2 | C |
| *Leucochloris* | 1 | 3/3 | 2/2 | 4 | 2-3 | B |
| *Microchera* | 1 | 1-3/2-3 | 2-3/2-3 | 4 | 3 | B |
| *Elvira* | 2 | 1-3/2-3 | 2-3/2-3 | 4 | 3 | A |
| *Eupherusa* | 2 | 1-2/2-3 | 2-3/2-3 | 3-4 | 3-4 | A |
| *Thalurania* | 2 | 1-3/2-3 | 1-2/2-3 | 3-4 | 2-3 | A |
| *Chalybura* | 2 | 0-1/0-1 | 1-2/1-2 | 3 | 2-3 | A |
| *Goldmania* | 1 | 1-3/3 | 2-3/3 | 3-4 | 3 | A |
| *Leucippus* | 2 | 1-2/1-2 | 2/2 | 4 | 3 | B |
| *Amazilia* | 7 | 2-3/2-3 | 2-3/2-3 | 3-4 | 2-4 | B |
| *Chrysuronia* | 1 | 3/3 | 2/2 | 4 | 2 | B |
| *Hylocharis* | 3 | 3/3 | 2/2 | 3-4 | 3 | B |
| *Damophila* | 1 | 3/3 | 2-3/2-3 | 4 | 3 | B |
| *Lepidopyga* | 2 | 3/3 | 2-3/2-3 | 4 | 2 | B |

## Notes:

[1] = Degree of tomial inrollment in males and females (*cf.* Fig. S1c).
[2] = Character states of the internal projections (*cf.* Fig. S1a).
[3] = Character states of the internal depressions (*cf.* Fig. S1b).
[4] = Prevailing tomial class *sensu* Stiles and Rico-Guevara (unpub. manusc.).

# Chapter 5

## Intraoral transport of nectar in hummingbird bills

**Abstract**

Hummingbirds are the most speciose group of vertebrate nectarivores. They have evolved bills with a variety of shapes and sizes to feed on nectar in flowers with corresponding corolla forms and lengths. We only recently discovered how hummingbirds use their tongues while feeding, but still do not understand how the nectar is moved from the tongue to the throat. The aim of this paper is to understand how hummingbird feeding apparati work in the context that has shaped their divergent evolution, drinking nectar. We tested several hypotheses and specific predictions through a "hypothesis flow chart" approach in order to reveal the true feeding mechanics of hummingbird drinking. To elucidate fluid transport inside the bill, we used backlighting techniques that allowed us to film the intraoral nectar flow in live hummingbirds. We were able to visualize nectar flow through the keratin, to track the nectar menisci and the tongue, and to follow bubble formation. We found that hummingbirds exploit hydrostatic pressure to move fluid inside the bill and we describe an unexpected role of the tongue base in nectar transport. Our results present the first evidence of maxillary bending in hummingbirds, in opposition to the predictions based on the lack of osteological traits that allow distal rhynchokinesis in other groups of birds. We propose that the combination of asynchronic movements at the bill tip and at the base, mediated through a bending zone, in coordination with tongue movements move the nectar from the tongue grooves to the bill base.

*Keywords*: Biomechanics; Drinking; Fluid transport; Hummingbirds; Nectar feeding

## Introduction

Opportunistic nectar consumption is widespread in the animal kingdom, probably because this highly energetic resource is relatively easy to find and has few or no defensive compounds. Efficiently securing enough of it to subsist on is more difficult. Nectarivores exhibit morphological modifications that increase their feeding efficiency at flowers. For insects and vertebrates those adaptations include proboscis and tongue elongation, respectively, and modifications of the head and mouthparts aimed at increasing the "reach" of the feeding apparatus, in response to elongation of the floral corolla. Studies of nectar-drinking insects indicate that adhesion and suction are the principal mechanisms involved in their nectar intake (see Kingsolver and Daniel 1995). In recent years there have been several studies of the morphology and mechanics of nectar feeding in bats and insects (*e.g*., Borrel 2007, Tschapka *et al*. 2008), but little recent attention has been given to nectarivorous birds. Hummingbirds evolved to feed on flowers well enough to make their living out of small volumes of nectar scattered over the landscape, and the resulting adaptations–hovering flight, high metabolism, body size reduction– entirely modified their lifestyle and biology. Well-documented coevolution between floral shape and bill morphology has produced a family of birds with elongate bills (from 5 to 110% of their body lengths) that range from recurved, through straight, to strongly decurved (up to more than $90^{o}$). Even though there is consensus that the remarkable biology of hummingbirds arises from feeding on flowers, little is known about *how* they feed on the floral nectar. The hummingbird tongue bifurcates distally (*i.e.* bifid, Darwin 1841), ending in two parallel semi-cylindrical tubes or grooves, formed by rolling of the thin tongue margin (Lucas 1891), the highest form of tubular tongue (Sundevall 1872 p. 87, Gadow 1883 p. 66).

The first step during nectar consumption is loading the tongue, which is protruded out of the bill into the nectar pool in the base of the flower, where it was suggested to function as tiny capillary tubes (Martin 1833, Lucas 1891, Scharnke 1931). The specifics of how the tongue tips and grooves are filled with nectar have been recently revealed to be quite different than historically thought (Rico-Guevara and Rubega 2011, 2012), but it is clear that once the tongue is loaded, and retracted into the hummingbird's mouth, separate mechanisms are involved in offloading, intraoral transport of nectar up the elongate bill, and deglutition of nectar.

Lucas (1891) pointed out that no vacuum can be formed at the base of the tongue, and Scharnke (1931) and Weymouth *et al*. (1964) emphasized that there is no connection between the distal semi-cylindrical grooves and the tongue base (*cf.* Gould 1861, p. 34); therefore the tongue cannot function as a "soda straw". Additionally, the dorsal slit and flexible walls of the distal grooves would prevent the formation of a vacuum and/or yield to collapsing of the structures respectively (*cf.* Kingsolver and Daniel 1983, pers. obs.). Hainsworth (1973) suggested that the grooves transport a relatively small amount of liquid from the nectar reservoir to the bill, and hypothesized that a substantial volume of nectar was channeled onto the beak along the tongue sides and top (*cf.* dog lapping, Crompton and Musinsky 2011), but his measurements could have been misled by low filming speeds (*cf.* Ewald and Williams 1982). Subsequently, a consensus was reached going back to the original ideas of Martin (1833): hummingbirds are "capillary feeders" and several biophysical models focused on tongue functioning have been developed (Kingsolver and Daniel 1983, Heyneman 1983, Kim *et al.* 2011, 2012, Kim and Bush 2012).

Relatively less attention has been paid to bill functioning and intraoral transport of the nectar from the tongue to the bill base, where it could be swallowed or passed to the crop. Based on direct observations and manipulation of museum specimens several authors proposed pioneering theories (Gadow 1883, Moller 1930, Döhling 1931, Scharnke 1931, Steinbacher 1935). In the late 1800's and early 1900's several theories about the drinking mechanism in hummingbirds were proposed, mostly based on inferences derived from the morphology of the feeding apparatus, but some also including observations of captive birds. Gadow (1883, p. 68) proposed that, alternating the use several oropharyngeal and hyobranchial muscles, a depression of the basal portion of the tongue could generate a vacuum between the tongue and the palate. Such a vacuum would move fluid from the tongue grooves into the throat; he called it the "suctorial apparatus" (Gadow 1883).

Moller (1930) suggested that the beak was used as a pump through a combined action of the tongue base moving diagonally downward and backward and capillary filling at the tongue tips. This pumping action inside the bill is allowed by the tomia overlap at the margin of the upper and lower jaws, making the internal bill space airtight; in this model, the tongue is envisioned as a piston and the bill as a cylinder (Moller 1930, Döhling 1931). Scharnke, who initially considered gravity as a main fluid driving force, eventually concurred with Moller after doing a more extensive morphological survey, and a literature review of observations on living hummingbirds (Scharnke 1931).

Steinbacher (1935) supported the idea of the tongue and bill tips forming an hermetic tube and proposed a role for the rapid pharyngeal movements observed in captive birds (*cf.* Döhling 1931) to create suction force at the base of the bill, consistent with Gadow's tongue depression vacuum hypothesis. A consensus hypothesis was achieved stating that intraoral transport of nectar in hummingbirds was accomplished by suction generated through a pumping action at the bill base created by tongue and pharynx movements, made possible by a sealed tube-like middle portion of the bill (*cf.* Böker 1937, Fig. 11 in Zusi 2013).

After a long lapse ended by the advent of high-speed videography, Ewald and Williams (1982) showed that the hummingbird tongue seems to be squeezed by the bill tips during protraction, presumably to force nectar off the tongue and into the oral cavity (Paton and Collins 1989), but none of these authors proposed a formal intraoral nectar transport mechanism. Finally, ten years ago, a study by Yanega and Rubega (2004) raised the possibility of mandibular spreading as an additional suction generator at the bill base. Some of these hypotheses for intraoral transport are mutually exclusive while others are compatible; therefore a systematic testing is required to untangle the hummingbird drinking mechanisms. The aim of this paper is to put together all the pieces of this puzzle, formulate a range of hypotheses with falsifiable predictions, and test them using cutting-edge technology and novel visualization methods.

We summarized the theories proposed to date in a "hypothesis flow chart" organizing them by being mutually exclusive or compatible (Fig. 1A). We complement our flow chart with diagrams of the hypothetical mechanisms to facilitate visual comparisons (Fig. 1B).

The most likely scenario is that a combination of mechanisms (as opposed to accepting only one hypothesis) is responsible for the intra-oral transport of nectar in hummingbirds. Our hypotheses flow chart starts with a distinction between mechanisms operating at the tip or at the base of the bill, for the most part a mechanism moving the fluid from the tip towards the base does not preclude the simultaneous operation of a mechanism pulling the fluid from the bill base. The only exception is intraoral capillarity, which is a hypothetical mechanism that would allow the fluid to move through the inside of the middle "cylindrical" portion of the bill purely through capillary action (Fig. 1B *bottom right*). A similar mechanism constitutes the first phase of the drinking process in some birds (*e.g.* pigeons, Zweers 1982). Intraoral capillarity would be incompatible with any mechanism that requires hermetic closing of the bill base because the column of fluid moved by capillarity would need to displace a column of air of equal volume, and if there is no air opening at the bill base such column of air cannot be displaced precluding capillarity. For intraoral capillarity to be a significant force for fluid displacement inside the bill, a period of time is required in which the bill tips and the middle portion of the bill are static, in order for the meniscus to traverse the bill and reach the base.

The alternative mechanism to move liquid from the bill tip backwards involves the dynamic constriction of the tongue by the bill tips, which offloads the nectar inside the oral cavity (Ewald and Williams 1982). If repeated multiple times consecutively, this squeezing could move the nectar through the bill by simply stacking nectar loads one after the other (like a stacking point pencil) from the bill tip backwards (Fig. 1B *top right*). We call this mechanism "hydraulic pushing".

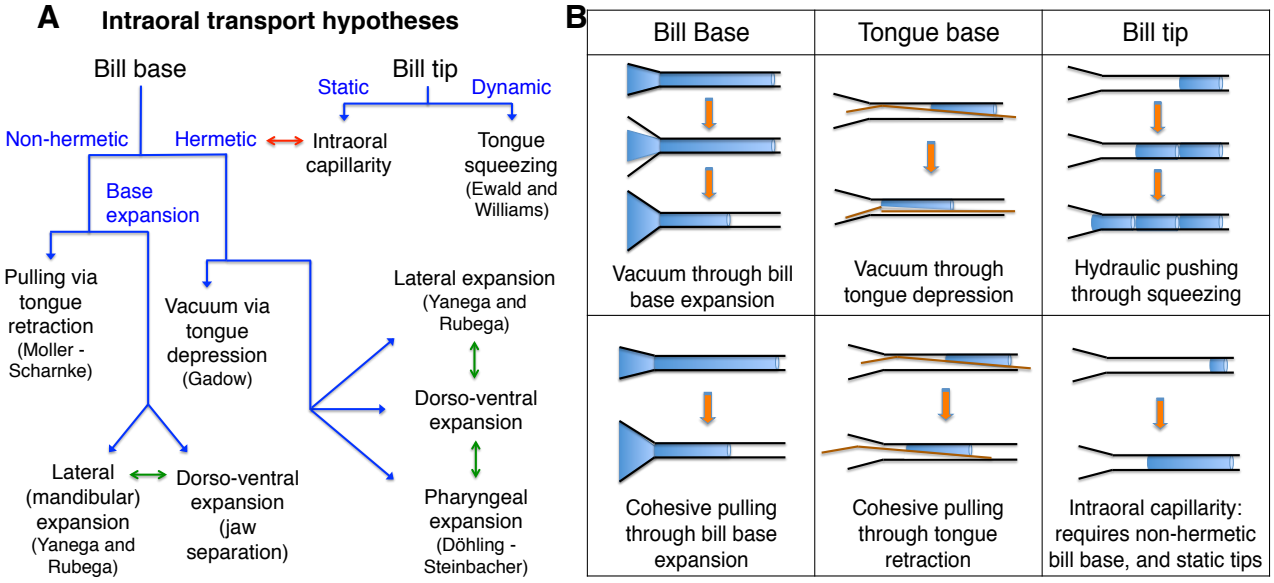



**Figure 1. Possibilities for intraoral transport. A)** Hypothesis flow chart of intraoral mechanisms in hummingbirds. The flow chart starts with a division between the intraoral mechanisms of nectar transport occurring near the bill tip (*right*) or near the bill base (*left*). Right angles following the blue lines from top to bottom indicate mutually exclusive hypotheses. Double-headed arrow near the top (red) denotes an extra pair of mutually exclusive (incompatible) hypotheses; *i.e.* intraoral capillarity and mechanisms that require a hermetic bill base. All the other double-headed arrows (green) designate compatible and complementary hypotheses. When appropriate, last names of the hypotheses proposers are given in parentheses below each hypothesis name. **B)** Hypothetical mechanism diagrams divided by the operating part of the feeding apparatus. In all diagrams the bill base is on the left (denoted by a funnel-like shape) and the bill tip is on the right, blue cylinders and areas inside the bill sketches represent the nectar, and in the middle panels the brown line inside the bill corresponds to the tongue. Orange arrows between diagrams designate non-scaled time steps. Hypothesis names correspond with the ones in the hypotheses flow chart.

Moving on to the mechanisms that could operate away from the tip and closer to the bill base, Ewald and Williams (1982) and Downs (2004) shared Moller's (1930) and Scharnke's (1931) idea that the base of the extruded tongue could adhere to the offloaded nectar load and bring it backwards when the tongue is retracted (Fig. 1B *bottom middle*).

The tongue base would function as a piston or a syringe plunger. We call this mechanism "cohesive pulling" since it takes advantage of the nectar properties as a liquid (adhesion and cohesion) in a way similar to the cohesion-tension mechanism that moves water across plant tissues (*cf.* Steudle 2001, Wheeler and Stroock 2008, but see Zimmermann *et al.* 2004). This cohesive pulling, which is controlled by tongue retraction, does not require hermetic sealing at the bill base. An alternative to this cohesive pulling through tongue retraction mechanism is that instead of the pulling being operated by tongue movements, it is instead operated by expansion at the bill base. If all the interstices at the basal buccal cavity are filled with nectar and the base expands, there is going to be a net backwards displacement of the liquid column (Fig. 1B *bottom left*). This mechanism, which would be similar to the suction feeding mechanism in fish (*cf.* Muller and Osse 1984), would also be a kind of cohesive pulling but in this case driven by bill basal expansion. There are two non-exclusive options to generate this expansion of the bill base; the base of the mandibular rami could bow and separate laterally (*cf.* Yanega and Rubega 2004), and/or the maxilla and mandible could separate dorso-ventrally, in both cases keeping the bill middle portion tight.

Up to this point, none of the aforementioned mechanisms has invoked suction. It is worth noting that we refer to suction here as the creation of a partial vacuum, in this case by lowering air pressure, to induce flow into it (pushing from the external pressure), such as in the case of "soda-straw suction". Cohesive pulling could be seen as a kind of suction but since the pressure differential would be almost immediately and continuously equalized, we do not consider it as proper suction. Ewald and Williams suggested the existence of a suction component by bulging in the throat region during tongue protrusion (*cf.* Döhling 1931, Steinbacher 1935).

This suction component could be grouped with possible suction being generated by the expansion of the bill base (lateral *cf.* Yanega and Rubega 2004 and/or dorsoventral), because suction generated one way would only reinforce suction generated in any other way.

These suction mechanisms require that the basal and the middle portions of the bill remain perfectly hermetic to allow for the vacuum production (Fig. 1B *top left*). The last mechanism is an alternative to the "bill base suction"; in this case the suction is generated by depression of the tongue creating a vacuum between its base and the palate (*cf.* Gadow, Fig. 1B *top middle*). A similar mechanism has also been proposed to operate in the group of birds with the most similar feeding apparatus to hummingbirds, the sunbirds (Gadow 1883, Liversidge 1967, Cheke and Mann 2008).

Lastly, distal rhynchokinesis (bill bending near the bill tip) could allow for the tongue to cycle in and out the bill while maintaining the rest of it as a nearly closed tube to improve nectar transport (*cf.* Rico-Guevara 2011, Zusi 2013). For hummingbirds, there are three ways to maintain the middle portion of the bill tightly closed while separating the tips: 1) by bending the maxilla and mandible simultaneously, 2) by bending the mandible while keeping the maxilla static as a support off of which the bendable lower bill portion is deflected, and 3) by bending the maxilla while keeping the mandible static as a support off of which the bendable upper bill portion is deflected.

Although Nitzsch (1816) originally proposed a form of rhynchokinesis in hummingbirds as an adaptation to nectar feeding, consisting of extensive flexion zones along the dorsal and ventral aspects of the upper jaw (see Bühler 1981), Zusi (1984) found no osteological evidence to support any rhynchokinetic capacity in hummingbirds. Moreover, he suggested that the apparent bill bending near the tip is an optical illusion enabled by the fact that the mandible is partially ensheathed inside the maxilla in lateral view; while rapidly opening and closing the bill tip, the differential overlapping of the tomia near the tip would suggest an equivocal bending point in the middle to distal portion of the bill (Zusi 1984).

Using the hypothesis flow chart, we designed a filming methodology that would allow us to navigate through the possible mechanisms, discarding possibilities through methodical observations and kinematic measurements. We studied the intraoral transport mechanism by filming (high-speed videography) hummingbirds feeding both in lateral and dorsal views, to decipher the dorsoventral bill motions noted previously (Rico-Guevara and Rubega 2011, Zusi 2013) and to assess the possibility that lateral spreading of the bill base (*cf.* Yanega and Rubega 2004) was contributing to the generation of suction forces. Additionally, we employed illumination techniques to visualize tongue base movements and quantify intraoral nectar flow.

**Methods**

The study of food transport in animals was greatly improved by the use of X-ray cinematography in physiological studies (*e.g.* Anker *et al.* 1967); radiography filming was used in conjunction with lead markers but was only available at relatively low recording speeds (*i.e.* 48 frames/sec, *e.g.* Zweers *et al*. 1977, Zweers 1982, Kooloos and Zweers 1989).

More recently X-ray Reconstruction of Moving Morphology (XROMM), a methodology combining 3D motion *in vivo* analysis with skeletal morphology from CT scans, has opened new avenues for the study of animal movement (*e.g.* Gatesy and Alenghat 1999, Brainerd *et al.* 2010) as well as feeding, in particular, taking advantage of high-speed capabilities (250 frames/sec, Dawson *et al.* 2011). However during pilot experiments with hummingbirds following the XROMM protocol, it became evident that the resolution of the cameras was not adequate for solving the questions pertaining to this study (Fig. S1).

Another cutting-edge visualization technique, using synchrotron X-rays, has been used successfully in animal physiology (*e.g.* Simon *et al.* 2010) with remarkable image resolution (2 μm, Greenlee *et al.* 2013). Unfortunately during preliminary assessment at the National Synchrotron Light Source at Brookhaven National Laboratory, we could not achieve fields of view large enough to study the intraoral transport in hummingbirds (always less than 5 mm in all the possible permutations). We also employed a thermal imaging camera (Flir Systems, Wilsonville, OR; Model A655sc, with a Macro-lens), intending to visualize cold nectar moving through a warmer body temperature hummingbird bill, but we did not register any change in the thermal profile of the bill. Additional alternatives like fluorescent dyes or radioactive tracers (*e.g.* for positron emission tomography) would greatly affect the nectar properties making it impossible to use those techniques to study nectar flow inside the bill. We were able to overcome all the difficulties of employing commonly used methods that turned out unsuitable for solving our questions by using a novel approach.

By trial and error, we were able to standardize a set of backlighting techniques that allowed studying intra-oral transport of nectar in free-living hummingbirds. We filmed Rufous-tailed Hummingbirds, a species that lacks melanin (an opaque pigment) from most of its red bill, a condition that makes it feasible to see through under the right illumination. This allowed us to track the movement of the tongue and nectar menisci, which is critical information in answering the question about how hummingbirds drink.

We filmed 27 species of free-living hummingbirds covering all the 9 currently recognized clades in the family (Table S1), at artificial feeders in lateral views to assess the bill movements. For a subset of those species, videos of birds feeding on artificial nectar (18.6% sucrose concentration) at feeders were obtained using synchronized high-speed cameras (TroubleShooter HR and Phantom Miro ex4), running up to 1260 frames/s (1280 x 512 pixels), positioned to capture orthogonal views, and coupled to macro lenses (Nikon 105mm f/2.8 VR). We worked in Colombia and Ecuador at four different elevations (0, 1000, 1700, 2400 m.a.s.l.) along the Andes mountains (all private reserves with the permission of their owners). We also used footage of captive Anna's Hummingbirds (*Calypte anna*) hosted in Dr. Douglas Altshuler's laboratory (at the University of California, Riverside). In total, we measured lick sequences of seven hummingbird species (two sites per species) belonging to three different clades (Table S2). For every bird, we randomly selected ten licks of different foraging bouts; videos (in lateral view) were converted into a series of image files using QuickTime Player Version 7.6.6. All images in each sequence were digitally enhanced equally by consistently adjusting the contrast and brightness using ImageJ 1.45p (Schneider *et al.*, 2012), in order to maximize the visibility of the bill contours.

A complete lick sequence was defined as the time from when the bill begins to open (start of the tongue protrusion), through the extension and immersion of the tongue tip in the nectar, and until the tongue is completely retracted and the bill completely closes again. For each lick sequence, we extracted a subset of 11 equally spaced frames to analyze in the lateral and dorsal views (yielding 10 time steps). The starting frame was selected as the one immediately preceding the first visualization of the tongue outside the bill, and the last frame was the corresponding one for the next lick, thus completing a full cycle.

(a) *Bill motion analyses*

For each of the 11 frames per lick, we digitally traced two bill contours, to create upper and lower bill profile lines, using tpsDIG2 (Rohlf 2008). The first line followed the culmen beginning distally at the maxillary tip and ending at the most proximal point of the exposed culmen (the point at which the feathers start). A line perpendicular to the bill axis was followed down from the exposed culmen's most proximal point (culmen base), and its interception with the ventral bill contour was used to place a point defining the most proximal point of the ventral bill profile. We traced the profile of the mandibular ventrum (defined here as the lower jaw contour, the ventral counterpart of the exposed culmen) from the most proximal point as defined above to the mandibular tip (Fig. 2). These bill profile lines were then resampled using tpsDIG2 so that each line had 21 equidistant semi-landmarks, respectively.

All corresponding points along the bill profiles were used to assign bill regions, beginning at the bill base (region 1) and ending at the bill tip (region 20), yielding 5% increment length steps along the bill. We also outlined the contour of the left and right maxillary edges (roughly matching the tomia) in the dorsal views obtaining two lateral bill profile lines. The most proximal points for these tomial profiles were determined as the points in which a line perpendicular to the bill axis and crossing the most proximal point of the exposed culmen (as defined above) intersected the maxillary edge.

The two tomial profiles were also resampled into 21 equidistant semi-landmarks using tpsDIG2, and all corresponding points were assigned a bill region matching the ones in lateral view. We assessed the separation between the maxilla and mandible, and the potential lateral mandibular spreading along the different bill regions throughout the lick cycle, by obtaining the coordinates for each semi-landmark and calculating the Euclidean distance between each corresponding pair of points in the culmen and ventrum (side view), and between tomial profiles (dorsal view).

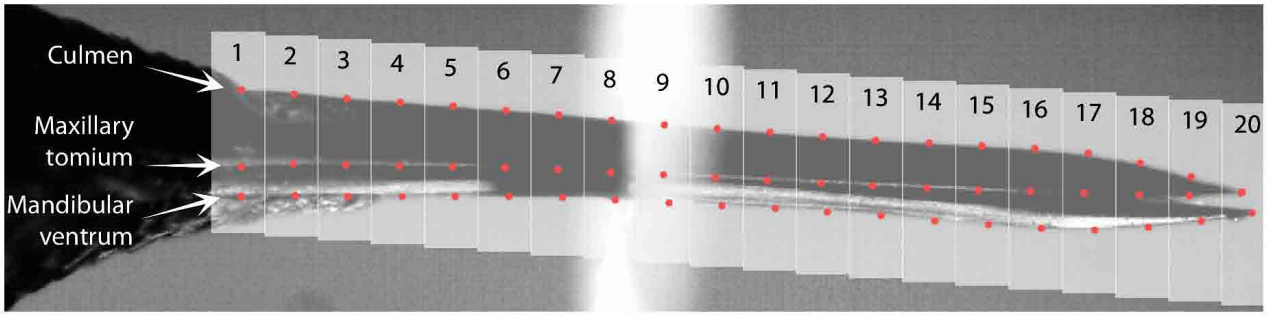



**Figure 2. Semi-landmark placement scheme for bill dorso-ventral motion analyses.** Still picture taken from a high-speed video of an Anna's Hummingbird (*Calypte anna*) drinking nectar. Numbered are the bill regions yielded by the semi-landmarks used in video digitizing. The white shadow between regions 8 and 10 corresponds to the artificial flower we used to guide the hummingbird while keeping it at a desired distance to the artificial nectar reservoir. We did not traced the mandibular tomium because it disappears behind the maxillary one (*i.e.* they overlap).

(b) *Tongue motion analyses*

We outlined the contour of the tongue length outside the bill in both the lateral and dorsal views (to account for deviations in any plane). The profiles of the tongue in both views were outlined from the maxillary bill tip to the tongue tip (Fig. 3). When the tongue bifurcated, the left fork was always sampled to retain consistency. The tongue profiles were initially sampled at a constant 8-millisecond rate, but when tongue visibility was limited (*e.g.* blur), additional images were measured at 2-millisecond intervals. Tongue length at each frame was calculated as the sum of the distances between each point along the tongue contour using the distance measurement analysis function in tpsDIG2.

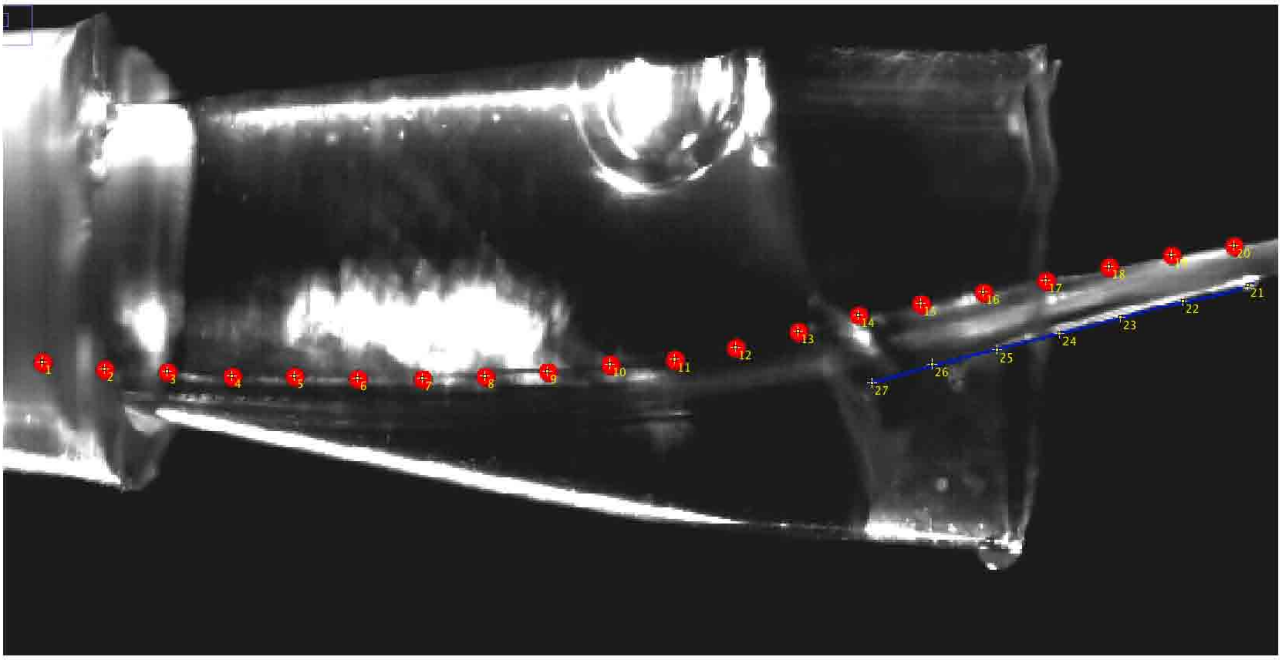



**Figure 3. Semi-landmark placement scheme for tongue displacement analyses.** Still picture taken from a high-speed video of a Rufous-tailed Hummingbird (*Amazilia tzacatl*) drinking nectar, showing the semi-landmarks we used to track the tongue motion. The tip of the tongue appears on the left at the bottom of the artificial nectar reservoir and the markers are equally spaced proximally, the bill tips are on the extreme right (markers 20 and 21). The air-nectar interface that the tongue is traversing is located between markers 13 and 14.

(c) *Intraoral tongue measurements*

In order to directly measure the motion of both the tongue and the nectar inside the bill during intraoral transport, we filmed free-living Rufous-tailed Hummingbirds (*Amazilia tzacatl*), which have paler bills than most species, feeding at nectar feeders using backlighting techniques. We positioned a flashlight (Energizer® Aluminum Alloy Waterproof Lithium LED Flashlight) below the feeder and pointing 45° away from the lens and 45° away from the base of the bill. We kept the hummingbird from noticing the light by using an opaque cardboard barrier. The inclinations (positioning of the flashlight) were intended to maximize light transmission through the keratin, in order to visualize their bills as translucent in the dorsal plane. When properly lit, both the tongue edges and the nectar appeared as shadows inside the backlit bill. Using these videos, broken out as images as described above, we measured the position of the tongue base (visible through the bill keratin) at the point of maximum protrusion of the tongue in all licks. We calculated the position of the tongue base as the Euclidean distance from the bill tip to the midpoint between the bill edges, which overlapped with the tongue base when it was the closest to the bil tip in each lick (maximum protrusion). We also measured the distances from the bill tip to the tongue tip outside the bill in both the lateral and dorsal views (as explained in Tongue motion analyses), for all licks.

We chose four lick sequences in which we were able to accurately visualize the tongue base with high confidence through the entire lick cycle, and measured the position of the tongue base as Euclidean distances from the bill tip. We compared the tongue base tracking to the tongue tip tracking by correlating the distance measurements to fully reconstruct the tongue movements in six additional licks (for a total of ten lick cycles).

Assuming that the tongue does not change in length (*i.e.* it not longitudinally stretchable, *cf.* Weymouth *et al.* 1964, Hainsworth 1973) and calculating the total tongue length in the sequences in which it was visualized with high confidence, we calculated the approximate position of the tongue base for the sequences in which it was difficult to visualize. We did this using the tongue protrusion distances measured in lateral and dorsal views to subtract them from the tongue total length; we use the remainder as the value of the Euclidean distance from the bill tip to the tongue base inside the bill. The complete retraction of the tongue into the bill was used as the minimum base distance (for future analyses) and was the last frame of each sequence; we started the next sequence when the tongue began protruding again.

(d) *Intraoral fluid tracking*

Using the videos in dorsal view of Rufous-tailed Hummingbirds with backlit bills, we tracked the progression of nectar intake through the rhamphothecal keratin; nectar and even bubbles inside of it were visible inside the bill (Fig. 4). Nectar showed in the lit bill as a shadowy mass with a distinct edge at the meniscus. Intraoral flow was measured as meniscal positions within the bill at a 2 millisecond sampling rate from the time in which a nectar meniscus was initially visible in the most distal point within the bill, and continuing through its proximal progression to the bill base.

In each image, the meniscus midpoint between the bill edges was sampled. To do this, two reference points were placed where the fluid came into contact with the inner bill walls; we traced a line between the reference points and resampled it to find a midpoint equidistant to each reference point. The midpoint of the meniscus was used to track the nectar flow using the maxillary bill tip as reference point (Euclidean distances to the menisci) through time.

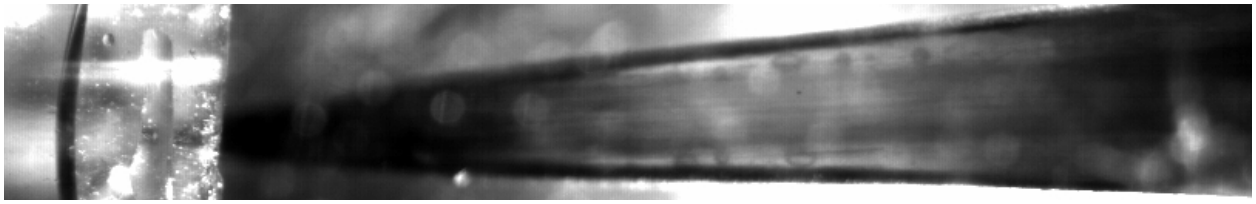


**Figure 4. Visualization of nectar flowing inside a backlit bill.** Still picture taken from a high-speed video of a Rufous-tailed Hummingbird (*Amazilia tzacatl*) drinking nectar; dorsal view. The artificial nectar reservoir is located on the left, and the backlighting makes the visualization of the nectar flow possible. Note the small bubbles trapped between the nectar and the palate (roof of the internal bill space). Even the tongue motion is detectable (by tracking the tongue base displacement) through the bill.

All filming activities were reviewed and authorized by the Institutional Animal Care and Use Committee at the University of Connecticut; Exemption Number E09-010.

## Results

(a) *Bill motion analyses*

We did not observe mandibular spreading (separation of the rami) in the dorsal views of hummingbirds drinking; this means that lateral expansion of the bill base is not a mechanism used by hummingbirds to generate suction in order to move the nectar from the bill tip to the throat. In lateral views, we did not observe the expected pattern of opening and closing of the upper and lower jaws hinging upon the naso-frontal and quadrato-mandibular articulations, respectively. Instead, we found asynchronous opening and closing between the bill base and the bill tip, while the middle portion of the bill stayed relatively fixed (*e.g.* Fig. 5). This asynchronous pattern can be described in terms of wave motions: tracking the separation of the bill tips through time generates a wave-like trace that completes a full oscillation during the licking sequence.

The separation of the bill base semi-landmarks can also be described in a wave trace through time, and since it also completes a full oscillation during the licking cycle; both waves (tips and base separation) have the same frequency. The bill tips and base "waves" are out of phase, which means that the separation of bill tips reaches its maximum right before the separation of the upper and lower bill bases reaches its minimum (almost in antiphase, Fig. 5).

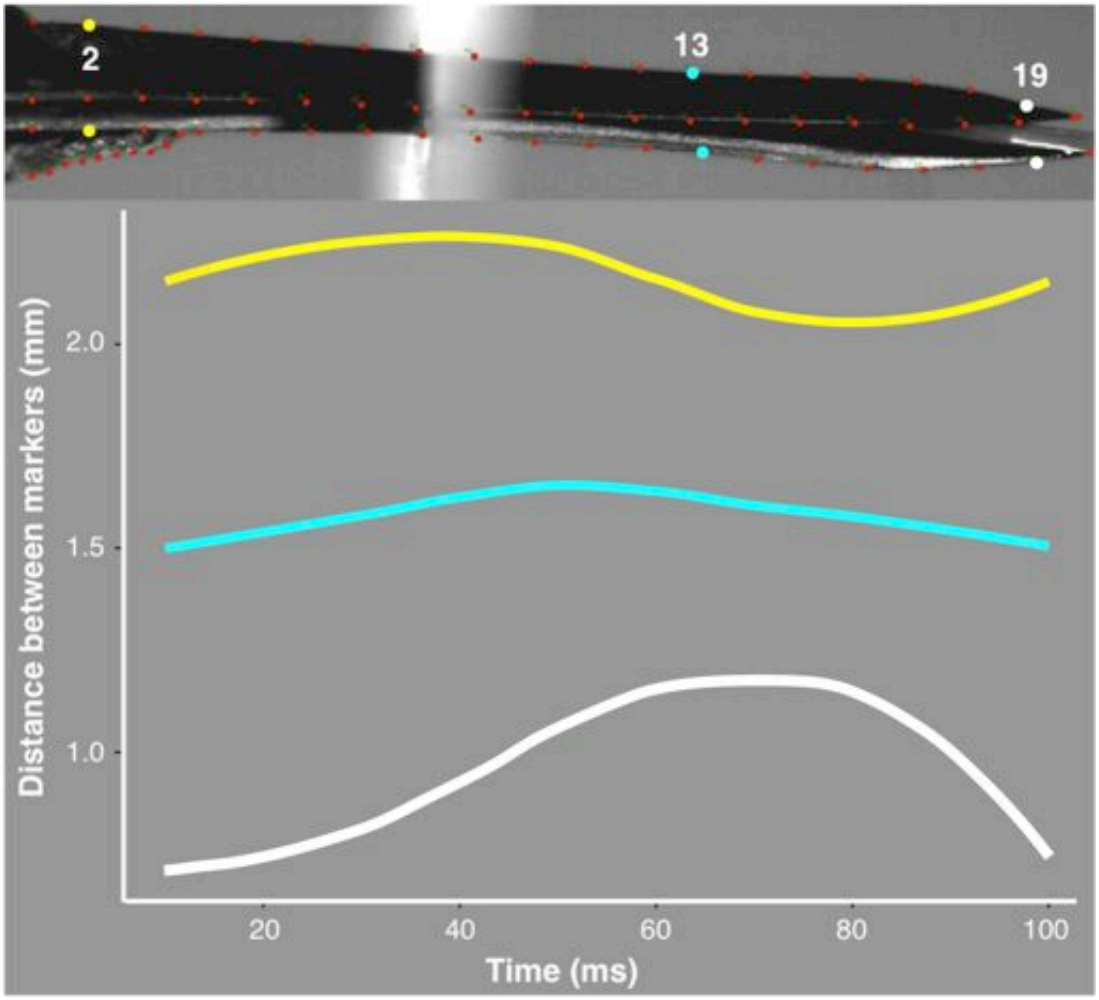



**Figure 5. Distances of separation between culmen and mandibular ventrum point-pairs over time for one individual of *Calypte anna,* for a representative licking cycle.** Only three selected point-pairs are shown (see high-speed video frame on top of the graph), point-pair 2 is the most cranially selected (yellow line at the top of the graph), and point-pair 19 the most rostrally selected one (white line at the bottom of the graph). We avoided selecting the absolute extremes (bill base and bill tip) in order to prevent biasing our interpretations by non-representative trends, however points near the tip and near the base show the same trends are the ones depicted in the graph above. We opted for showing point-pair 13 because this is the zone in which the asynchronous trends between the bill base and tip regions seem to transition (Figs. S1 and S2), hence suggesting a bending zone.

Since the bill base and tips are moving asyncrhonically (Fig. S1), there should be a region in the middle portion of the bill that serves as a hinge enabling the decoupling of the proximal and distal jaw separation (or as a pivot for the maxilla to swivel on top of the mandible, Fig. S2). In order to define the apparent bending zone that permits the asynchronous waves at the distal and proximal regions of the bill, we plotted spatio-temporal areas of separation along the bill length and through the lick cycle (Fig. 6).

Using ten randomly selected licks from different foraging bouts for a single Anna's Hummingbird we obtained an average lick sequence for each one of the point-pairs along length of bill. Given that the timing of each lick was slightly different, we standardize every time-step to a percentage of time throughout the lick cycle. Based on the differential patterns of separation between the point-pairs along the bill and across time, we determined that a bending (or a pivotal) zone is likely to exist around point-pairs 11 to 13 (*e.g.* Fig. 6).

In order to simplify the peaks and valleys of the surface contours depicted in Fig. 6 and thus allow for more straitghforward interspecific comparisons, we analized the ranges of separation per point-pair along the licking cycle. For each culmen-ventrum point-pair along the bill (*e.g.* the three selected in Fig. 5) we calculated the minimal and maximal separation throughout the licking cycle, the difference between these is the range of separation.

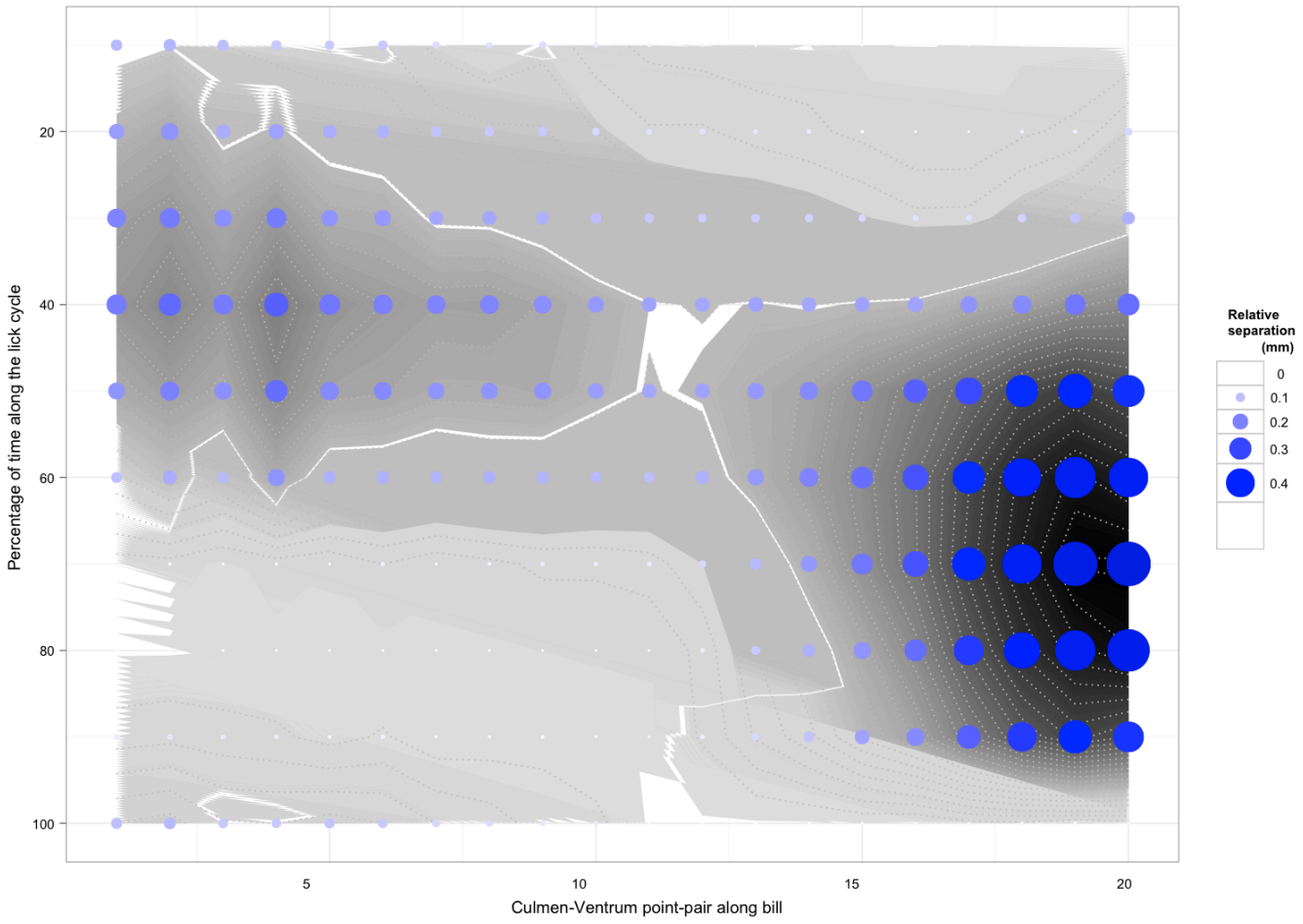



**Figure 6**. **Distances of separation between culmen and mandibular ventrum point-pairs along length of bill (cranial-rostral) over time for one individual of *Calypte anna,* for the average licking cycle.** All point-pairs are shown. In the X-axis, the bill tip is in the right (*cf.* Fig. 5), and in the Y-axis the percentage of time along the licking cycle indicates the start at the top and the end at the bottom. Bubble size and color are proportional to the distance between culmen and ventrum, relative to the minimum distance at a given point-pair across time intervals. Large points (blue) represent more separation, and the smallest points (white) represent minimal separation. Note that towards the middle-distal region (point-pairs 11 and 12) the maximum separation along the entire cycle is smaller than maximum separations towards the base or the tip. This suggests zones of high stability (minimal separation), around which the bill bends, mostly rostrally, but also cranially to a lesser extent. The grey surface contours depict spatio-temporal areas of separation; dark areas indicate large separation and light areas small separation. Note the white fracture in the middle indicating the confluence of low separation valleys (between point-pairs 11 and 13), pinpointing the bending zone.

In order to allow comparisons across species, we equaled the highest range of separation among the point-pairs to 100% (Fig. 7). The interpretation of these data coincides with the one in Fig. 6 for *Calypte anna*; there is a higher range of separation between markers at the base of the bill compared to the middle region and around the point-pair 13 there is a marked increase in separation range rising consistently until the bill tip. In other words, around the middle of the bill, there is less separation between markers than at the base or tip, thus, the middle region of the bill is maintained relatively shut while the base and tip separate independently (*cf.* Figs. 5 and 6).

We observed a similar pattern for all the species studied; the middle bill region is separating less when compared to the base or the tip. It is interesting to note that the species with the shortest bill (*Myrmia micrura*) showed increased separation in the middle region when compared to all the other species, and the species with the longest bill (*Phaethornis baroni*) showed the largest relative separation of the bill base among the species studied (Fig. 7).

To make a multispecies comparison of the asynchronous wave patterns that may be involved in the intraoral transport process, we scaled the lateral bill movement graphs (*e.g.* Fig. 5) to 100% of the maximum bill thickness measured per species on the Y-axis, and to a percentage of time out of the absolute total time of the lick cycle on the X-axis (Fig. 8).

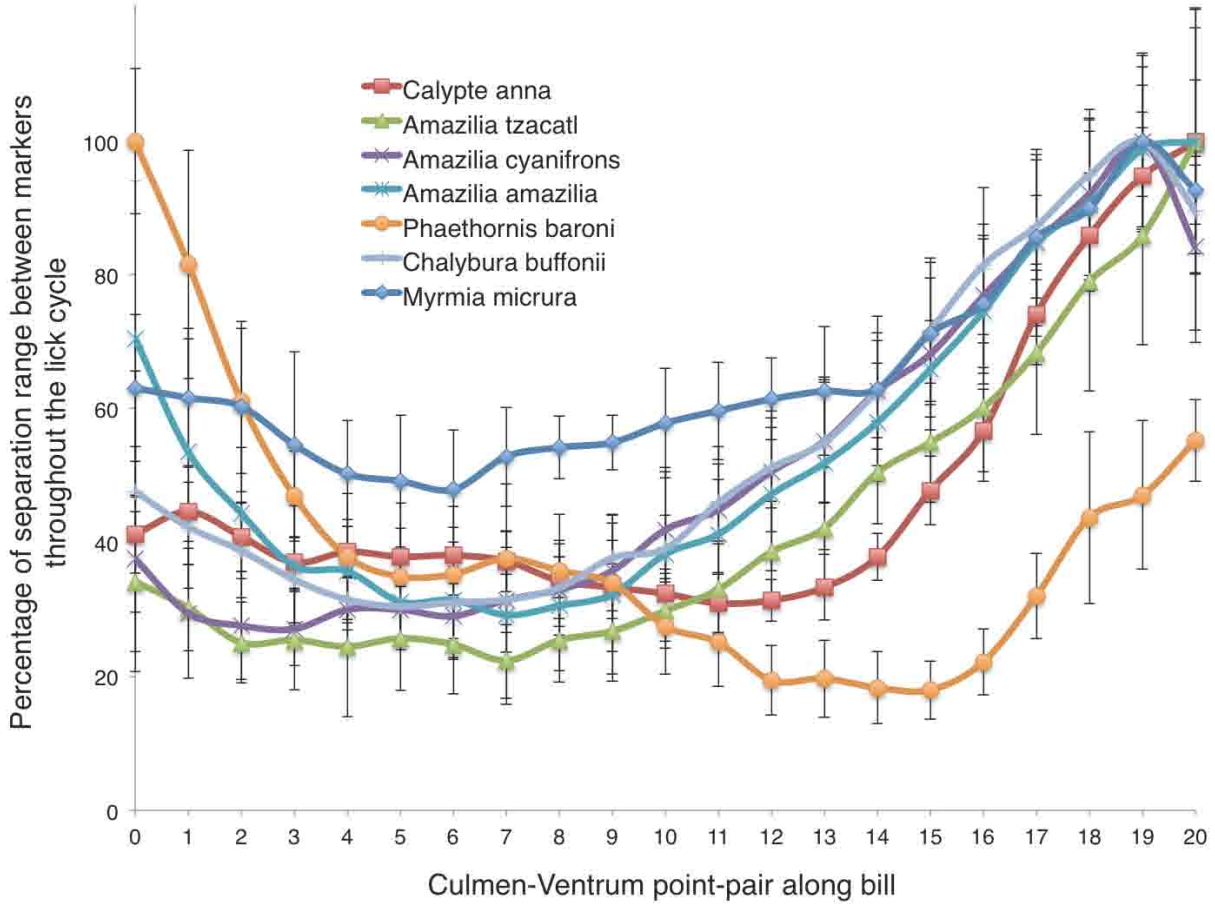



**Figure 7. Ranges of separation (proportional to maximum point-pair range per species) among culmen and mandibular ventrum point-pairs for six species of hummingbirds.** We obtained the range per point-pair by substracting the maximum and mimimum separation values along the lickin cycle. We used ten randomly selected licks from different foraging bouts and report average patterns (with corresponding standard deviations) per species.

We detected the same out-of-phase, equal frequency, pattern of bill tip and base expansion was registered in all of the species we filmed, with some variation in the amplitude of the wave-like trace and in the locations of the peaks and valleys. The bill-base wave was most pronounced (had largest amplitude) in the Baron's Hermit (*Phaethornis baroni*) and the Short-tailed Woodstar (*Myrmia micrura*) and was least pronounced (lowest amplitude) in the Amazilia Hummingbird (*Amazilia amazilia*). The bill tip separation was proportionally greatest in the Rufous-tailed Hummingbird (*Amazilia tzacatl*) and smallest in the Baron's Hermit, which was also the species with the least pronounced (smallest amplitude) tips wave pattern.

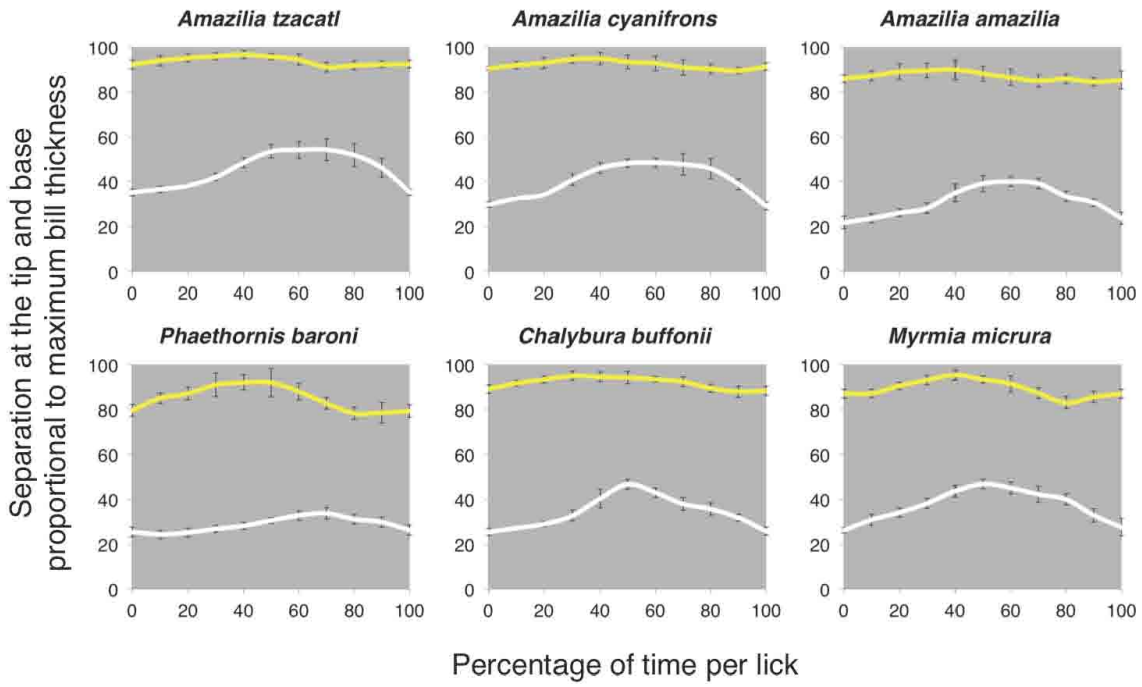



**Figure 8. Distances of separation (proportional to maximum bill thickness) between culmen and mandibular ventrum point-pairs over proportion of time per lick for six species of hummingbirds.** Only two selected point-pairs are shown (*cf.* Fig. 5): point-pair 2 (yellow lines always on the top of each graph, corresponding to the bill base) and point-pair 19 (white lines below the yellow ones, corresponding to the bill tip). We do not present data on Anna's Hummingbird because it follows the same trend in Fig. 5 and a more detailed analysis is presented in Figs. S1 and S2. Using ten randomly selected licks from different foraging bouts we obtained an average wave pattern for each point-pair (with corresponding standard deviations for each time step) per species.

We found consistency among species in the pattern of asynchronous bill tip and base movements (Fig. S3). The maximum separation between the semi-landmarks at the bill base (base wave peak) always occurred at a time between 30% and 50% of the lick cycle duration, and the base wave valley always occurred at a time between 70% and the 90% of the lick cycle duration (Fig. S3). Conversely, the bill tip wave peaks always occurred between 50% and 70% of the lick cycle duration, and all the licks were synchronized using the bill tip valleys (minimum separation of the bill tips) at the start and end of each lick sequence (see Methods: Bill motion analysis).

(b) *Tongue motion analyses*

We found that the tongue is protruded at a time between 0% and 60% of the duration of the licking cycle; during the remaining time it is being retracted with no pauses between changes of direction. From 0% to 40% of the duration of the cycle the bill tips are kept close together (Fig. 5), *i.e*., during most of the tongue protrusion time. While examining individual videos we noticed that the bill tips are close together during the entire extrusion of the tongue grooves, once the base of the grooves reaches the bill tip, the bill tips are forced open by the change in thickness of the tongue and, if the bottom of the nectar reservoir is not detected by the hummingbird, the tongue is protruded further (*e.g.* Movie S1). Therefore, the tongue grooves are squeezed through a small aperture at the bill tips for as long as they can be extruded. While the tongue is being retracted the bill tips are kept far enough apart (the aperture is just big enough) to allow the tongue loaded with nectar to enter the bill (Movie S1).

(c) *Intraoral tongue measurements*

We were able to time-match measurements of the bill base and tips separation with those of the intraoral displacement of the tongue base (Fig. 9). Right before the tongue base is the closest to the bill tip (maximum protrusion) the bill base achieves maximum separation, in other words the internal volume of the bill at the bill base is the largest right before the tongue base is the farthest from the bill base. Similarly, the volume at the bill base is minimal when the tongue is retracting inside the bill.

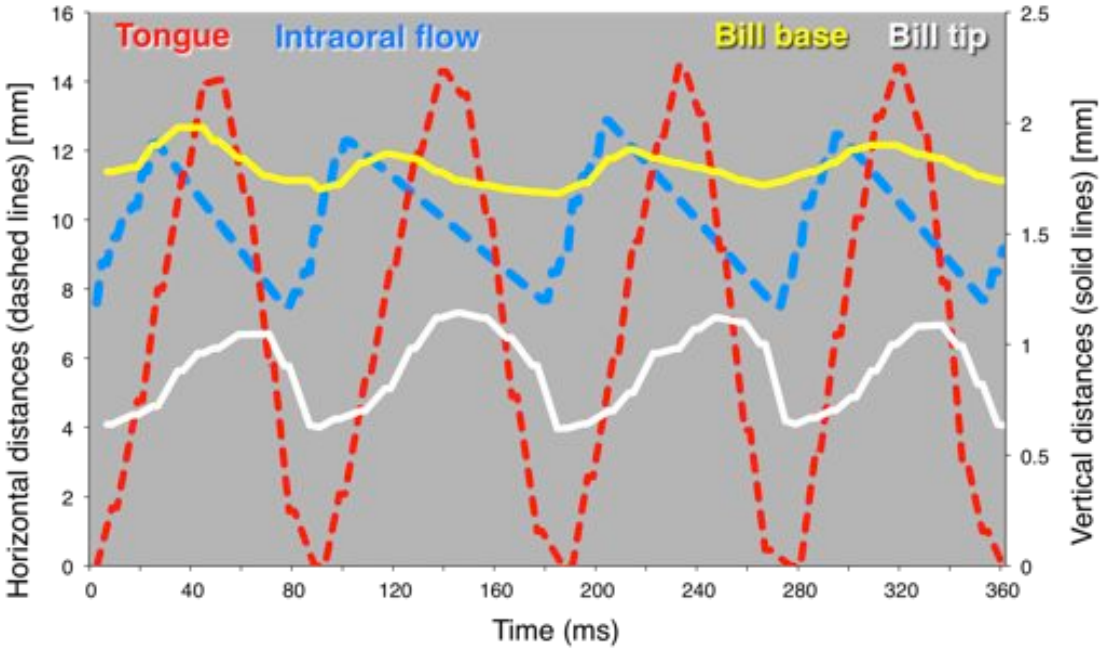



**Figure 9. Bill and tongue movements in relation to intraoral flow for four consecutive licks of a Rufous-tailed Hummingbird (*Amazilia tzacatl*).** The *X-axis* displays the continuous time in milliseconds. The *left Y-axis* portrays the tongue movement (red dashed line) varying from 0 (tongue entirely inside the bill) to 14 millimeters (maximum protrusion); and the intraoral flow of nectar (blue dashed line), measured by following the separation between the proximal nectar meniscus and the bill tip inside the bill. The *right Y-axis* shows the dorso-ventral separation in millimeters at the bill base (yellow solid line on top) and at the bill tip (white solid line at the bottom).

(d) *Intraoral fluid tracking*

We found that upon tongue retraction the nectar starts flowing inside the bill (Movie S2, Fig. 9), and continues filling the internal bill space while the tongue is squeezed by the bill tips (Fig. 10A-D). Once the tongue is maximally protruded and the bill is filled with nectar, the retraction of the tongue displaces the nectar column inside the bill backwards (Fig. 10E-F), a meniscus forms at the tongue base (due to the splitting of the column) suggesting that the tongue wings are dragging the liquid towards the throat (Movie S2).

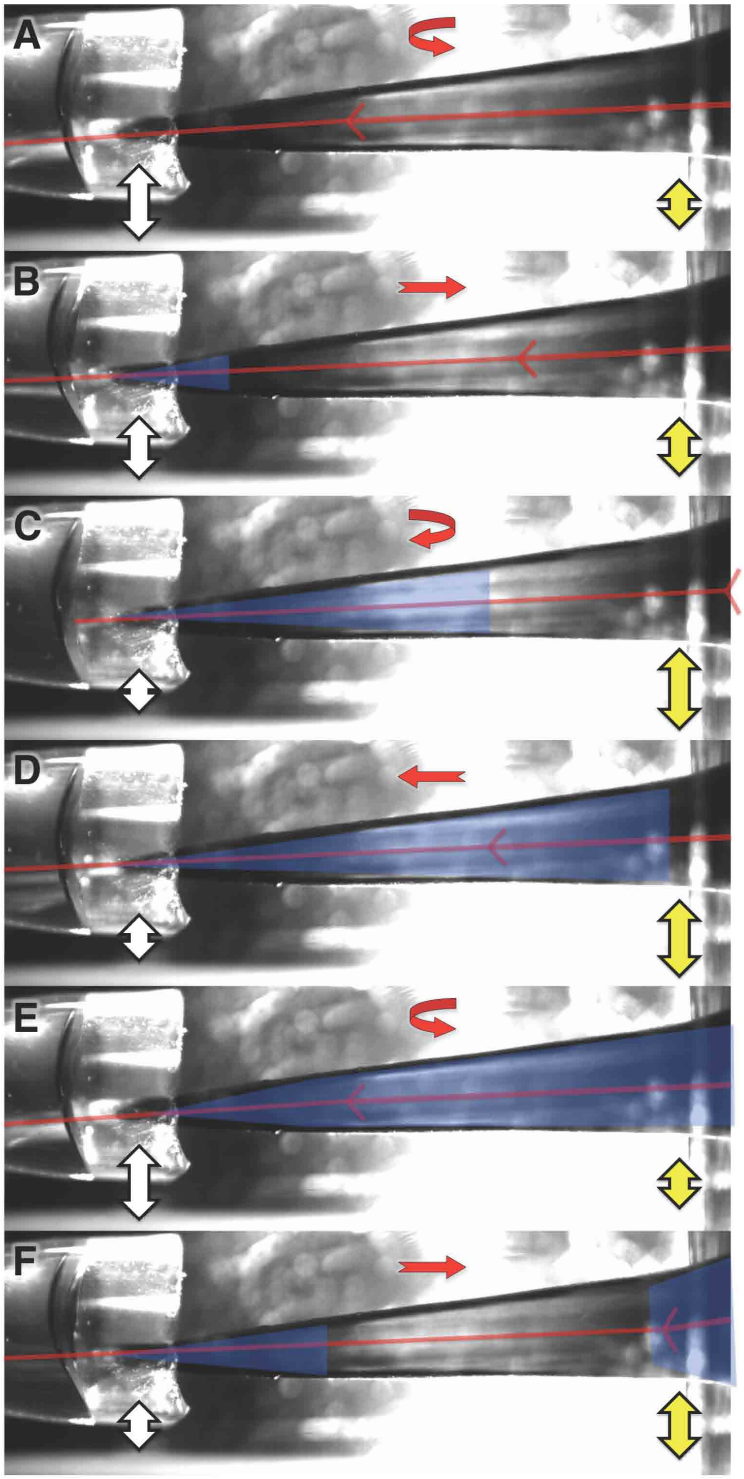



**Figure 10. Frames from a high-speed video of a Rufous-tailed Hummingbird (*Amazilia tzacatl*) drinking nectar**. Dorsal views with overlaying shapes picturing the movements of each one of the elements involved in the intraoral transport process. Red line in the middle of the bill represents the tongue, and the V shape crossing the red line represents the tongue wings at its base. Red straight arrows on top of the bill indicate the direction of the tongue movement (pointing to the left: protrusion, to the right: retraction), and red curved arrows represent the moments in which the tongue changes direction in the reciprocating cycle. Blue shadow inside the bill depicts the nectar flowing intraorally. White double-headed arrows on the left symbolize the dorso-ventral separation of the bill tips, and yellow double-headed arrows denote the dorso-ventral separation of between maxilla and mandible at the bill base (Fig. 9).

Therefore, the retraction of the tongue resets the proximal meniscus, due to the splitting of the nectar column, and this intraoral flow cycle is repeated over consecutive licks (Fig. 9). Proximal displacement of the nectar column by the tongue base is accompanied by a dorso-ventral expansion of the bill base (Figs. 9 and 10), and filling of the bill with nectar is coincident with a reduction of the separation between the bill tips (Figs. 9 and 10).

We tracked the meniscus at the proximal front of the nectar column inside the bill, and found that it was synchronized with the movement of the tongue base (Movie S3, Fig. 10). A single nectar load requires one lick cycle to be squeezed out of the tongue grooves into the oral cavity (filling most of the bill length); subsequently, an additional lick cycle is required to transport the nectar load to the bill base, following the movement of the tongue base backward. Thus, a total of two licks are required to move a single nectar load from the tongue to the throat (Fig. 10, Movie S3). We predict that for shorter protrusion distances more than two licks would be required to transport a single load.

**Discussion**

Now, we have finally found the missing piece of the long-standing feeding mechanics puzzle: how the nectar is unloaded from the tongue (after it is collected) and then moved through the bill to the throat. Since we were able to track menisci inside the bill, we could thus distinguish filled and empty spaces (including small and large bubbles) throughout the licking cycle.

We summarize the intraoral transport steps as follows: 1) the tongue collects the nectar from the pool and tongue retraction starts (Fig. 10A); 2) the bill starts closing, and this action compresses the tongue, releasing nectar inside the bill (a meniscus moves proximally from the tip, Fig. 10B-C); 3) as protrusion starts the tongue is squeezed out through a narrow aperture between the bill tips (the full nectar load is released inside the bill, Fig. 10C-D); 4) the tongue moves backwards after collecting the next load of nectar, and the tongue wings move the nectar already inside the bill (the previous load) towards the throat (Fig. 10E-F); 5) simultaneously the jaws separate dorsoventrally at the bill base, increasing the space available for the incoming load (Fig. 10E-F).

The intraoral transport of a single nectar load is a two-step process; the first load stays inside the bill until the tongue base pushes it backwards when the second one comes (comparte panels B and F in Fig. 10), and so forth. This mechanism could explain why hummingbirds extend their tongues far beyond their bill tips after foraging bouts (pers. obs.), they are emptying the tongue grooves by extruding them completely and at the same time they are cleaning the inside of their bills by pushing backwards any remnants with their tongue wings. Future confirmation of our results on the Rufous-tailed Hummingbird would be possible working with other species with red bills (lacking melanin in some regions) varying in length and curvature; *e.g.* Spangled Coquette (*Lophornis stictolopha*), White-tailed Goldenthroat (*Polytmus guainumbi*) and Sapphires (*Hylocharis spp.*).

We found asynchronous base-tip motion (*e.g.* Fig. 7) on 27 hummingbird species (Table S1). In order to maximize nectar uptake efficiency, offloading as much nectar as possible from the tongue is paramount for maintaining the maximum loading capacity across consecutive licks. After squeezing the tongue to unload the nectar inside the bill, the liquid must be transported towards the throat in order to be swallowed; if this process is not rapid and complete, the rate at which the bird can add additional nectar to the intraoral space is limited, and thus so is its overall intake rate. In hummingbirds, this intraoral transport must be coordinated with tongue squeezing at rates up to 20 times per second. A bending (or pivot) zone in the middle region of the tip has the potential to coordinate independent movements of the bill base and tips, by allowing the tips to open and close (nectar on- and off-loading) while keeping the middle region of the bill tightly closed (improving intra-oral transport).

Christian Nitzsch, back in 1816, puzzled by an apparent movement of the hummingbird (*Trochilus*) bill tip, restricted to the maxilla, believed that opening the whole bill while drinking from flowers would be unnecessary and inappropriate (Nitzsch 1816). Opening at the bill tips only was thought to optimize the feeding process by coupling bill and tongue movements (*cf.* Moller 1930, 1931, 1932); and anatomically, the hummingbird bill was thought to have extensive flexion zones along the maxilla (*cf.* Bühler 1981). However, in the most exhaustive comparative morphological survey on avian rhynchokinesis (bending of the distal portions of the bill), Zusi (1984) did not find osteological evidence supporting this kind of bill bending in hummingbirds, and suggested that the apparent bending near the bill tip is an optical illusion caused by the mandible being partially ensheathed inside the maxilla in lateral view.

In the most recent review, and after studying our videos (Rico-Guevara and Rubega 2011), Zusi (2013) concluded that distal rhynchokinesis seems to be happening at least in few species of hummingbirds. Throuhgout a detailed study of rhynchokinesis in hummingbirds (which will be presented elsewhere) we found that bill bending is ubiquitous in all the main clades (unpub. data).

Birds have evolved a form of cranial kinesis that confers the ability to move the maxilla with respect to the cranium through narrow bending zones of thin bone (*i.e.* cranio-facial hinge; Simonetta 1960; Bock 1964; Bout & Zweers 2000; Holliday and Witmer 2008). However, amidst the large variety of functional adaptations, some bird families have evolved the capability to bend their bills beyond the cranio-facial hinge (*s.s.* prokinesis), such that the bill itself bends, either along the maxilla – *s.s.* rhynchokinesis (Zusi 1984) – or along the rami of the mandible (*e.g.* Bühler 1981; Yanega and Rubega 2004, Field *et al.* 2011). Although all birds possess kinetic skulls to some degree or other, rhynchokinesis is mostly expressed in shorebirds (*e.g.* the Charadriiformes). Their abilities to open and close the tips of the bill independently of the base have traditionally been associated with their substrate-probing feeding habits (Zusi 1984; Zweers and Gerritsen 1997; Gussekloo *et al.* 2001), their unique surface-tension transport mechanism (*e.g.* phalaropes, Rubega and Obst 1993), and have recently been tied to enhancing feeding efficiency on small aquatic prey in *Calidris* scolopacids (Estrella and Masero 2007). However, bill bending is considerably less well understood outside of shorebirds, and few other clades are known to possess this ability.

One of the few clades capable of extensive bill bending is the Cypselomorphae, which contains the Caprimulgiformes (*e.g.* nighthawks), Apodiformes (*e.g.* swifts and hummingbirds), and Aegotheliformes (*e.g.* nightjars). The families in this group are aerial insectivores that use mandibular bending for gape expansion to increase prey-catching area (Bühler 1981; Smith *et al.* 2011). As noted above, this clade includes hummingbirds whose ancestors were short-billed aerial predators resembling modern cypselomorphs such as swifts (Apodidae). To be able to take advantage of nectar as a food resource, hummingbird tongues have become elongated and protractible. Furthermore, through coevolution with increasingly specialized ornithophilous plants (*i.e.* insect exclusion to optimize avian pollination), their beaks became long and slender. Additionally, their tongues became highly efficient liquid trapping devices (Rico-Guevara and Rubega 2011) closely coupling to, and paralleling, bill morphology. Interestingly, such an ostensibly high degree of morpho-functional specialization for nectarivory has not compromised their abilities to effectively perform aerial insectivory, in large part because of mandibular bending. Yanega and Rubega (2004) demonstrated that ventral flexion of the mandible enhances aerial prey capture success. Similarly, here we propose that a form of distal rhynchokinesis operating in the maxilla (Figs. 5 and 6) may confer distinct advantages for enhancing nectar feeding efficiency (Figs. 9 and 10).

We found that the tongue base has an important role for the intraoral transport of nectar. Hummingbirds have two flaps at either side of the base of the tongue, also called “tongue wings” (Sharnke 1931; Weymouth *et al*. 1964). In most birds the tongue wings are part of a larger structure called “papillary crest” and are triangular, spiny, and directed proximally; helping to move food items towards the throat (reviews Parchami *et al.* 2010, Erdoğan and Iwasaki, 2013).

In hummingbirds the capillary crest is reduced to the tongue wings or flaps, and our videos demonstrate that when the tongue is being protruded the flaps fold towards the center of the tongue; and when the tongue is being retracted (moving proximally) the flaps open almost perpendicularly to the tongue axis (Movies S2, S3). The "opened tongue flaps" act as paddles, dragging fluid towards the bill base, as the tongue is moving backwards. The whole system works in accord with flow dynamics, in much the same way that valves in the vascular system work, promoting unidirectional fluid flow in the direction of the pharynx. Given that the tongue wings or flaps are made of connective tissue and a sheath of keratin (Weymouth *et al.* 1964), there are no tendons or muscle attachments to make them able to be controlled independently. Hence, the birds do not need to expend muscular action for the opening and closing of the flaps; drag and resistance do all the work for them.

Navigating our initial hypothesis flow chart (Fig. 1A), we found that the bill base does not seem to be hermetic (in some videos a light between mandible and maxilla is evident, *e.g.* Fig. S4, Movies S4 and S5) during the drinking process. This observation discards the hypotheses involving the generation of a vacuum at the bill base, between the tongue and the palate near the bill base (Gadow 1883), or at throat region (Döhling 1931, Steinbacher 1935). In a similar way, we did not find evidence for cohesive pulling via tongue retraction (Moller 1930, Scharnke 1931), a process analogous to a syringe mechanism, in which the barrel is the bill and the plunger is the tongue. In the case of drinking in hummingbirds we found that the tongue (plunger) is pulled faster than it would be necessary to prevent rupturing the nectar column, the end result is that only a small meniscus follows the front of the tongue base after the column splits (Fig. 10).

In contrast, we found that the fluid is moved proximally on the backside of the tongue base, *i.e.* the nectar is not being pulled but it is being pushed (Movie S2). We did not find evidence of lateral mandibular expansion at the bill base (*e.g.* Yanega and Rubega 2004) during drinking, but we did find dorso-ventral separation between the mandible and maxilla that probably creates cohesive pulling through expansion of the bill base (Fig. 1B).

We did not find that the region near the bill tips stays static allowing for intraoral capillarity to be the main driving force, instead we did find support for Ewald and Willams' hypothesis (1982) of tongue squeezing near the bill tip. In conclusion we found that the nectar is transported intraorally by a combination of dorso-ventral expansion at the bill base, the tongue wings pushing the fluid backwards, and the hydraulic pushing through extruding the tongue at the bill tip.

Understanding the intraoral transport mechanism opens the doors to explore its ecological and evolutionary implications, for instance the potential cost of having enlongated bills in terms of the time and energy invested moving fluid through a longer tube. Long bills could be beneficial to hummingbirds by facilitating probing inside long corollas (Wolf *et al.* 1972) and maintaining small distances between nectar and bill tips. This length coupling may yield greater rates of licking squeezing more efficiently nectar loads off of the tongue (Ewald and Williams 1982). This hypothesis is in accordance with the fact that bills of hummingbirds tend to be similar in length to the corollas of the flowers they usually feed on (Wolf *et al.* 1972, 1976).

Since foraging efficiency (caloric value obtained relative to the caloric costs for obtaining) ought to be improved through evolution (*e.g.* Schoener 1971, Tullock 1971, Houston and McNamara 2014), individuals of a given hummingbird species (or sex) should be more efficient (nectar intake rate) while feeding on flowers matching their bill length and shape (*e.g.* Wolf *et al.* 1972, Temeles and Roberts 1993, Temeles *et al.* 2000, 2010). However, tests of the "bill-corolla matching hypothesis" have produced conflicting results; it would be expected that long-billed species were more efficient at long flowers and short-billed hummingbirds were more efficient at short flowers, but in fact previous research has failed to support the second prediction (*e.g.* Hainsworth 1973; Montgomerie 1984, Temeles and Roberts 1993, Temeles 1996). In other words, under experimental conditions: Longer-billed birds feed more quickly from longer flowers than shorter-billed birds, but shorter-billed birds do not feed more quickly from shorter flowers than longer-billed ones (review in Temeles 1996). Longer bills, by probing deeper inside corollas, achieve smaller distances between the bill tip and the nectar than shorter bills. Smaller bill tip – nectar distances allow for higher licking rates (Ewald and Williams 1982), and elevated nectar extraction efficiency (Hainsworth 1973, Hainsworth and Wolf 1976, Montgomerie 1984, Grant and Temeles 1992, Temeles and Roberts 1993, Temeles 1996).

Temeles (1996) proposed a drawback for possessing longer bills: Longer-billed hummingbirds seem to make more insertion errors when feeding in narrow flowers compared to shorter-billed hummingbirds. Insertion errors would increase handling time (total time of floral visit) and therefore render diminished net energy gain per visit (*cf.* Temeles 1996). Nevertheless, there has not been a proposal for a negative influence of longer transport times in long-billed species as a drawback for its acquirement.

A close match between shorter bills and shorter corollas should allow for shorter transit times from the nectar source to throat. Therefore having a long bill could be costly and sometimes inconvenient. From a biomechanical point of view, bills should be just as long as absolutely needed, longer bills could access to a wider floral spectrum however this should increase the difficulty of transport from bill tip to throat. The mere existence of hummingbirds with really short bills (assuming a derived condition, which seems to be supported *cf.* McGuire *et al.* 2014) could be a clue about how transport efficiency can affect bill length.

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## Literature cited


Anker, G. C., Simons, J., & Dullemeijer, P. (1967). An apparatus for direct X-ray cinematography exemplified by analysis of some respiratory movements in *Gasterosteus aculeatus*. *Experientia*, *23*(1), 74-77.

Bock, W. J. (1964). Kinetics of the avian skull. *Journal of Morphology*, *114*(1), 1-41.

Böker, H. (1937). Einführung in die vergleichende biologische Anatomie der Wirbeltiere. Gustav Fischer, Jena. Vol. 1, xii, 228 p.

Borrell, B. J. (2007). Scaling of nectar foraging in orchid bees. *Am. Nat.* 169(5): 569-80.

Bout, R. G., & Zweers, G. A. (2001). The role of cranial kinesis in birds. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *131*(1), 197-205.

Brainerd E.L., Baier D.B., Gatesy S.M., Hedrick T.L., Metzger K.A., Gilbert S.L. & Crisco J.J. (2010). X-ray reconstruction of moving morphology (XROMM): precision, accuracy and applications in comparative biomechanics research. *J. Exp. Zool.* 313A:262–279.

Bühler, P. (1981). Functional anatomy of the avian jaw apparatus. *Form and function in birds*, *2*, 439-468.

Cheke, R. A. & Mann, C. (2008) *Family Nectariniidae (Sunbirds)*. In: del Hoyo, Josep, Elliot, Andrew andChristie, David A., (eds.) Handbook of the birds of the world: Penduline-tits to Shrikes. Handbook of the Birds of the World, Volume 13 . Lynx Edicions, Barcelona, Spain, pp. 196-243.

Collins, B. G. (2008). Nectar intake and foraging efficiency: responses of honeyeaters and hummingbirds to variations in floral environments. *The Auk* 125(3): 574-587.

Crompton, A. W. & Musinsky, C. (2011) How dogs lap: ingestion and intraoral transport in *Canis familiaris*. *Biol Lett* 7(6):882–884.

Darwin, C. R. ed. (1841). *Birds Part 3 of The zoology of the voyage of H.M.S. Beagle*. by John Gould. Edited and superintended by Charles Darwin. London: Smith Elder and Co.

Dawson, M. M., Metzger, K. A., Baier, D. B., & Brainerd, E. L. (2011). Kinematics of the quadrate bone during feeding in mallard ducks. *The Journal of experimental biology*, *214*(12):2036-2046.

Döhling, Fritz. (1931) Die Kolibris lecken und saugen ihre Nahrung. Ornitholog. Monatsbericht 39.

Downs, C. T. (2004). Some preliminary results of studies on the bill and tongue morphology of Gurney's Sugarbird and some southern African sunbirds. *Ostrich-Journal of African Ornithology*, *75*(3):169-175.

Erdoğan, S., & Iwasaki, S. I. (2014). Function-related morphological characteristics and specialized structures of the avian tongue. *Annals of Anatomy-Anatomischer Anzeiger*, *196*(2):75-87.

Ewald, P.W. & W. A. Williams. (1982). Function of the bill and tongue in nectar uptake by hummingbirds. *Auk* 99:573-576.

Estrella, S. M., & Masero, J. A. (2007). The use of distal rhynchokinesis by birds feeding in water. *Journal of Experimental Biology*, *210*(21):3757-3762.

Field, D. J., Lin, S. C., Ben-Zvi, M., Goldbogen, J. A., & Shadwick, R. E. (2011). Convergent evolution driven by similar feeding mechanics in balaenopterid whales and pelicans. *The Anatomical Record*, *294*(8):1273-1282.

Gadow, H. (1883). On the suctorial apparatus of the Tenuirostres. Proceedings of the Zoological Society of London. 62-69.

Gatesy, S.M. & T. Alenghat. (1999). A 3-D computer-animated analysis of pigeon wing movement. Amer. Zool. 39, 104A.

Gould, J. (1861). An introduction to the Trochilidae: Or family of humming-birds. London: Printed by Taylor and Francis.

Grant, V., & Témeles, E. J. (1992). Foraging ability of rufous hummingbirds on hummingbird flowers and hawkmoth flowers. *Proceedings of the National Academy of Sciences*, *89*(20):9400-9404.

Greenlee, K. J., Socha, J. J., Eubanks, H. B., Pedersen, P., Lee, W. K., & Kirkton, S. D. (2013). Hypoxia-induced compression in the tracheal system of the tobacco hornworm caterpillar, Manduca sexta. *Exp. Biol. 216*(12):2293-2301.

Gussekloo, S. W., Vosselman, M. G., & Bout, R. G. (2001). Three-dimensional kinematics of skeletal elements in avian prokinetic and rhynchokinetic skulls determined by Roentgen stereophotogrammetry. *Journal of Experimental Biology*, *204*(10), 1735-1744.

Hainsworth, F. R. (1973). On the tongue of a hummingbird: its role in the rate and energetics of feeding. *Comp. Biochem. Physiol.* 46A: 64-78

Haisworth, F. R., & Wolf, L. L. (1976). Nectar characteristics and food selection by hummingbirds. *Oecologia 25*(2):101-113.

Heyneman, A. J. (1983). Optimal sugar concentrations of floral nectars: dependence on sugar intake efficiency and foraging costs. *Oecologia* 60:198-213.

Holliday, C. M., & Witmer, L. M. (2008). Cranial kinesis in dinosaurs: intracranial joints, protractor muscles, and their significance for cranial evolution and function in diapsids. *Journal of Vertebrate Paleontology 28*(4):1073-1088.

Houston, A. I., & McNamara, J. M. (2014). Foraging currencies, metabolism and behavioural routines. *Journal of Animal Ecology 83*(1):30-40.

Kim W., Gilet T. and J. W. M. Bush. (2011). Optimal concentrations in nectar feeding. *Proc Natl Acad Sci USA* 108:16618-16621.

Kim W., & Bush, J. W. M. (2012). Natural drinking strategies. *J Fluid Mech* 705:7–25.

Kim, W., Peaudecerf, F., Baldwin, M. W., & Bush, J. W. (2012). The hummingbird's tongue: a self-assembling capillary syphon. *Proceedings of the Royal Society B: Biological Sciences* 279(1749):4990-4996.

Kingsolver, J. G. and T. L. Daniel. (1983). Mechanical determinants of nectar feeding strategy in hummingbirds: Energetics, tongue morphology, and licking behavior. *Oecologia* 60: 214–226.

Kooloos, J. G. M., & Zweers, G. A. (1989). Mechanics of drinking in the mallard (Anas platyrhynchos, Anatidae). *Journal of morphology*, *199*(3):327-347.

Liversidge, R. (1967). The tongues and feeding methods of sunbirds, white-eyes, and sugarbirds. In: Skead CJ (ed) The Sunbirds of Southern Africa, also Sugarbirds, White-eyes and the Spotted Creeper. pp 27–32. Balkema, Cape Town

Lucas, F. A. (1891). On the structure of the tongue of hummingbirds. *Proc. U. S. Nat. Mus.* 14:169-172.

Martin WCL (1833) The Naturalist's Library: A General History of Humming-Birds or the Trochilidae, ed W Jardine (H.G. Bohn, London), Vol 41.

McGuire, J. A., Witt, C. C., Remsen Jr, J. V., Corl, A., Rabosky, D. L., Altshuler, D. L., & Dudley, R. (2014). Molecular phylogenetics and the diversification of hummingbirds. *Current Biology*, *24*(8):910-916.

Metscher B. D. (2009). MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC Physiology* 9(11):1-14.

Moller, W. (1930). Über die Schnabel- und Zungenmechanik blütenbesuchender Vögel. I. *Biol. Generalis* VI:651-726.

Moller, W. (1931). Bemerkungen zu Scharnke's Mitteilung 'Die Nektaraufnahme mit der Kolibrizunge'. *Ornithol. Monatsber*. 39:135-138.

Moller, W. (1932). Die Zungen der kostarizensischen Zuckervögel. Z. *Mikr.-anat. Forsch.* 28:363-417.

Montgomerie, R. D. (1984) Nectar extraction by hummingbirds: Response to different floral characters. *Oecologia* 63:229–236.

Muller, M., & Osse, J. W. M. (1984). Hydrodynamics of suction feeding in fish. *The Transactions of the Zoological Society of London*, *37*(2):51-135.

Nitzsch, Christian L. (1816). Über die Bewegung des Oberkiefers der Vögel. *Deutsches Archiv für die Physiologie* 2:361-380.

Parchami, A., Dehkordi, R. A. F., & Bahadoran, S. (2010). Scanning electron microscopy of the tongue in the golden eagle Aquila chrysaetos (Aves: Falconiformes: Accipitridae). *World Journal of Zoology* 5:257-263.

Paton, D. C., & Collins, B. G. (1989). Bills and tongues of nectar-feeding birds: A review of morphology, function and performance, with intercontinental comparisons. *Australian Journal of Ecology* 14(4):473-506.

Rico-Guevara, A. and M. A. Rubega. (2011). The hummingbird tongue is a fluid trap, not a capillary tube. *Proc Natl Acad Sci USA* 108:9356–9360.

Rico-Guevara A. and M. A. Rubega. (2012). Hummingbird feeding mechanics: Comments on the capillarity model. *Proc Natl Acad Sci USA* 109:E867.

Rohlf FJ (2010) *TPSDig2 Version 2.16* (Department of Ecology and Evolution, Stony Brook University, New York).

Rubega, M. A., and Obst, B. S. (1993). Surface-tension feeding in phalaropes: discovery of a novel feeding mechanism. *The Auk* 110(2):169-178.

Scharnke, H. (1931). Beiträge zur Morphologie und Entwicklungsgeschichte der Zunge der Trochilidae, Meliphagidae und Picidae. *Journal of Ornithology* 79(4):425-491.

Schneider, C. A., Rasband, W. S., & Eliceiri, K.W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9:671-675.

Schoener, T. W. (1971). Theory of feeding strategies. *Annual review of ecology and systematics* 2:369-404.

Simon, M. A., Woods Jr, W. A., Serebrenik, Y. V., Simon, S. M., van Griethuijsen, L. I., Socha, J. J., ... & Trimmer, B. A. (2010). Visceral-locomotory pistoning in crawling caterpillars. *Current biology*, *20*(16):1458-1463.

Simonetta, A. M. (1960). On the mechanical implications of the avian skull and their bearing on the evolution and classification of birds. *Quarterly Review of Biology* 35(3):206-220.

Smith, M. L., Yanega, G. M., & Ruina, A. (2011). Elastic instability model of rapid beak closure in hummingbirds. *Journal of theoretical biology* 282(1):41-51.

Steinbacher, G. (1935). Der Trinkakt der Kolibris. *Ornithologische Monatsberichte* 43:11-15.

Steudle, E. (2001). The cohesion-tension mechanism and the acquisition of water by plant roots. *Annual review of plant biology* 52(1):847-875.

Stiles, F.G. (1975). Ecology, flowering phenology, and hummingbird pollination of some Costa Rican Heliconia species. *Ecology* 56: 285-301.

Stiles, F. G. (1981). Geographical aspects of bird-flower coevolution, with particular reference to Central America. *Annals of the Missouri Botanical Garden* 68:323-351.

Sundevall, C. J. (1872). *Methodi naturalis avium disponendarum tentamen: Försök till fogelklassens naturenliga uppställnung*. Samson & Wallin.

Temeles, E. J., & Roberts, W. M. (1993). Effect of sexual dimorphism in bill length on foraging behavior: an experimental analysis of hummingbirds. *Oecologia* 94(1):87-94.

Temeles, E. J. (1996). A new dimension to hummingbird-flower relationships. *Oecologia* 105(4):517-523.

Temeles, E. J., Pan, I. L., Brennan, J. L., & Horwitt, J. N. (2000). Evidence for ecological causation of sexual dimorphism in a hummingbird. *Science* 289(5478):441-443.

Temeles, E. J., Miller, J. S., & Rifkin, J. L. (2010). Evolution of sexual dimorphism in bill size and shape of hermit hummingbirds (Phaethornithinae): a role for ecological causation. *Philosophical Transactions of the Royal Society B: Biological Sciences* 365(1543):1053-1063.

Tschapka, M., Sperr, E. B., Caballero-Martinez, L.A., & Medellin, R. A. (2008). Diet and cranial morphology of *Musonycteris harrisoni*, a highly specialized nectar-feeding bat in Western Mexico. *J Mamm* 89:924-932.

Tullock, G. (1971). The coal tit as a careful shopper. *American Naturalist* 105:77-80.

Vogel, S. (2011). Surface tension helps a tongue grab liquid. *Proc Natl Acad Sci USA* 108: 9321–9322.

Wheeler, T. D., & Stroock, A. D. (2008). The transpiration of water at negative pressures in a synthetic tree. *Nature* 455(7210):208-212.

Weymouth R.D., Lasiewski, R.C., & Berger, A. J. (1964). The tongue apparatus in hummingbirds. *Acta Anatomica* 58:252-270.

Wolf, L. L., Hainsworth, F. R., & Stiles, F. G. (1972). Energetics of foraging: rate and efficiency of nectar extraction by hummingbirds. *Science* 176(4041):1351-1352.

Wolf, L. L., Stiles, F. G., & Hainsworth, F. R. (1976). Ecological organization of a tropical, highland hummingbird community. *The Journal of animal ecology* 45:349-379.

Yanega, G. M., & Rubega, M. A. (2004). Feeding mechanisms: Hummingbird jaw bends to aid insect capture. *Nature* 428(6983):615-615.

Zimmermann, U., Schneider, H., Wegner, L. H., & Haase, A. (2004). Water ascent in tall trees: does evolution of land plants rely on a highly metastable state?. *New Phytologist 162*(3):575-615.

Zusi, R. L. (1984). A functional and evolutionary analysis of rhynchokinesis in birds. Smithsonian Institution Press.

Zusi, R. L. (2013). Introduction to the Skeleton of Hummingbirds (Aves: Apodiformes, Trochilidae) in Functional and Phylogenetic Contexts. *Ornithological Monographs* 77:1-94.

Zweers, G. A. (1982) Drinking of the Pigeon (*Columba livia* L.) *Behaviour* 80:274-317

Zweers, G. A., Gerritsen, A. F., & van Kranenburg-Voogd, P. J. (1977). Mechanics of feeding of the mallard (*Anas platyrhynchos L.*; Aves, Anseriformes): the lingual apparatus and the suction-pressure pump mechanism of straining. S. Karger.

Zweers, G. A., & Gerritsen, A. F. C. (1996). Transitions from pecking to probing mechanisms in waders. *Netherlands Journal of Zoology* 47(2):161-208.

## Supplementary Figures

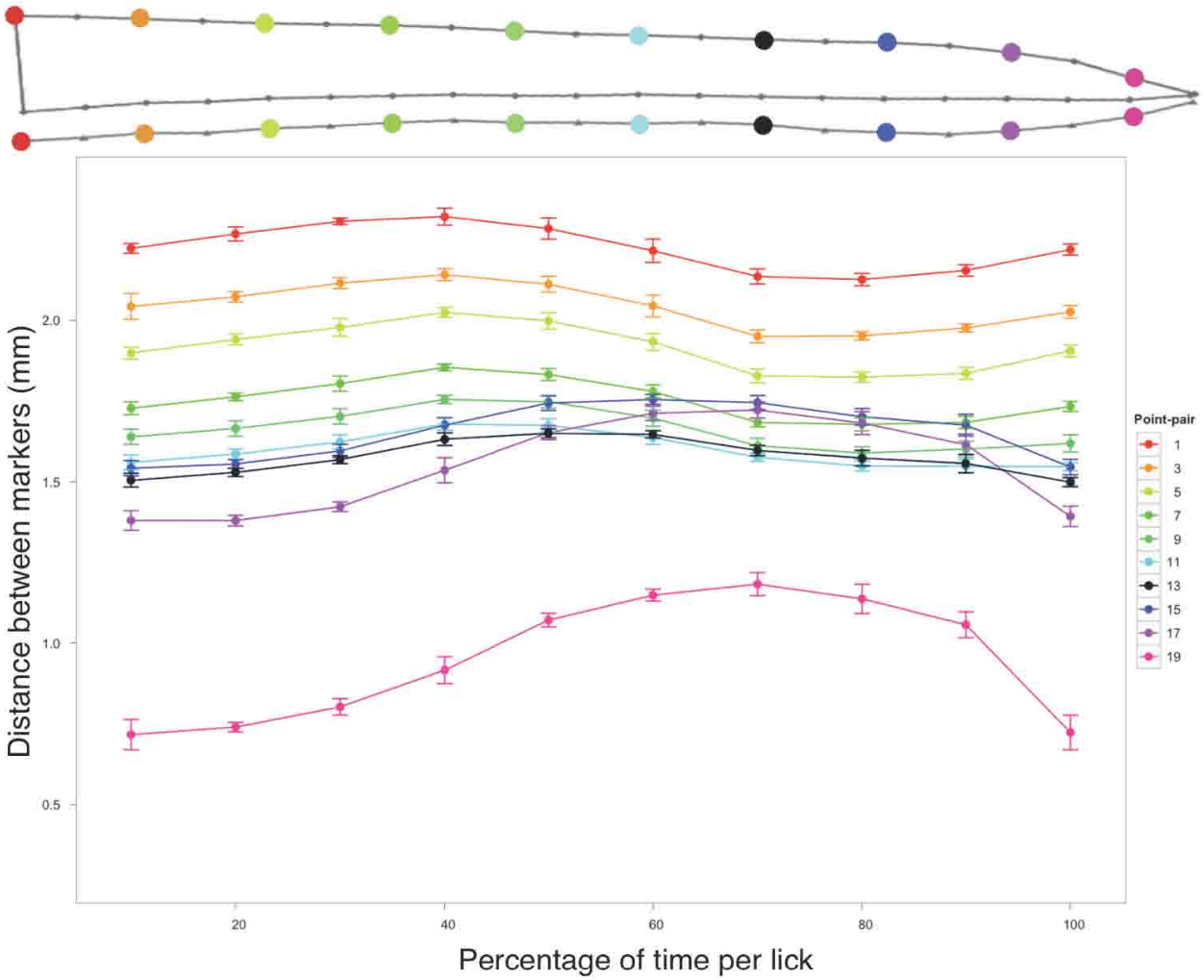



**Figure S1. Distances of separation between culmen and mandibular ventrum point-pairs over time for *Calypte anna*.** Only odd point-pairs are shown. The point-pairs scheme on top of the graph was generated from XY coordinates taken from a high-speed video frame (*cf.* Fig. 5), and the colors correspond to the lines in the graph. Using ten randomly selected licks from different foraging bouts we obtained an average movement pattern for each point-pair with corresponding standard deviations for each time step. Note that point-pairs 1-11 follow the same motion pattern although the absolute displacement is greater in the most proximal points, which suggest that the basal bill motion is buffered towards the middle of the bill in a hinge (or pivot) zone. In a similar way, the most distal point-pairs (15-19) follow the same pattern but the absolute change in separation is greater distally (compare 19 *vs.* 15), pointing towards the same hinge zone. Point-pair 13 appears as intermediate between the asynchronous patterns at the bill base and tip.

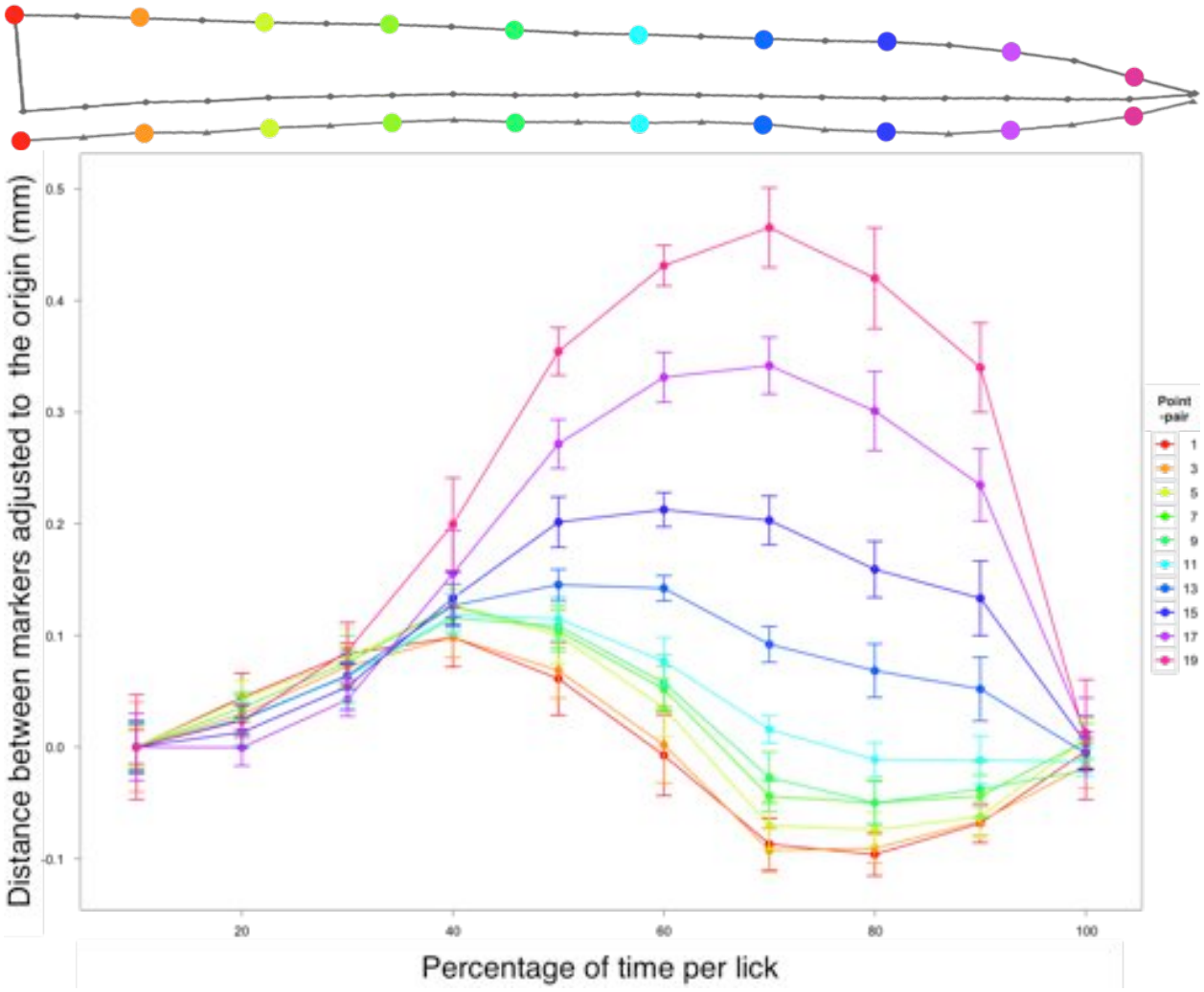



**Figure S2. Distances of separation between culmen and mandibular ventrum point-pairs over time for *Calypte anna*, adjusted to the origin.** In order to control for differential bill thickness along the bill, we made the average for the first time step zero for all the point-pairs. The resulting graph indicates the relative movements of each point-pair, and clearly shows that the absolute opening at the distal region is larger than the decoupling at the basal region. Note that the asynchrony between basal and distal regions maximizes around 70% of the licking cycle, right after the tongue reaches maximum protrusion and it is retracted dragging the nectar backwards. Finally, it is evident how point-pair 13 is intermediate between the asynchronous patterns at the bill base and tip, designating a bending or pivotal (maxilla swiveling over the mandible) zone.

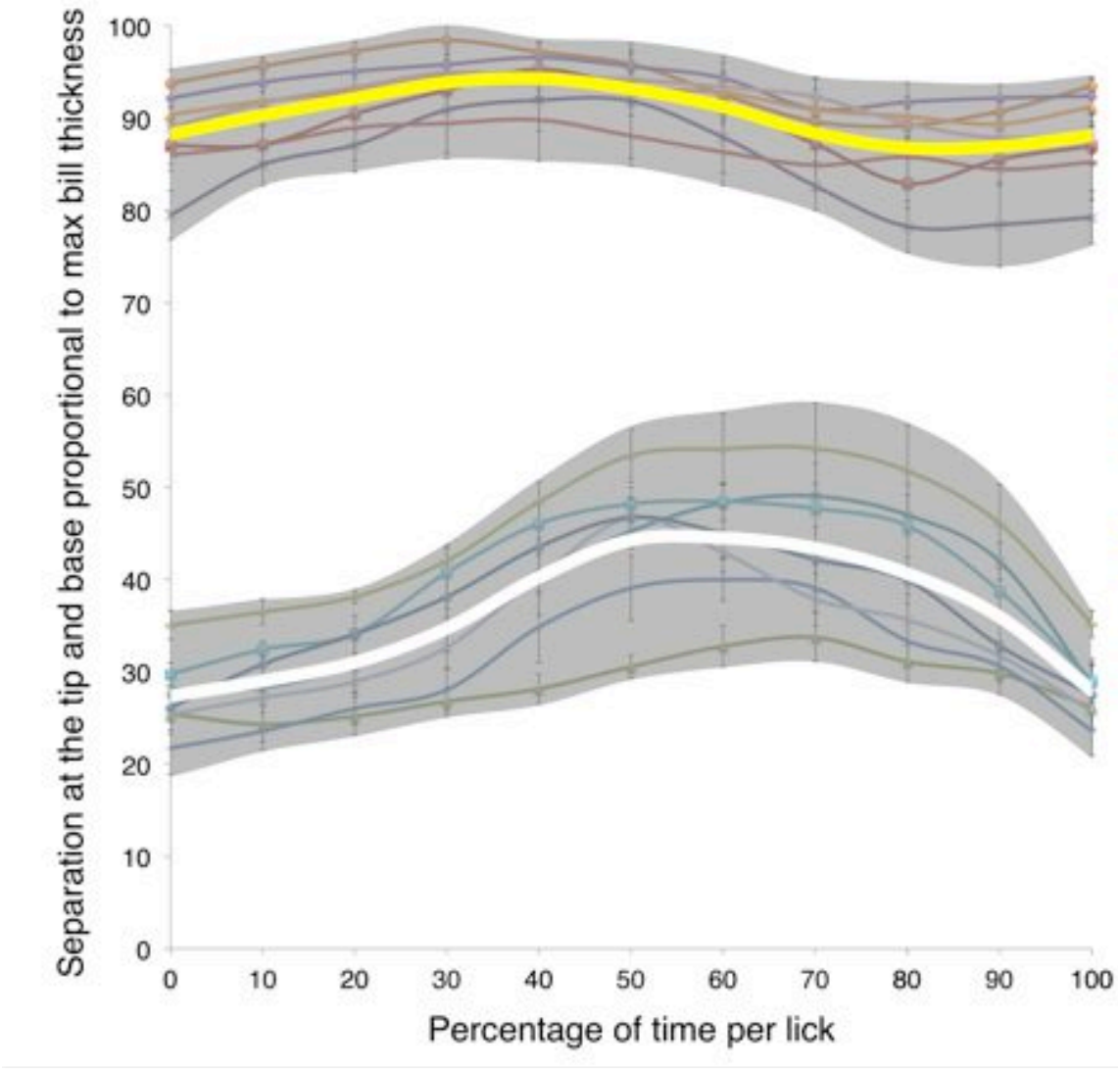



**Figure S3. Distances of separation (proportional to maximum bill thickness) between culmen and mandibular ventrum point-pairs over proportion of time per lick showing the overall average for seven species of hummingbirds.** Two selected point-pairs are shown (*cf.* Fig. 5): point-pair 2 (lines grouped at the top of the graph) and point-pair 19 (lines at the bottom). Data behind the grey shadow represent the averages per species with standard deviations (*cf.* Fig. 8). Thick yellow (top) and white (bottom) lines represent the average patterns for the base and tip movements respectively.

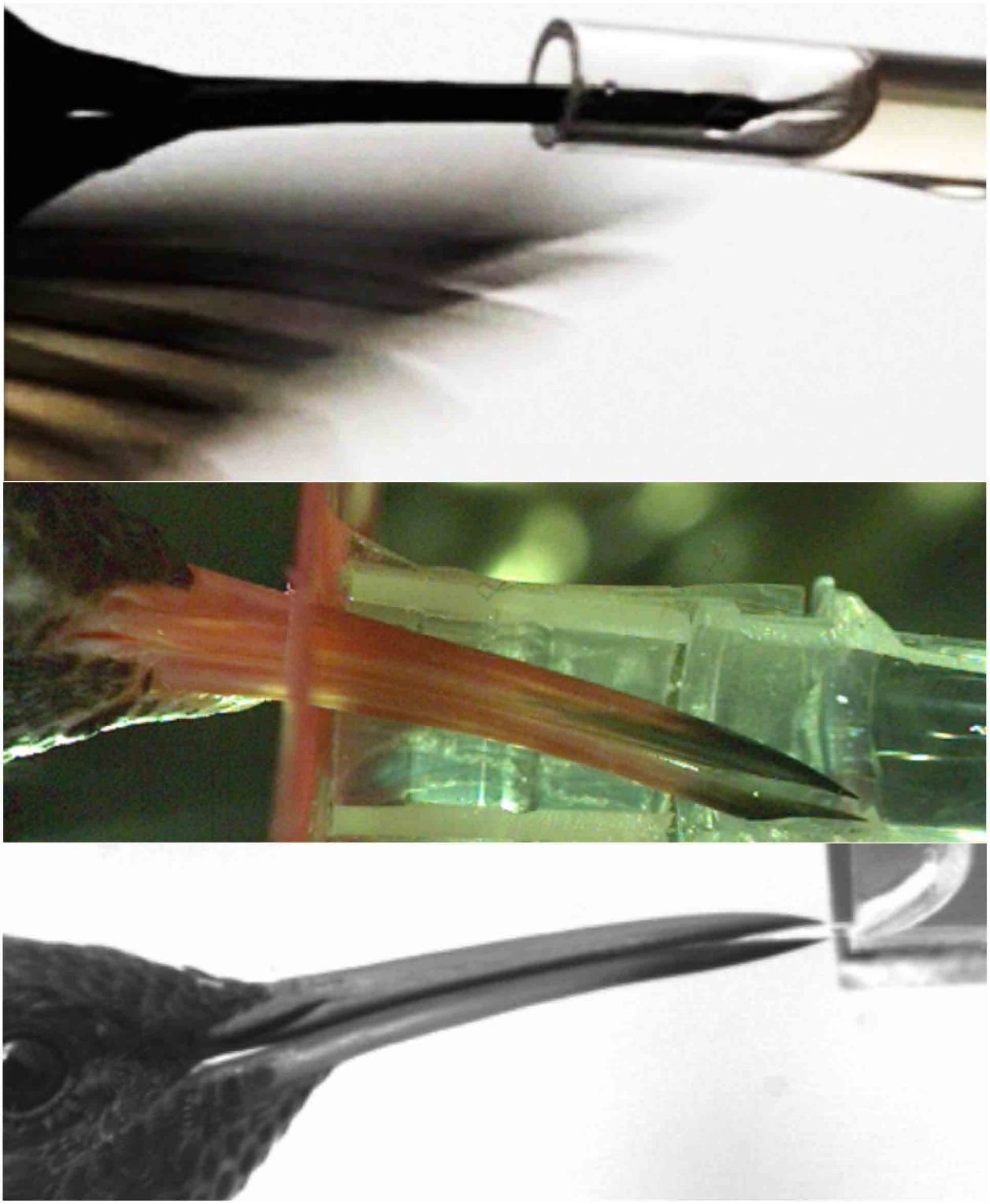
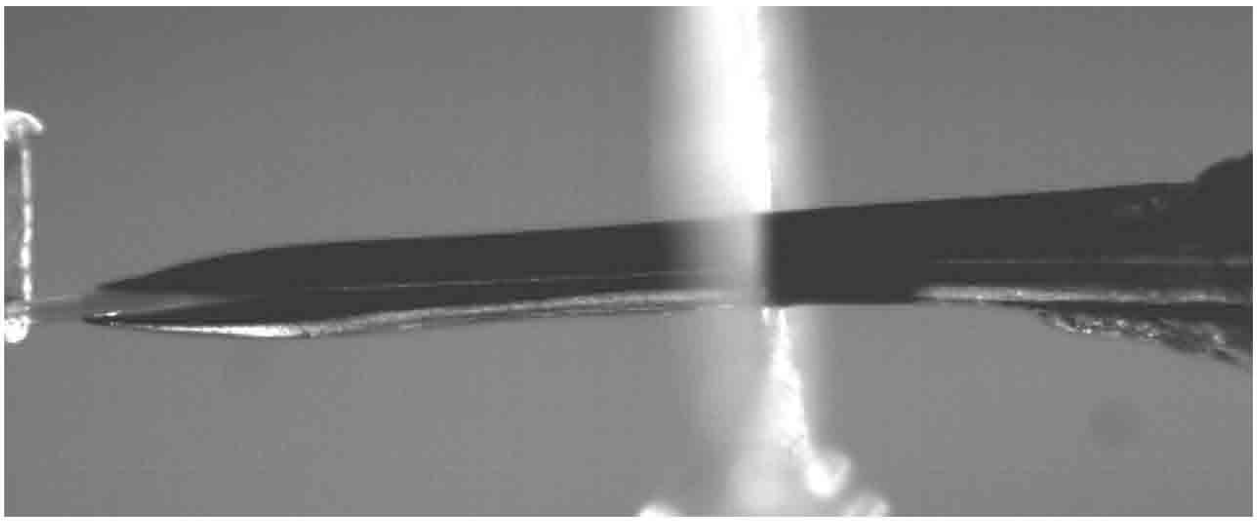
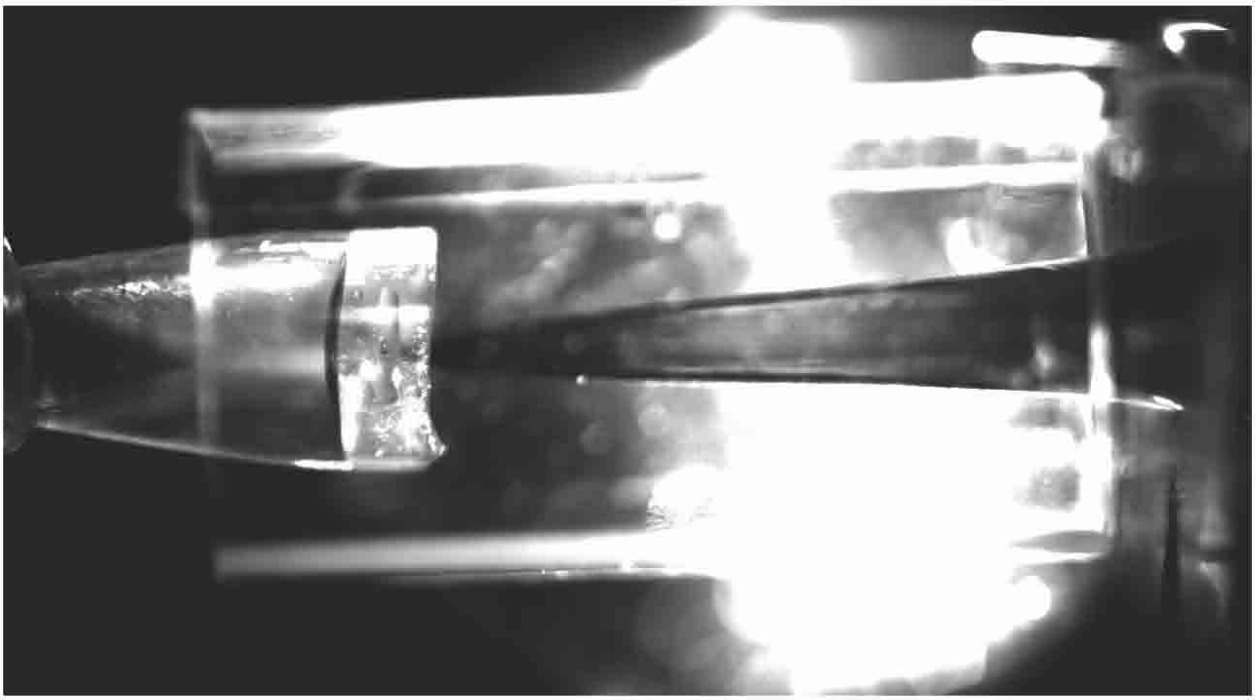


**Figure S4. Light (opening) at the base of the bill of hummingbirds drinking nectar.** Still frames taken from high-speed videos of free-living birds feeding from artificial feeders. The upper image shows a Violet-Throated Starfrontlet (*Coeligena violifer*), the middle one an Amazilia Hummingbird (*Amazilia amazilia*), and the lower one shows an Indigo-capped Hummingbird (*Amazilia cyanifrons*).

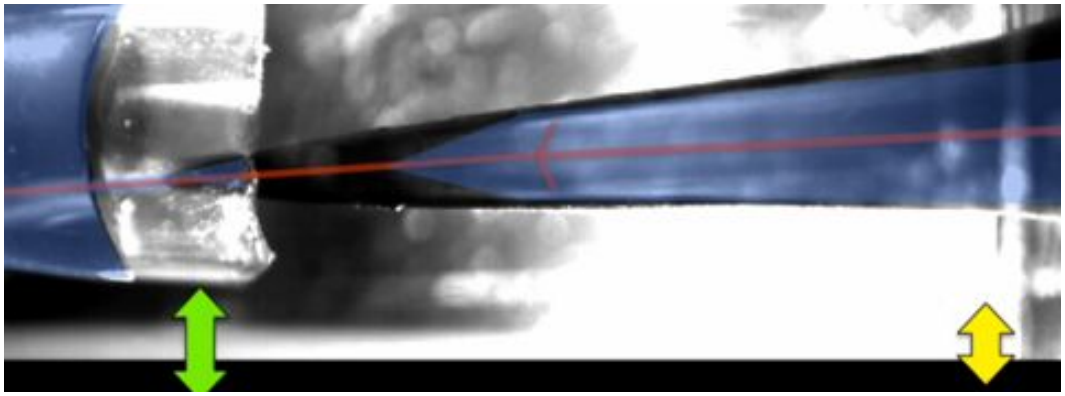
## Supplementary Movies



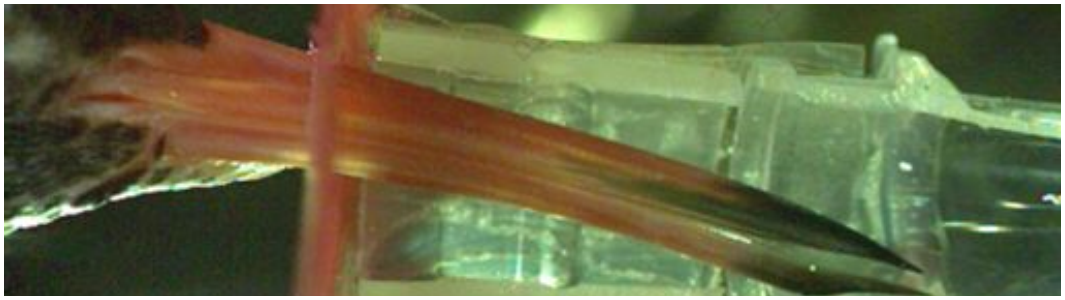
**Movie S1. Lateral view of a hummingbird drinking nectar.** High-speed video (1000 fps) of an Anna's Hummingbird (*Calypte anna*), the base of the bill is on the right and the nectar reservoir on the left. The diffuse shadow in the middle is the flat, flower-shaped guide that served to control the hummingbird positioning to achieve orthogonal views.



**Movie S2. Dorsal view of a hummingbird drinking nectar.** High-speed video (500 fps) of a Rufous-tailed hummingbird (*Amazilia tzacatl*), the nectar reservoir is located on the left. Note how the tongue tip splits inside the nectar, bending downwards and sideways; this is one of the reasons for using orthogonal angles in order to accurately track the displacement of the tongue. Achieving this backlit view allowed us to track the nectar menisci inside the bill.



**Movie S3. Animated overimposition of mechanics data on the dorsal view of a hummingbird drinking nectar.** High-speed video of a Rufous-tailed hummingbird (*Amazilia tzacatl, cf.* Movie S2) including shapes and shades obtained from the bill and tongue motion analyses. Red line in the middle of the bill represents the tongue, and the V shape crossing the red line represents the tongue wings at its base. Blue shadow inside the bill depicts the nectar flowing intraorally. Green double-headed arrow on the left symbolizes the dorso-ventral separation of the bill tips, and yellow double-headed arrow on the right denotes the dorso-ventral separation of between maxilla and mandible at the bill base.



**Movie S4. High-speed video (1000 fps) of an Amazilia Hummingbird (*Amazilia amazilia*) drinking nectar.** In this video, a space between maxilla and mandible at the base of the bill is noticeable. In rare occasions a drop of nectar escapes through this opening.

**Movie S5. High-speed video (1000 fps) of an Indigo-capped Hummingbird (*Amazilia cyanifrons*) drinking nectar.** The light at the base of the bill is a space between maxilla and mandible that is maintained while the bird is drinking.

## Supplementary Tables

### Table S1. Hummingbird species included in the bill motion analyses.

| # | Clade | Genus | species | Females | Males |
|---|---|---|---|---|---|
| 1 | Topazes | *Florisuga* | *mellivora* | 2 | 3 |
| 2 | Hermits | *Rhamphodon* | *naevius* | 3 | 3 |
| 3 | | *Phaethornis* | *baroni* | 1 | 0 |
| 4 | Mangoes | *Colibri* | *coruscans* | 2 | 1 |
| 5 | | *Anthracothorax* | *nigricollis* | 2 | 1 |
| 6 | Coquettes | *Lophornis* | *chalybeus* | 3 | 3 |
| 7 | | *Metallura* | *tyrianthina* | 1 | 1 |
| 8 | Brilliants | *Eriocnemis* | *vestita* | 0 | 2 |
| 9 | | *Aglaeactis* | *curpipennis* | 2 | 3 |
| 10 | | *Coeligena* | *bonapartei* | 0 | 1 |
| 11 | | *Coeligena* | *violifer* | 3 | 0 |
| 12 | | *Heliodoxa* | *leadbeateri* | 0 | 1 |
| 13 | | *Boissonneaua* | *flavescens* | 2 | 3 |
| 14 | Giant | *Patagona* | *gigas* | 1 | 0 |
| 15 | Mt. Gems | *Lampornis* | *clemenciae* | 0 | 2 |
| 16 | Bees | *Chaetocercus* | *mulsanti* | 2 | 0 |
| 17 | | *Myrmia* | *micrura* | 4 | 0 |
| 18 | | *Calypte* | *anna* | 0 | 3 |
| 19 | | *Archilocus* | *colubris* | 2 | 1 |
| 20 | Emeralds | *Thalurania* | *glaucopis* | 1 | 2 |
| 21 | | *Eupetomena* | *macroura* | 0 | 2 |
| 22 | | *Clytolaema* | *rubricauda* | 0 | 1 |
| 23 | | *Amazilia* | *amazilia* | 2 | 2 |
| 24 | | *Amazilia* | *tzacatl* | 2 | 3 |
| 25 | | *Amazilia* | *cyanifrons* | 2 | 2 |
| 26 | | *Thalurania* | *colombica* | 2 | 0 |
| 27 | | *Chalybura* | *buffonii* | 0 | 2 |

### Table S2. Hummingbird species included in the tongue displacement analyses.

| **Clade** | **Genus** | **species** | **Country** | **Females** | **Males** |
|---|---|---|---|---|---|
| Topazes | *Florisuga* | *mellivora* | Colombia | 1 | 1 |
| Hermits | *Phaethornis* | *baroni* | Ecuador | 1 | 0 |
| Bees | *Calypte* | *anna** | USA | 1 | 1 |
| | *Myrmia* | *micrura* | Ecuador | 2 | 0 |
| Emeralds | *Amazilia* | *amazilia* | Ecuador | 1 | 2 |
| | *Amazilia* | *tzacatl* | Colombia | 1 | 1 |
| | *Amazilia* | *cyanifrons* | Colombia | 0 | 1 |

* Captive filming