The diversity of sticky frog tongues

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Aims

Frogs are well known for their use of sticky tongues to capture elusive prey items. The movements of the tongues are extremely fast and single feeding events usually happen within tenths of a second[1-3]. The forces acting on the tongues during feeding can be multiple times the body weight of the animals[4]. Tongue feeding in frogs is diverse, comprising species that only slightly protrude the tongue out of the mouth (i.e. tongue pulling) and species that fire ballistic tongues towards distant targets[5] (Fig. 1). While the kinematics of tongue movements in different species of frogs have received some attention in the past[5], little is known on how the different tongue feeding modes relate to anatomical differences. Further, although previous studies mentioned an unusual interdigitation of muscle fibers from different tongue muscles in frogs[6], the three-dimensionality of this muscle fiber arrangement could not be resolved due to limitations in the methods used (manual dissection and histology). Here we used micro-CT imaging to explore the three-dimensional organization of frog tongues. The aim of this study is to provide detailed descriptions of frog tongue anatomy in relation to differences in the feeding modes of frogs.

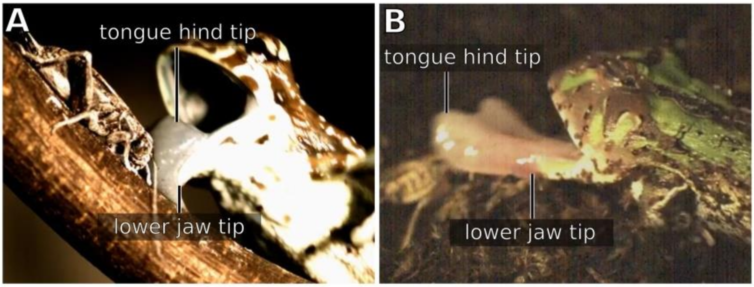

Figure 1: Tongue feeding modes in frogs. Single high-speed video frames taken at 1.000 frames per second. **A:** Tongue pulling. The tongue is slightly protruded over the tip of the lower jaw. **B:** Tongue projection. The tongue is flipped out of the mouth by a rotational movement.

Method

We used a total of nine specimens from the following frog species for micro-CT imaging: *Bombina variegata*, *Bufo bufo*, *Ceratophrys ornata*, *Discoglossus pictus*, *Litoria caerulea*, *Megophrys nasuta*, *Oophaga histrionica*, *Rana* (*Lithobates*) *pipiens*, and *Rhacophorus prominanus*. Tongue pulling is found in *B. variegata*, *D. pictus*, and *L. caerulea*, while the remainder species use ballistic tongue projection[5,6]. For all species, except for *B. bufo* and *R. pipiens*, we captured three micro-CT scans: (1) of the entire animals; (2) of isolated tongues; (3) of small pieces of tongue tissue (approximately 2x2x2mm) that we dissected from the tongues. For *B. bufo* and *R. pipiens*, we only had isolated tongue specimens available and thus did not prepare micro-CT scans of the entire animals.

Prior to micro-CT imaging, we stained the specimens with LUGOL's iodine potassium iodide solution to enhance the x-ray contrast of soft-tissues, especially of the musculature. LUGOL's has previously been demonstrated to allow for good contrast within soft tissues in micro-CT scans of biological specimens[7,8]. For staining, the specimens were first transferred to distilled water by stepwise decreasing the concentrations of the ethyl alcohol in which the animals were stored (70%, 50%, 30%, aqua dest.). We then submerged the specimens in LUGOL’s solution, which penetrated the specimens by diffusion and replaced the aqua dest.. The progress of tissue penetration can be estimated by daily visual inspection of the staining solution. In the beginning of the staining step, the brownish LUGOL’s solution fades out quickly because it is thinned by the aqua dest. emerging from the specimen. In this case, we replaced the thinned staining solution. Once the specimen is saturated, the coloration of the LUGOL’s does not change anymore. Depending on the sizes of the frogs, this process took between ten and fourteen days.

For micro-CT scanning, we placed the stained specimens in containers that we filled with aqua dest.. Entire frog specimens were put in plastic cans with a diameter of 5cm and a height of 7cm; isolated tongues were mounted inside plastic tubes with 2cm diameter (Falcon tubes); pieces of tongue tissue were placed into pipet tips and wrapped with laboratory film (Parafilm) to prevent leakage.

We used a Skyscan 1172 desktop micro-CT scanner for micro-CT imaging. Frogs were scanned with a resolution of 26.6µm (low resolution setting) at a source voltage of 100kV, a current of 100µA, and with an aluminum plus copper filter in front of the x-ray detector. At this resolution, the field of view of the Skyscan 1172 is approximately 2.5cm wide, which was too narrow to capture the width of entire frog specimens. Thus, we had to apply the *Camera offset* function to double the width of the field of view. Further, five subscans along the long-axis of the specimen had to be combined by using the *Oversize scan* option to visualize the entire animals. For micro-CT scans of isolated but intact tongues, we applied a source voltage of 70kV, a current of 139µA, and used an aluminum-cupper filter in front of the x-ray detector. Small pieces of tongue tissue were scanned at a source voltage of 40kV, a current of 250µA, and without any filter. We used the low-resolution setting of the x-ray detector (i.e. 1000 x 668 pixels) for all scans of intact tongues; for fragments of tongue tissue, we used the high-resolution mode (i.e. 4000 x 2672 pixels). Volumetric 3D data was then reconstructed with the NRecon software and visualized with the volume rendering software CTVox. For segmentation of the micro-CT data sets, we imported the volumetric data as stack of TIFF images into the third party software Amira 5.4.2 (Visage Imaging).

Results

In all frogs examined herein, the body of the tongue mainly consists of two muscles: the m. genioglossus that moves the tongue out of the mouth (tongue protractor) and the m. hyoglossus that will pull the tongue back to its resting position inside the mouth (tongue retractor). The m. genioglossus originates from the rostral tip of the lower jaw; the m. hyoglossus has its origin on the tongue skeleton (i.e. the hyoid) rostral to the shoulder girdle (Fig. 2). Inside the tongue, the fibers of these two muscles are interwoven which will help to cause an equal distribution of forces over the surface when the tongue is in contact. Besides the general architecture of frog tongues, we found notable variations in the shape of the two tongue muscles between species. The m. genioglossus has a broader region of origin along the inner (lingual) face of the lower jaw in frogs that show tongue pulling than in frogs with projectile tongues. In frogs with ballistic tongue projection, the m. hyoglossus is elongated and appears curved when the tongue is in its resting position. In frogs that use tongue pulling for prey capture, the m. hyoglossus has shorter fibers with a straight course (Fig. 2).

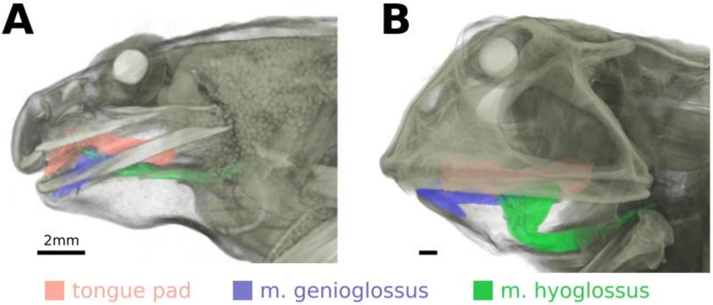

Figure 2: Micro-CT imaging of the head region of frogs. Lateral views. **A:** *Bombina variegata*. **B:** *Ceratophrys ornata*. In *B. variegata*, the fibers of the tongue retracting muscle m. hyoglossus run straight, while in *C. ornata*, the fibers of this muscle are curved. *B. variegata* uses tongue pulling for prey capture; *C. ornata* uses tongue projection.

Two types of hair-like outgrowths cover the tongue surface: the filiform and fungiform papillae. Filiform papillae are known to be the places of mucus production while the fungiform papillae acts as chemoreceptors[9]. The sizes of these papillae, their aspect ratios, and their density show high interspecific variation (Fig. 3), which does not seem to correlate closely with the feeding mode. Further, we found notable differences in the organization of the deeper layers underneath the tongue surface: some species have a layer with presumably fluid filled lacunae between the surface papillae and the muscle body of the tongue while in other species, the muscle fibers extend towards the tongue epithelium (Fig. 3).

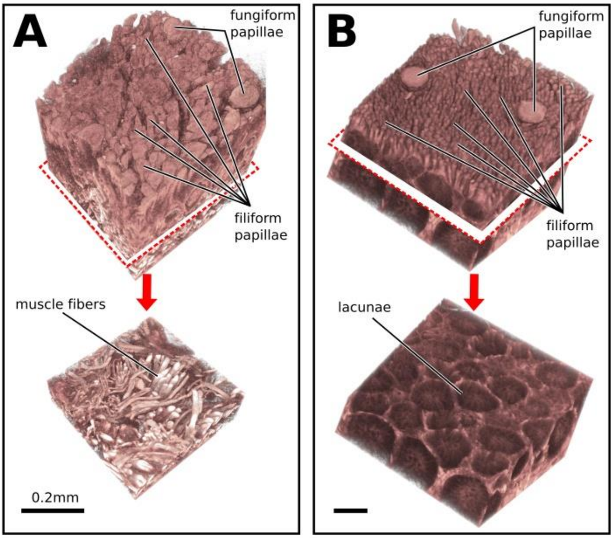

Figure 3: High-resolution micro-CT imaging of frog tongue surfaces. **A:** *Ceratophrys ornata*. **B:** *Megophrys nasuta*. The tongue surface is covered by filiform and fungiform papillae. Please, note interspecific differences in the shape, amount, and distribution of these papillae as well as in the underlying tissue layers.

Conclusion

Micro-CT imaging allowed us to reveal a high degree of diversity in the anatomy of frog tongues on multiple levels ranging from the muscle attachment in the animal towards the microscopical organization of the tongue. Some of the macroscopic variation, e.g. in the shape of the tongue muscles, can be related to differences in the tongue feeding mode. However, the tongue diversity described herein might also reflect evolutionary and developmental patterns that have to be further explored in the future.

References:

1. Gans, C., & Gorniak, G. C., “How does the toad flip its tongue? Test of two hypotheses” Science, 216:1335–1337, 1982
2. Nishikawa, K. C., & Gans, C., “Mechanisms of tongue protraction and narial closure in the marine toad *Bufo marinus*” J Exp Biol, 199: 2511–2529, 1996
3. Nishikawa, K. C., & Roth, G., “The mechanism of tongue protraction during prey capture in the frog *Discoglossus pictus*” J Exp Biol, 159: 217–234, 1991
4. Kleinteich, T., & Gorb, S. N., “Tongue adhesion in the horned frog *Ceratophrys* sp.” Scientific Reports, 4: 5225, 2014
5. Nishikawa, K. C., “Feeding in frogs” In “*Feeding: Form, function, and evolution in tetrapod vertebrates*” (Schwenk, K ed.), Academic Press, 117–147, 2000
6. Regal, P. J., & Gans, C., “Functional aspects of the evolution of frog tongues”, Evolution, 30: 718–734, 1976
7. Metscher, B. D., “MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues”, BMC Physiol, 9: 11, 2009
8. Metscher, B. D., “MicroCT for developmental biology: a versatile tool for high-contrast 3D imaging at histological resolutions”, Develop Dyn, 238: 632–640, 2009
9. Iwasaki, S. I., “Evolution of the structure and function of the vertebrate tongue”, J Anat, 201: 1–13