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# Comparison of Isometric Contractile Properties of the Tongue Muscles in Three Species of Frogs, *Litoria caerulea, Dyscophus guinetti,* and *Bufo marinus*

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*ABSTRACT* Previous studies show that anurans feed in at least three different ways. Basal frogs have a broad tongue that shortens during protraction and emerges only a short distance from the mouth. Some frogs have long, narrow tongues that elongate dramatically due primarily to inertia from mouth opening, which is transferred to the tongue. A few species have a hydrostatic mechanism that produces tongue elongation during protraction. This functional diversity occurs among frogs that share the same two pairs of tongue muscles. Our study compares the isometric contractile properties of these tongue muscles among three frog species that represent each feeding mechanism. Nerves to the paired protractors and retractors were stimulated electrically in each species to record the force properties, contraction speeds, and fatigabilites of these muscles. Few differences were found in the isometric contractile properties of tongue muscles, and the greatest differences were found in the retractors, not the protractors. We propose that the unique arrangement of the tongue muscles in frogs results in a retractor that may also be coactivated with the protractor in order to produce normal tongue protraction. Inertial effects from body, head, and jaw movements, along with clear differences that we found in passive resistance of the tongues to elongation, may explain much of the behavioral variation in tongue use among species. J. Morphol. 242:107–124, 1999. 

KEY WORDS: Anura; tongue muscles; contractile properties

Study of the feeding behavior of frogs provides an opportunity to examine the relationship between behavioral variation and a diverse and specialized set of derived musculoskeletal and neural features. All frogs use their tongues to contact and retrieve their prey; nonetheless, there is considerable variation in tongue action across anurans. Nishikawa ('99) described this diversity in terms of three basic feeding modes: one primitive for anurans (involving mechanical pulling of the tongue) and two derived forms (inertial elongation or hydrostatic elongation of the tongue).

Descriptions of anuran feeding behaviors include kinematic analyses of tongue, jaw, and body movements using high speed video (e.g., Nishikawa and Roth, '91; Nishikawa and Gans, '96) and electromyography (EMG) (Gans and Gorniak, '82a; Matsushima et al., '85). Studies of neural control also reveal a diversity of sensory feedback (Anderson and Nishikawa, '97) and neuroanatomical patterns (Nishikawa, '97) among anuran species. Despite the large literature on these and other aspects of feeding behavior in frogs, there have been no studies on the contractile properties of the tongue muscles involved in feeding. This study compares the isometric contractile properties (force, speed, fatigability) of the tongue muscles in three species of frogs that use their tongues differ-

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ently during feeding. Our hypothesis is that differences in contractile properties of the tongue muscles for the three feeding styles will correlate with differences in feeding behavior among frogs.

In frogs that have a mechanical pulling tongue (e.g., *Ascaphus* [Nishikawa and Cannatella, '91], *Discoglossus* [Nishikawa and Roth, '91], and *Hyla* [Deban and Nishikawa, '92]), the tongue is relatively short and broad; it shortens as it is protracted so that the fully extended tongue is shorter than at rest (≈60% of jaw length). Maximum protraction velocities (40 cm/sec) and accelerations (15 m/sec$^2$) are moderate (Nishikawa, '99). Frogs with mechanical pulling tongues are sit-and-wait predators that feed on moderate to large-sized prey, and tongue protrusion is often coupled with lunging of the body to provide a longer reach to the prey. Jaws are commonly used to assist in capturing prey and the forelimbs aid in bringing the prey into the mouth.

Derived from the mechanical pulling tongue is the inertially elongating tongue, that evolved multiple times in different frog lineages (e.g., bufonids [Nishikawa and Gans, '96]; phyllomedusine hylids [Gray and Nishikawa, '95]). Inertial tongues are relatively long but relatively small in mass and can be protruded up to 180% of jaw length (Nishikawa and Gans, '96). Elongation occurs primarily because of the inertia that is transferred to the tongue pad from the rapidly opening jaws. Maximum tongue accelerations can exceed 300 m/sec$^2$ at the tip, reaching maximum velocities of over 270 cm/sec. Many frogs with inertial tongues feed on relatively small prey and rely entirely on tongue prehension to bring prey into the mouth (Valdez and Nishikawa, '97; Nishikawa, '99).

A second derived tongue type has been found so far only among microhylid and hemisotid frogs (Nishikawa et al., '99). These species have hydrostatic tongues in which a muscular hydrostat (Kier and Smith, '85) provides the mechanism for elongation (see below). These tongues may be protruded up to 200% of jaw length. In the only species described, the termite specialist *Hemisus marmoratum* (Ritter and Nishikawa, '95), tongue protrusion is quite slow (maximum velocity of 15 cm/sec; maximum acceleration of 0.5 m/sec$^2$).

In this study, we examined one species of frog representing each of these tongue types to compare the isometric force, contraction and relaxation speeds, and fatigability of their tongue muscles. Our major goal was to explore the connection between contractile properties of the tongue muscles and differences in tongue function among representatives of the three tongue types. We expected, for instance, that if force were an important factor in overcoming static inertia for rapid tongue protraction, then the inertial elongator would have the largest protractor muscles. However, because inertial elongators feed on relatively small prey, we might also expect them to have the smallest retractor muscles. Presumably the protraction speed of the tongue is derived at least in part from the speed of contraction of the protractor muscles. Thus, the rapid inertial elongator should have the fastest contraction time. We also expected that fatigability of the muscles might differ. Because inertial elongators must generate protractive force very rapidly to produce such great accelerations, we expected their tongue muscles to be comprised of larger motor units that tend, in frogs, to be more fatigable than smaller motor units (Peters, '94).

We expected a strong correlation between structure and function in a behavior as fundamental as feeding, so that the contractile properties reported here would likely reflect feeding adaptations among species. However, no single species may fully represent all of the attributes of a particular feeding type. In addition, phylogenetic relationships may play a role in many differences between species. In fact, we found relatively few differences in contractile properties among species. Future studies of a wide variety of species are necessary to determine the generality of the results reported here, and to evaluate our preliminary interpretations.

## MATERIALS AND METHODS

### *Tongue structure and function*

In frogs, the tongue is attached anteriorly in the mouth near the mandibular symphysis. As it protracts, it rotates over the symphysis and flips upside down on top of the prey. Thus, the dorsal surface at rest becomes the ventral surface of the fully protracted tongue. Retraction pulls both the tongue and prey back into the mouth, and flips the tongue back into its resting position.

The tongues of all frogs are composed of only two muscles, both with extrinsic origins

(Regal and Gans, '76; Horton, '82) (Fig. 1). The tongue protractors are the paired genioglossus medialis (GG), which originate near the mandibular symphysis. The fused heads of GG consist of parallel fibers that lie on the dorsal side of the tongue at rest (Fig. 1). As GG fibers enter the tongue pad, they fan out and end on connective tissues just beneath the epithelial layer of the tongue pad (Regal and Gans, '76; Gans and Gorniak, '82a). Based on this morphology, as well as from denervation and stimulation studies (Gans and Gorniak, '82a; Deban and Nishikawa, '92; Nishikawa and Gans, '92; Ritter and Nishikawa, '95), it is inferred that contraction of GG elevates the tongue pad and pulls the tongue toward the mandibular symphysis. Tongue retractors consist of the paired hyoglossus (HG) muscles that originate ventrally on the posteromedial process of the hyoid apparatus and lie on the ventral side of the tongue at rest. When the tongue is at rest, the HG fibers course anteriorly toward the tongue base, but on entering the body of the tongue, they bend back 180° upon themselves (Fig. 1). As in GG, fibers of HG are parallel over most of their length, but on entering the tongue pad they fan out to interdigitate with the fibers of GG. Thus, HG elongates and straightens as the tongue protracts; its contraction is assumed to return the tongue to rest.

Frogs with hydrostatic tongues have a compartment of dorsoventrally oriented fibers in the center of each genioglossus muscle, medial to its parallel fibers (Nishikawa et al., '99). These dorsoventrally oriented fibers insert on a tendinous sheath that surrounds the compartment. As these fibers shorten, they would pull the dorsal and ventral sides of the sheath closer together, thus flattening the tongue. Because any fluid-based structure maintains a constant volume as shape changes, if the tongue flattens it must also elongate. In hydrostatic tongues, the parallel fibers of GG are presumed from their morphology to initiate protraction by rotating the tongue pad into an anteriorly directed position. The hydrostatic mechanism should then provide protrusion of the tongue and may be essential in its aiming (Ritter and Nishikawa, '95).

### *Specimens*

The isometric contractile properties of tongue muscles were studied in three anuran species, each representing one of the

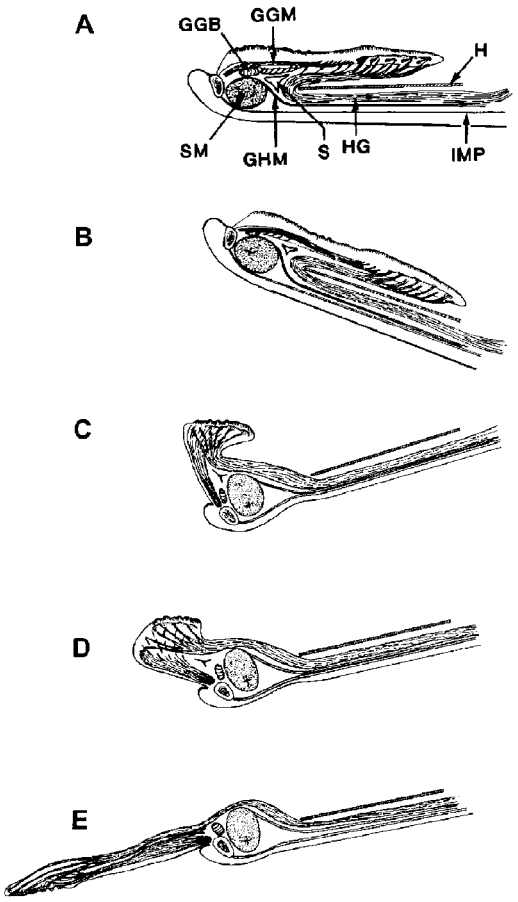


Fig. 1. A schematic sagittal section through the tongue and lower jaw of a typical anuran shows the anatomy and actions of the tongue protractor (genioglossus medialis [GGM]; in the text simply referred to as genioglossus [GG]) and retractor (hyoglossus [HG]) muscles). Genioglossus originates near the mandibular symphysis and projects posteriorly along the dorsal surface of the tongue just beneath the epithelium. HG fibers originate on the posteromedial process of the hyoid apparatus and lie ventral to the hyoid (H) for most of their length. When the tongue is at rest (**A**), HG fibers bend sharply around the anterior end of the hyoid apparatus to enter the base of the tongue. In the tongue pad, HG fibers fan out to interdigitate with those of GGM. Panels **B** through **E** show how tongue protraction proceeds from the resting position (**A**). The mandible first elevates (**B**) and then as it lowers (**C–E**) the tongue moves forward in the mouth (**C**), flips out over the jaw tip (**D**) and comes to lie dorsal-side down at full protraction (**E**). Thus, genioglossus first shortens during protraction (**C,D**) and in some species then elongates as the tongue extends beyond the jaw tip (**E**). Hyoglossus is straightened and, in some species, elongates during protraction. GGB, genioglossus basalis; GGM, genioglossus medialis; GHM, geniohyoideus medialis; H, hyoid apparatus; HG, hyoglossus; IMP, intermandibularis posterior; S, lymphatic sinus; SM, submentalis. Redrawn from Gans and Gorniak ('82a).

three basic tongue types. The protractor (GG) and retractor (HG) muscles of the tongue were sampled in different experiments for each species. Care was taken to size-match within species so that there was no difference in body mass or tongue mass between individuals sampled for GG vs. those sampled for HG.

*Litoria caerulea* is a hylid tree frog with a mechanical pulling tongue. Its natural diet is unknown. However, most hylids that have been studied appear to be dietary generalists (Toft, '81). Eleven specimens of *Litoria*, ranging in body mass from 24.7–61.5 g ($\bar{x}$ = 41.0 ± 3.1 g) were used to study the protractor (n = 6) and retractor (n = 5) muscles. The inertial tongue was studied in *Bufo marinus*. Marine toads in nature feed on a wide variety of insect prey. However, ants and termites make up a large proportion of their natural diets (Zug and Zug, '79; Toft, '81; Emerson, '85). Twelve specimens of *Bufo*, averaging 66.8 ± 5.5 g body mass (range, 40.5–101.1 g) were used; six GG and six HG samples were obtained. Hydrostatic tongues are thought to be present only in hemisotid and microhylid frogs (Nishikawa, '99). We chose not to test the tongue muscles in the well-studied species, *Hemisus marmoratum*, because of its small body size. Instead, we chose one of the largest microhylids to study, the Madagascar tomato frog, *Dyscophus guinetti*. The natural diet of *Dyscophus* is also unknown. Whereas many microhylids appear to specialize on ants and termites (Toft, '81; Emerson, '85), and we have found that many microhylid frogs will eat only ants or termites in the laboratory, it appears that *Dyscophus* is more of a dietary generalist in that it readily ate crickets and waxworms in captivity. Specimens of *Dyscophus* ranged in size from 29.3–61.1 g, with a mean body mass of 39.8 ± 2.9 g. Genioglossus was sampled in seven individuals and HG in six. All three species were purchased from Glades Herpetology Suppliers (Ft. Myers, FL). Individuals were housed in terraria and fed on a diet of waxworms and crickets.

Prior to examination of their tongue muscles, the feeding behaviors of the animals were observed to document the extent of tongue protraction and its general use in prey capture. While they fed, specimens were videotaped at 120 fps using a video camera (Display Integration Technologies, Santa Clara, CA) and a Panasonic AG-6300 VCR. Tapes were subsequently digitized and analyzed using Peak Performance software. Special attention was paid to describing tongue protraction in *Litoria caerulea* (seven sequences from three individuals) and *Dyscophus guinetti* (seven sequences from four individuals) since a great deal of kinematic information was already available on *Bufo marinus* (Nishikawa and Gans, '96).

### *Measurement of contractile properties*

Individuals were anesthetized using tricaine methane sulfonate (MS222) either by immersion (0.2% solution) or subcutaneous injection (200 mg/kg body mass). Once anesthetized, the animals were weighed. Then the neurocranium was removed by cutting from the back corners of the jaws across the craniocervical junction with bone shears. Thus, the entire top of the head was removed to expose the tongue lying between the mandibles. This allowed free access to the tongue from both anterior and posterior.

It was impossible to isolate the tongue muscles from the body of the tongue without doing considerable damage to the muscles. Thus, we left the tongue intact and stimulated the muscles independently via their nerves. It is possible that differences in the amounts of nonmuscular tissue among species may have affected our results. However, these effects would also exist naturally among species.

Two lengths were measured as references to standardize between individuals and species. Resting tongue length ($L_o$) was measured from the mandibular symphysis to the tip of the tongue pad when the tongue lay in its normal resting position between the mandibles. Tongue resting length ($L_o$) approximates the position of the protractor muscles at the length at which they begin contracting and, so, is a good measure of resting muscle fiber length in GG. Since there is much variability in maximum tongue elongation, and since we believe that HG may function in both protraction and retraction (see Discussion), we decided to use as its reference a length also equivalent to the resting length of the tongue. Because the HG fibers are folded back when the tongue is at rest (Fig. 1), we measured HG reference length ($L_{HG}$) by extending the tongue to a passively stable position, i.e., rotated forward over the mandibular symphysis and lying at rest. In this position, $L_{HG}$ was measured from the anterior edge of the glottis to the tip of the tongue pad. The goal was to straighten the HG muscles without stretching

them, so that $L_o$ and $L_{HG}$ would reflect functionally equivalent lengths of the two muscles when the tongue is at rest. While $L_{HG}$ may not be a resting length in the classic sense, it can be measured conveniently and repeatably both within and between species.

In order to attach the tongue to the transducer for measurement of contractile properties, a piece of 2–0 suture silk was tied through the tongue pad just posterior to its tip. This undoubtedly damaged some of the terminal fibers in both GG and HG. Though we cannot know what percent of the total muscle force was compromised by this tie, we estimate that it was acceptably small, and, because the tie was made as close to the same place as possible in all animals, its effect on our data was consistent across individuals and species.

Both GG and HG are innervated by the hypoglossal nerve (Fig. 2). This nerve emerges from the posterior brain stem and dives ventrally to the posterior corner of the mandible. It then courses anteroventrally along the lateral surface of the rectus cervicis and becomes superficial on the throat, lying within the belly of the geniohyoideus muscle just dorsal to the intermandibularis muscle. Posteriorly, the hypoglossal nerve gives off a medial branch into the sternohyoideus. Anteriorly, as it becomes more superficial, it gives off the primary nerve branch into HG (Fig. 2). This arises about one-quarter of the mandible length forward from the jaw joint. The nerve then gives off several small medial branches into the geniohyoideus before arborizing at its anterior end into multiple branches to both medial and basal heads of GG (Fig. 2).

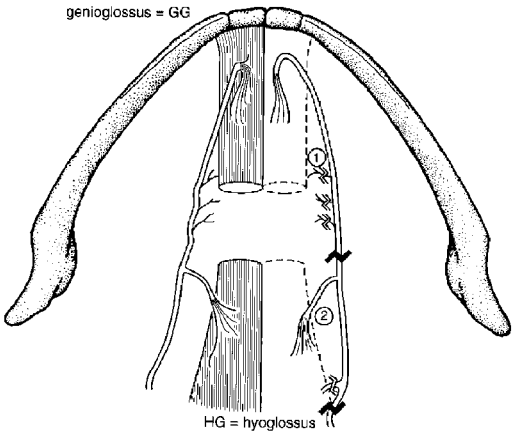


Fig. 2. A ventral view of the lower jaw shows the two tongue muscles, genioglossus (GG) and hyoglossus (HG) (each cut and partially removed from the figure), and their pattern of innervation by the hypoglossal nerve. Shown on the left, the main nerve into HG branches medially about one-quarter of the jaw-length forward from the jaw joint. Anterior to this branch point, several small nerves branch into the geniohyoideus (muscle not shown). Near the tip of the lower jaw, the hypoglossal nerve bends medially and sharply posterior to enter the base of GG, where it arborizes into many small branches. The right side shows that in all experiments the hypoglossal nerve was severed distal to the HG branch (upper dark cut mark). In experiments on HG, the nerve was also severed behind the corner of the jaw (lower dark cut mark) to produce an isolated piece via which to stimulate the HG muscle (piece #2). In GG experiments, the distal portion (piece #1) of the nerve was attached to the electrode. The light cut lines indicate where the branches to sternohyoideus (lower) and geniohyoideus (upper) were severed. Redrawn from Duellman and Trueb ('86).

In separate experiments, bilateral contractions of the GG or HG muscles were elicited by electrical stimulation of appropriate segments of the hypoglossal nerves. In each surgery, the nerves to both right and left sides were dissected near the corner of the jaw joint. The hypoglossal nerve was transected distal to the branch point of the HG nerve branch (Fig. 2). In experiments testing HG, the distal portion of the hypoglossal nerve (the part serving GG) was reflected away from the experimental area. The proximal hypoglossal nerve was then severed near the jaw joint so that an ≈1 cm length of the cleared nerve could be attached to the electrode. Thus, a segment of the hypoglossal nerve from the jaw joint out to and including the HG branch was isolated from both upstream and downstream connections so stimulation led to contraction of the HG muscle only. In experiments on GG, the distal portion of the nerve (separated from proximal connections, including the HG branch), ≈1.5 cm in length, was free to be attached to the electrode.

Because the animals had to be placed belly-down for experimental contraction of the tongue muscles, and these nerves lie on the ventral surface of the throat, access to the nerve segments with simple hook electrodes was difficult and resulted in unstable contact. Thus, bipolar electrodes were made using two pieces of coated stainless steel wire (diameter 0.003 in., California Fine Wire, Garden City, CA) that were each bared in a small spot (≈1 mm in length) and held in contact with the nerve by sleeving both the nerve and wires inside small lengths (2–4 mm) of polyethylene tubing (PE 50,

Intramedic). Each wire was secured separately on the nerve so that contact points could not touch. The wires were attached to a stimulating cable via gold connectors soldered to the wire tips and plugged into matching connectors on the cable. This method provided stable contact for stimulation of the muscles via the nerves, verified by the consistency of the EMGs recorded simultaneously with muscle contraction.

In the experiments on *Dyscophus,* it was not possible to isolate the axons to the longitudinal GG fibers from those serving the central compartment of transverse fibers in the hydrostatic tongue. Thus, both compartments were stimulated. The effect of this would be to underestimate the whole muscle forces of GG in our results (since the hydrostatic compartment is antagonistic to the longitudinal fibers). The effects on contraction and relaxation times should be minimal because the mean length of the transverse fibers (number of sarcomeres in series) is likely to be much shorter than the longitudinal fibers. Thus, the speed characteristics of the longitudinal fibers should have predominated in the results. This complication is discussed further below in the context of interpreting the results.

Once surgical preparation of the muscles and nerves was completed with the attachment of the electrodes, a clamp was placed on the hyoid apparatus to immobilize the origin of the HG muscles. The frog was placed in a heavy metal frame and the hyoid clamp was secured to it. A second clamp was attached distally to the left mandible as close to the symphysis as possible. This stabilized the origin of the GG muscles. For examination of the GG muscles, the force transducer was placed posterior to the head and the muscles stimulated to pull from the resting position of the tongue ($L_o$). In measuring properties of HG, the transducer was placed in front of the head and measurements were taken relative to the resting HG length ($L_{HG}$). Thus, in both cases the muscles were stimulated to pull from positions approximating their natural actions in either protracting or retracting the tongue.

All experiments were done at room temperature (22 ± 2°C). Electrical stimuli (0.1 msec duration) of supramaximal voltage (2.5X threshold) were delivered to the nerves from a Grass S88 square wave stimulator. Single twitches or tetanic trains (each pulse 0.1 msec duration; at 80 pps for 670 msec train duration) were elicited while the tongue was attached to a Grass FT03 isometric force transducer. Signals were sent via a carrier amplifier (Grass TM501–1) into a Tektronix TD310 digital oscilloscope. EMG signals from the muscles were sent into a Grass high impedance probe and amplified through a Grass P511 AC amplifier. These signals were simultaneously displayed on the oscilloscope and good traces were printed using an HP LaserJet III printer.

Contraction (CT) and half-relaxation (Tw ½R) times were measured from single twitches. The CT was the time (in msec) from the stimulus trigger point to the peak of twitch force; the Tw ½R time was measured from the peak of twitch force to the point at which the signal fell to one-half peak force. In addition, relaxation times from the maximal isometric tetani were measured (TT ½R) as the time from offset of the stimulus train to the point where the signal fell to half of peak tetanic force.

Isometric twitch and tetanic forces were recorded over a range of muscle lengths estimated from the videotapes of feeding behavior for each species. The force transducer was mounted on a rack and pinion so lengths could be changed in millimeter increments over this natural range of muscle lengths. From the basic reference length of $L_o$ (for GG) or $L_{HG}$ (for HG), muscles were stimulated to produce twitch and tetanic contractions every 2 mm to their maximum natural lengths. Lengths at these 2 mm increments were later converted into percent of $L_o$ or $L_{HG}$ to construct a length–tetanic tension curve for each individual and each muscle.

In addition to the active isometric muscle tensions, passive tensions of the whole tongue were also measured in each species. Before moving the rack and pinion from one length to the next in each experiment, tongue length was slackened by moving the rack and pinion so that the tongue was flaccid. Baseline on the transducer signal was zeroed and then the muscle length was set to the next value to be tested. The displacement of the baseline due to passive resistance was then recorded at each length from $L_o$ or $L_{HG}$ (as appropriate) to the maximum lengths.

Once muscle tensions over the natural range of lengths were determined, further tests on each muscle were done at the length at which maximum isometric tetanic tension occurred. These tests included two measures of how stimulation parameters affect force production. First, a series of tetanic stimuli was used to plot a force–frequency

curve (Peters, '94). Trains of stimuli of constant train duration (670 msec) were applied at varying pulse frequencies from 5–80 pps (at 5-pulse intervals from 5–40 pps and at 60 and 80 pps), and the peak tension at each frequency was recorded. To prevent neural or muscular fatigue during this test, each muscle was rested for 2 min between tetanic trains. In addition to varying with stimulus frequency, force will also vary with the duration of a train of stimuli. Thus, a force–duration test was done using tetanic trains of constant pulse frequency but variable train duration. We chose 30 pps as the standard pulse rate. This is a frequency that is at or slightly above the fusion frequency for each of these muscles. Tetani at 30 pps were elicited at train durations of 50, 100, 150, 200, 300, and 670 msec. Again, the muscles were rested for at least 2 min between trains to minimize the effects of fatigue. In reporting both force–frequency and force–duration data, the amount of force generated at each frequency or for each duration was plotted in percent of maximum force seen during those tests. Thus, the relative amounts of force and their rates of generation could be compared between muscles and species.

Fatigue was tested using a modified version of that described by Burke et al. ('71), designed to reveal muscle fatigue without depressing transmission at the neuromuscular junction (evidenced by stable EMG signal throughout the test) (Peters, '94). The muscle nerves were stimulated with repetitive trains at a stimulus rate of 30 pps, once every 2 sec, for a duration of 200 msec. The test was applied for a total of 4 min. In a first analysis, a fatigue index (FI) was calculated as the sum of forces produced over the first 2 min divided by the sum over the whole 4 min (X100) (Burke, '81; Peters, '94). Thus, an FI of 50 would indicate equal force produced in both halves of the test, i.e., no fatigue. FIs greater than 50 indicate greater force produced in the first half of the test, and lower forces in the second half, i.e., fatigue of varying amounts. For instance, an FI of 60 indicates a decline in force of about 40–50% between the first and last halves of the test, depending on the rate of decline. An FI $>70$ normally corresponds to a decrease in force of $>80\%$ over the 4-min test.

While valuable as a general indicator of fatigue, the FI does not give details as to whether the force fell gradually and continuously, or whether it remained high and then dropped precipitously to a low level. To give a better indication of the dynamics of the fatigue, we grouped the force traces into 20-sec bins (10 stimulus trains each) and averaged the force over each 20-sec interval. The average force during the first 20-sec interval served as the 100% value against which the rest of the bins were compared. The mean forces in percent of maximum were plotted over the 4-min test to show the profile of fatigue for each muscle.

### *Data Analysis*

The data were analyzed using Statview II and SuperAnova on a MacIntosh IIci computer. Because at least some contractile properties, such as maximum force, were expected to vary with body size, we first performed a log–log regression for all variables against both body mass and tongue mass. For all variables, tongue mass explained a greater proportion of the variation than body mass, and therefore tongue mass was used as the covariate in all subsequent analyses. Tongue mass was compared among species with one-factor ANOVA. Morphometric data (Table 1) were analyzed using one-factor ANCOVA. Model effects were species as the main factor, tongue mass as the covariate, and their interaction. Student-Newman-Keuls (SNK) post-hoc tests were used to determine which species differed from each other. Contractile properties (Table 2) of the tongue muscles were compared using two-factor ANCOVA. The model effects were species, muscle (GG vs. HG), and species $\times$ muscle, with tongue mass as the covariate. *P*-values were computed after removing all nonsignificant interactions involving the covariable. Again, species were compared using SNK post-hoc tests. For the force–frequency (Fig. 4), force–duration (Fig. 5), and fatigue over time (Fig. 7) data, two-factor ANCOVAs were performed separately for each interval value of the ordinate variable. For the length–tension data, a two-way ANOVA was performed. For all tests, type III sums of squares were used in computing *P*-values and the sequential Bonferroni test was applied to the *P*-values to account for the fact that multiple measurements were made on each frog. The experiment-wide $\alpha$-value was $P < 0.05$ for all tests.

In order to compare the length–tension curves across species, it was necessary to fit an equation to each curve and extrapolate to interval values of tongue length. This was necessary because the actual reference lengths (in mm) differed among individuals,

and the percentage length increments differed slightly among individuals as well. For instance, in a frog with an $L_o$ of 25 mm, the 2 mm increments represent 8% changes in $L_o$; if $L_o$ = 27 mm, the 2 mm increments produced $L_o$ increments of 7.1%. Thus, the actual forces were not tested at comparable lengths in percent of $L_o$ or $L_{HG}$ among frogs. In order to get a mean length–tension curve, we plotted the individual length–tetanic tension curve for each muscle in each frog with a best polynomial curve fit using least squares regression. Once each individual length–tension curve was plotted using the actual length increments, forces were extrapolated that corresponded to lengths in 10% increments from shortest (100% $L_o$ or $L_{HG}$) to longest natural lengths (from 180–210% of $L_o$ for GG, depending on species; for HG, maximum lengths ranged from 150–180% of $L_{HG}$, depending on species; see Results). These values for individual frogs were then averaged at each length to produce a mean length–tetanic tension curve for each muscle in each species. Values at each length-interval were then compared in an ANCOVA (using tongue mass as covariate) to test for differences in force over the entire range of tongue lengths.

## RESULTS

The video sequences of each species confirm most of the assumptions about their appropriate use as examples for each feeding style. In *Litoria*, the pattern of tongue and jaw movement in prey capture, and the maximum tongue velocities and accelerations (15–48 cm/sec and 5–9.5 m/sec$^2$; Table 1) were similar to those found in *Hyla* and other species with mechanical pulling tongues (e.g., Deban and Nishikawa, '92). In *Bufo marinus*, the inertially elongating tongue has been studied in the most detail (Gans and Gorniak, '82a,b; Nishikawa and Gans, '96). Its ballistic tongue protraction produces extremely high maximum velocities and accelerations (250–272 cm/sec and 250–310 m/sec$^2$; Table 1).

There have been no previous studies of tongue use in *Dyscophus*. However, several lines of evidence suggest that *Dyscophus* possesses a hydrostatic tongue. In unpublished data from six microhylid species representing five subfamilies, denervation of one side of the tongue resulted in bending toward the inactivated side. In addition, histological studies have found both transverse and dorsoventral muscle fibers within GG of a species of *Gastrophryne* (Cannatella, pers. comm.) and in *Dyscophus* (this study). Other unpublished observations from more than 20 species of microhylids indicate their ability to aim the tongue in three dimensions. All of these preliminary data support the conclusion that microhylids, as a group, typically have hydrostatically elongating tongues (see also Nishikawa, '99). *Dyscophus guinetti* has most of the kinematic characteristics typical of hydrostatic elongators (long protrusion, asynchronous tongue, and jaw

*TABLE 1. Morphometric comparisons of Litoria, Dyscophus and Bufo specimens ($\bar{x} \pm SE$)*

| | *Litoria* (n = 11) | *Dyscophus* (n = 13) | *Bufo* (n = 12) | *P*-values for overall effects of:[a] species | Tongue mass | spXtm |
|---|---|---|---|---|---|---|
| Body mass (g) | 41.0 ± 3.1 | 39.8 ± 2.9 | 66.8 ± 5.5* | 0.05 | 0.0001 | 0.0001 |
| Tongue mass (g) | 0.56 ± 0.04 | 0.81 ± 0.10 | 0.75 ± 0.05 | 0.0001[b] | | |
| Tongue mass/body mass (%) | 1.35 ± 0.03* | 1.96 ± 0.15* | 1.14 ± 0.05* | 0.001[b] | | |
| Jaw length (mm) | 19.7 ± 0.5 | 20.2 ± 0.6 | 23.0 ± 0.6* | 0.2 | 0.0001 | 0.02 |
| Resting tongue length ($L_o$) (mm) | 21.1 ± 0.6* | 23.6 ± 0.9* | 29.4 ± 0.7* | 0.03 | 0.0001 | 0.6 |
| Resting HG length ($L_{HG}$) (mm) | 25.5 ± 1.4* (n = 5) | 36.1 ± 2.2* (n = 6) | 45.5 ± 2.2* (n = 6) | 0.005 | 0.0001 | 0.05 |
| Maximum tongue velocity (cm/sec) | 48 (n = 7) (range, 15–48) | 220 (n = 7) (range, 120–220) | 272[c] (range, 250–272) | | | |
| Maximum tongue acceleration (m/sec$^2$) | 9.5 (n = 7) (range, 5–9.5) | 144 (n = 7) (range, 48–144) | 310[c] (range, 250–310) | | | |

[a]*P*-values derived from 1-factor ANCOVA (Type III SS) using tongue mass as covariate (see Materials and Methods).
[b]*P*-values derived from 1-factor ANOVA.
[c]From Nishikawa and Gans ('96).
*Indicates significant differences among species (from Student-Newman-Keuls post-hoc test). An asterisk on one species only indicates that it differs from both of the other species. Asterisks on all three species indicate that all differed significantly from each other.

movements, aiming ability). However, unlike the well-studied *Hemisus marmoratum* (Ritter and Nishikawa, '95), *Dyscophus* has relatively fast maximum velocities and accelerations (120–220 cm/sec and 48–144 m/sec$^2$; Table 1). The slow elongation and aiming of the tongue in *Hemisus* is clearly accomplished entirely by the hydrostatic mechanism (Ritter and Nishikawa, '95). However, because of the speed of this movement in *Dyscophus*, it is probable that inertia may also play a significant role in tongue protrusion.

Table 1 shows the morphometric measurements for each species. Specimens of *Bufo marinus* were significantly larger in body mass than the other two species. However, the tongue masses did not differ significantly among species (Table 1). Thus, *Bufo* has the smallest ratio of tongue mass to body mass. The jaw length in *Bufo* was somewhat longer than in *Dyscophus* or *Litoria*. *Bufo* also had the longest $L_o$ and $L_{HG}$, but in both cases *Dyscophus* was also significantly longer than *Litoria*. Thus, tongue reference lengths varied significantly such that *Litoria*<*Dyscophus*<*Bufo*.

### *Force properties*

Few differences among species were found in the isometric force properties of the protractor. The major significant differences were found in HG, with *Bufo* having the smallest maximum tetanic force. The HG in *Bufo* and *Dyscophus* was similar in that greater force was produced at lower stimulus rates and durations. Finally, *Bufo's* HG not only produced the smallest forces over its natural range of length change, but the length at which maximum force was reached was relatively much shorter when compared with the other species.

Table 2 summarizes the maximum isometric force data. No significant differences were found among species in either the maximum twitch forces or tetanic forces of GG. In HG, *Dyscophus* had a significantly larger mean twitch force than either *Bufo* or *Litoria* (which did not differ from each other), and its mean tetanic force was also largest, but not significantly different from *Litoria*. HG in both *Dyscophus* and *Litoria* had significantly larger mean tetanic forces than in *Bufo*. The mean maximum tetanic force of HG was much larger than GG in *Dyscophus* and *Litoria*, but, because of its small force, HG did not differ from GG in *Bufo*.

The mean isometric tetanic forces at other lengths over the entire length–tetanic ten-

*TABLE 2. Isometric contractile properties of tongue muscles in Litoria, Dyscophus and Bufo ($\bar{x} \pm SE$)*

| | Genioglossus | | | Hyoglossus | | | *P*-values for overall effects of:[a] | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Lit* (n = 6) | *Dysc* (n = 7) | *Bufo* (n = 6) | *Lit* (n = 5) | *Dysc* (n = 6) | *Bufo* (n = 6) | Species | Muscle | Tongue mass |
| Maximum twitch force (g) | 3.7 ± 0.4 | 3.7 ± 0.6 | 4.8 ± 0.5 | 8.4 ± 1.6 | 34.9 ± 7.1* | 13.7 ± 1.9 | 0.3 | 0.8 | 0.002 |
| (N) | 0.036 | 0.036 | 0.047 | 0.082 | 0.342* | 0.134 | | | |
| Maximum tetanic force (g) | 39.0 ± 3.6 | 24.7 ± 2.6 | 24.6 ± 2.0 | 68.8 ± 10.9 | 84.4 ± 12.7 | 38.6 ± 3.6* | 0.4 | 0.9 | 0.0001 |
| (N) | 0.382 | 0.242 | 0.241 | 0.675 | 0.828 | 0.379* | | | |
| | | * | | | | | | | |
| Twitch/tetanus ratio (%) | 9.3 ± 0.6 | 15.1 ± 1.5 | 19.3 ± 1.1 | 12.3 ± 1.8* | 39.7 ± 2.5 | 35.5 ± 3.1 | 0.03 | 0.006 | 0.8 |
| Length at max. tension | 168 ± 4.4 | 182.4 ± 5.9* | 158.4 ± 4 | 140.5 ± 3.6* | 159 ± 4.1* | 124.4 ± 2.9* | 0.2 | 0.004 | 0.001 |
| | | ($\%L_o$) | | | ($\%L_{HG}$) | | | | |
| Contraction time (msec) | 55.5 ± 1.8* | 36.4 ± 1.2* | 44.2 ± 1.6* | 60.2 ± 3.3* | 79.3 ± 2.0* | 92.0 ± 4.3* | 1.0 | 0.004 | 0.6 |
| Tw ½ relax time (msec) | 43.7 ± 1.3* | 32.6 ± 2.0 | 27.5 ± 1.3 | 70.2 ± 4.6* | 99.5 ± 5.2 | 110.3 ± 4.2 | 0.6 | 0.0004 | 1.0 |
| TT ½ relax time (msec) | 248.8 ± 21.0 | 253.5 ± 7.1 | 145.1 ± 7.4* | 249.0 ± 25.0 | 488.7 ± 18.3* | 251.3 ± 18.3 | 0.1 | 0.0001 | 1.0 |
| Fatigue index | 53.9 ± 0.6* | 62.8 ± 2.0 | 59.5 ± 2.8 | 58.8 ± 2.3 | 60.1 ± 1.3 | 64.3 ± 1.0 | 0.7 | 0.2 | 0.3 |

[a]*P*-values derived from 2-factor ANCOVA (Type III SS) using tongue mass as covariate (see Materials and Methods).
*Indicates significant differences among species within each muscle (based on Student-Newman-Keuls post-hoc test). Single asterisks indicate one species differing from the other two. Asterisks on all three values indicate that all differ significantly. Bar designates which two values differ from each other.

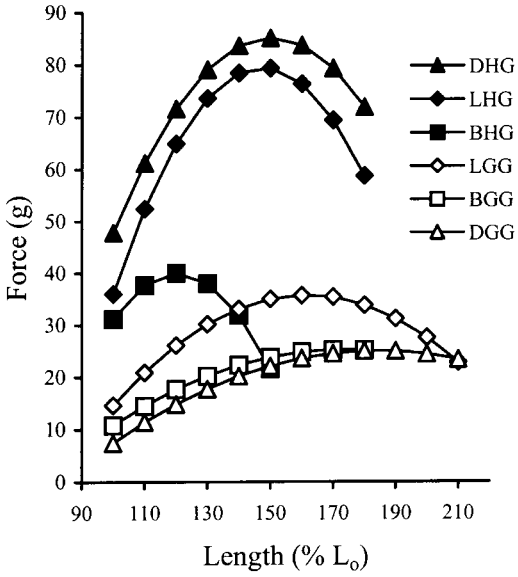


Fig. 3. The active length–tension curves for GG and HG in each of the three species are shown relative to their appropriate reference lengths. For the HG muscles, this is $L_{HG}$ and for GG we used the tongue resting length, $L_o$ (see text for details). These are mean curves extrapolated from the actual length–tension curves of each muscle in each species. There were no significant differences in force at any length among the GG muscles. LHG and DHG were significantly different from the GGs at all lengths. Forces in BHG were not significantly different from those in the GG muscles. At lengths $\geq$120% of $L_{HG}$, BHG force was significantly less than the force of the other HG muscles. LGG, *Litoria* genioglossus (n = 6); LHG, *Litoria* hyoglossus (n = 5); DGG, *Dyscophus* genioglossus (n = 7); DHG, *Dyscophus* hyoglossus (n = 6); BGG, *Bufo* genioglossus (n = 6); BHG, *Bufo* hyoglossus (n = 6).

sion curves (Fig. 3) showed a similar pattern among species as did the maximum tetanic forces (Table 2). None of the forces at comparable lengths differed significantly among the three species for GG; the HG forces in *Litoria* and *Dyscophus* were equivalent over all lengths. In *Bufo*, tetanic forces of HG did not differ from those in *Dyscophus* or *Litoria* at the two shortest lengths. However, at maximum-force length and longer, HG forces in *Bufo* were significantly smaller than the HG forces in the other two species, and did not differ from the forces produced by the GGs. Note that the GG curves are flatter than HG curves, i.e., there is less difference between minimum and maximum forces over the natural length changes in GG than in HG (Fig. 3).

In looking at the mean lengths at which the muscles reach maximum tetanic force (Table 2, Fig. 3), *Dyscophus* reaches maximum force at the longest relative lengths for both GG (182.4 $\pm$ 5.9% of $L_o$) and HG (159 $\pm$ 4.1% of $L_{HG}$). In both *Bufo* and *Litoria*, GG reaches maximum force at equivalent lengths (168 $\pm$ 4.4% and 158.4 $\pm$ 4% of $L_o$, respectively). For HG, *Dyscophus* reaches maximum force at the longest length, *Litoria* is intermediate (140.5 $\pm$ 3.6% of $L_{HG}$), and *Bufo* reaches maximum force at the shortest length of all (124.4 $\pm$ 2.9% of $L_{HG}$). Thus, in a comparison of the length–tetanic tension curves, the most striking result is the small force produced by HG in *Bufo* and the fact that it reaches its maximum tetanic force at the shortest relative length.

The force–frequency results are shown in Figure 4. Overall, the differences among species in GG were less pronounced than in HG. The general pattern was for HG in *Dyscophus* and *Bufo* to reach higher percents of their maximum force at lower frequencies than in *Litoria*. For GG, there were no significant differences in the amount of force produced at any stimulus frequency between *Bufo* and *Litoria*. In the mid-range of stimulus frequencies (25–40 pps), however, *Dyscophus* GG produced significantly less force than in *Bufo* or *Litoria* (Fig. 4A), though the magnitude of the difference was not great. So again, there are relatively few differences in GG among the species. The greatest significant differences were seen in HG (Fig. 4B). At all frequencies from 5–30 pps, *Litoria* produced the lowest percent of maximum force, *Dyscophus* the most, and *Bufo* was intermediate. At 35–40 pps, the forces were equivalent in all three species; at high frequencies (60–80 pps), *Dyscophus* and *Bufo* produced a lower percent of maximum force than did *Litoria*. Thus, the muscles that produce large forces at lower frequencies reach their maximum and then the force falls off at the higher frequencies.

The force–duration data (Fig. 5) show a pattern similar to the force–frequency data. At a constant stimulus frequency (30 pps), the differences in percent of maximum force produced at each stimulus duration between the GG or HG muscles in the three species were relatively small. In each case where significant differences occurred, *Litoria* produced the lowest percent of maximum force. The magnitude of these differences was less in GG (Fig. 5A) than in HG (Fig. 5B), and differences were not as marked as for the force–frequency data (Fig. 4).

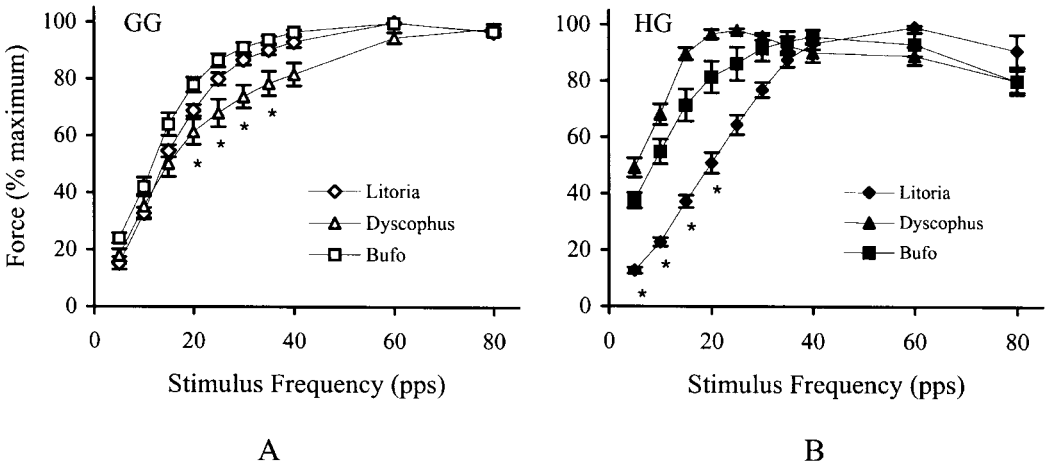


Fig. 4. The force (in % of maximum) produced by each muscle at varying stimulus frequencies ($\bar{x} \pm$ SE). Relatively small differences were found among the GG muscles (**A**). Though *Dyscophus* produced significantly less force than the other two species, the magnitude of the difference was small. Differences in force with frequency were greater among the species for HG (**B**). From 5 to 35 pps, *Litoria* produced the least, *Bufo* was intermediate, and *Dyscophus* produced the most HG force. Past 40 pps, *Dyscophus* and *Bufo* produced less force than did *Litoria*.

The ratios between the maximum twitch force and maximum tetanic force (Tw/TT) for each muscle in the three species are shown in Table 2. This ratio is a measure of activation of the muscle with a single twitch relative to the maximum tetanic force. It should parallel the force–frequency and force–duration results in that the amount of force generated in a single twitch should be a higher percent of the maximum tetanus in those muscles that are most activated at lower frequencies or shorter durations. This is indeed the case for our tongue muscles. The Tw/TTs for GG are similar among species as were the force–frequency and force–duration data. In *Litoria*, the Tw/TT also did not

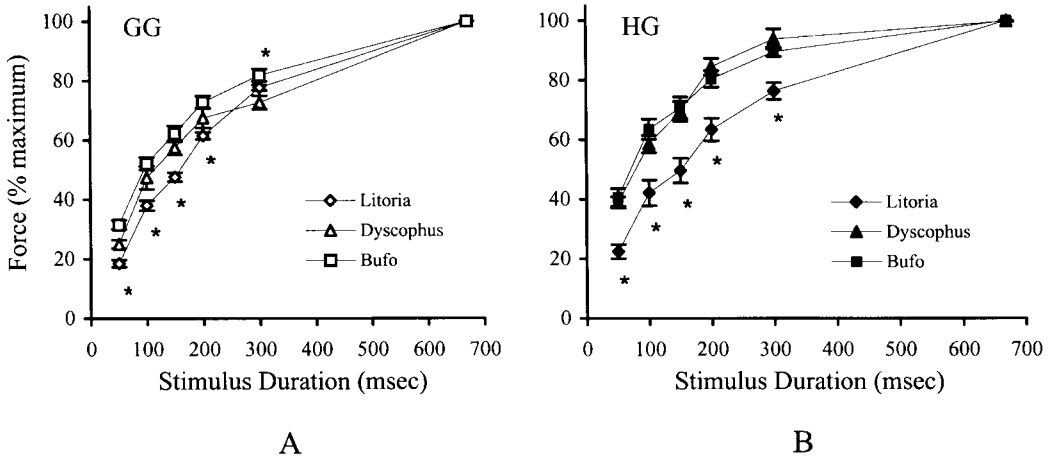


Fig. 5. The force (in % of maximum) produced by each muscle at varying stimulus durations ($\bar{x}$ SE). The differences among GGs (**A**) were slight; the only statistically significant differences show that *Litoria's* GG produced somewhat less force at the shortest durations (50–200 msec) than in the other two species. The forces produced by HG (**B**) in *Dyscophus* and *Bufo* were identical over the range of stimulus durations, but *Litoria's* HG produced less force at all frequencies from 50–300 msec.

differ between GG and HG. The major significant differences occur in the Tw/TT of HG in which *Dyscophus* and *Bufo* both have much larger ratios than *Litoria*. This, again, reflects the high percent of maximum force activated in HG with low stimulus input in both *Dyscophus* and *Bufo*.

Passive tension was measured during the length–tension tests in all of the experiments. Since we could not isolate the individual muscles, the passive tension readings are for the whole tongues in each species. There was no significant difference in the passive readings within a species during tests of either GG or HG at any given length (all calculated in percent of the tongue's resting length — $L_o$), so all passive data within a species could be lumped together at each equivalent length. As for the length–tension data (Fig. 3), since actual percentage tongue lengths varied among individuals, the amount of passive tension from $L_o$ to 170% $L_o$ (at 10% intervals) was extrapolated from the individual data and mean tensions at each length interval were calculated and compared in an ANOVA. In each species, the passive tension at $L_o$ was zero (Fig. 6). For *Litoria*, the mean maximum passive force was significantly greater than in the other two species (12.5 ± 1.4 g; ≈32% of maximum GG tetanic tension); in *Dyscophus* and *Bufo*, the mean maximum forces were not significantly different from each other (3.9 ± 1.3 g [15.8% max. GG tetanic force] and 3.05 ± 0.24 g [12.4% max. GG tetanic force], respectively). *Dyscophus* did not differ significantly in passive tension from *Bufo* at any of the lengths over the natural range. At almost all lengths, however, *Litoria* produced significantly greater passive tension than either of the other two species (Fig. 6). The passive tension in *Litoria* was 1.4 times (at shortest lengths) to >4 times (at maximum length) greater than in the other two species.

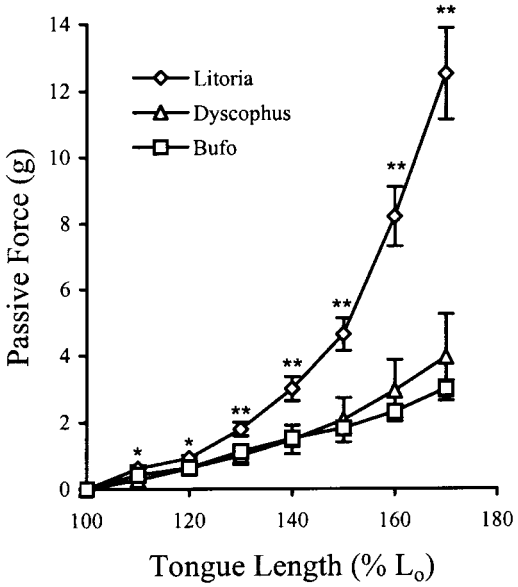


Fig. 6. The passive force produced by the whole tongue as it was elongated from $L_o$ to 170% of $L_o$ is shown for each species ($\bar{x}$ SE). The force at $L_o$ was unmeasurable in all three species. *Litoria's* passive tension increased most rapidly from 120–170% of $L_o$. *Litoria* differed significantly only from *Dyscophus* at 110% and from *Bufo* at 120% of $L_o$ (single stars); at all longer lengths, *Litoria* was significantly different from both of the other species (double stars). *Bufo* and *Dyscophus* did not differ significantly from each other at any length.

*Contraction and relaxation times*

The mean contraction and relaxation times measured from the twitch, and relaxation times from tetani at maximum-force length in each muscle are shown in Table 2. Contraction times for both GG and HG differed among all three species. In GG, *Dyscophus* was fastest, *Bufo* intermediate, and *Litoria* slowest. A nearly opposite pattern was seen in HG, with *Litoria* fastest, *Dyscophus* intermediate, and *Bufo* slowest. In *Litoria*, the CTs of GG and HG did not differ significantly from each other, but in *Dyscophus* and *Bufo*, GG contracted significantly faster than HG. The pattern among species in twitch relaxation was similar (Table 2): for GG, Tw ½R in *Litoria* was significantly slower than in *Dyscophus* or *Bufo*, but for HG, *Litoria's* Tw ½R was significantly faster. The Tw ½Rs in *Dyscophus* did not differ significantly from *Bufo* in either GG or HG.

As Table 2 shows, the TT ½R times did not differ significantly among four of the six muscles: GGs in both *Litoria* and *Dyscophus*, as well as HGs of *Litoria* and *Bufo* all had very consistent tetanic relaxation times at around 250 msec. The outliers are GG in *Bufo*, which had a significantly faster mean TT ½R, and HG in *Dyscophus*, which had a significantly slower TT ½R.

*Fatigue*

Fatigability of the muscles was compared in two ways; using a fatigue index (FI) (Table 2), and by plotting the profile of fatigue over the full 4-min test (Fig. 7). Very few differences were found among species or between

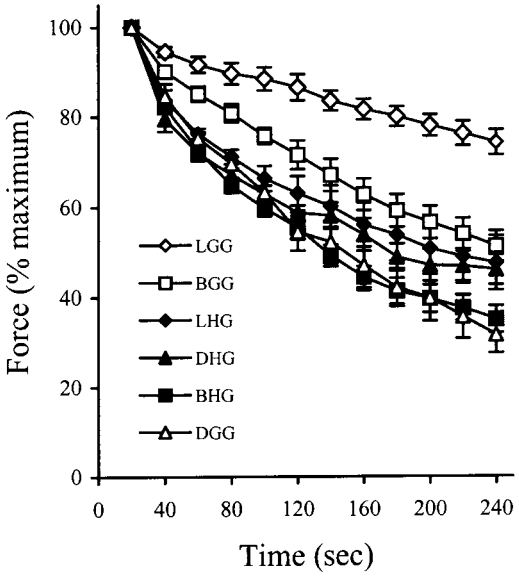


Fig. 7. Profiles of fatigue in each muscle for each species are plotted over the 4-min fatigue test. The average force during each 20-sec interval from 0–240 sec was calculated in percent of maximum (which always occurred during the first 20 sec). The force in LGG remained significantly higher than in any of the other muscles or species throughout the test. Force in BGG was less than in LGG but significantly greater than the others up to 140 sec. After 140 sec, BGG fell to levels equivalent to the other muscles. Over the entire test, there was no significant difference between DGG and all three HG muscles; HG muscles did not differ among species either. LGG, *Litoria* genioglossus; LHG, *Litoria* hyoglossus; DGG, *Dyscophus* genioglossus; DHG, *Dyscophus* hyoglossus; BGG, *Bufo* genioglossus; BHG, *Bufo* hyoglossus.

muscles. When summarized in an FI, the only muscle that was significantly less fatigable than all the others was GG in *Litoria* (Table 2). The other GGs and all of the HGs had equivalent FIs of around 60 (moderate fatigue). Information from the profiles (Fig. 7) provides a better view of what happened during the fatigue test. As expected from the FI, the least fatigable muscle was GG in *Litoria*. Its mean force fell by only about 20% over the 4 min. During the first 2 min, GG in *Bufo*, though more fatigable than in *Litoria*, held force better than the other muscles, but by 4 min, it had fallen to the same level as the GG in *Dyscophus* and all three of the HG muscles. The *Dyscophus* GG and all three HGs followed identical profiles, falling to about 70% of original force by the first minute, to 60% of original force by the second minute, and to between 40–50% of the original force by the third and fourth minutes (Fig. 7).

## DISCUSSION

This study describes isometric whole-muscle contractile properties of the tongue protractor and retractor muscles in three frog species. While no one species necessarily has all the characteristics of its feeding type, we feel that our three species are representative of the diversity found in frogs. In general, we found very few differences in isometric contractile properties that might explain the diversity of tongue use in these frogs. Since we cannot replicate the dynamic changes in length, shortening speed, and inertia that must naturally occur, isometric tests at least provide comparable conditions under which to test the muscle properties. We do not have data to directly compare the shortening velocities of the muscles (no isotonic data). Since tongue mass is the same in all three species, we can assume that all experience the same initial loading, but if there are differences in the force–velocity properties of the tongue muscles, then power production could differ markedly between species. Future studies incorporating force–velocity properties will provide a more complete understanding of the relationship between muscle contractile properties and tongue use.

In summary, our major results show that 1) differences in tongue use cannot be explained by differences in the isometric whole-muscle force properties of the GG; 2) a small tongue retractor muscle (HG) in *Bufo* accords well with its feeding on small prey and may result in a relatively smaller tongue mass that would be adaptive in an inertial elongator (see below); 3) the minor differences in contraction times of GG do not explain the hugely disparate velocities and accelerations of the tongue during protraction in these species; and 4) differences in passive tension suggest that resistance to elongation of the tongue is least in those species that elongate the tongue greatly (*Dyscophus* and *Bufo*) and greatest in the mechanical puller that does not normally elongate the tongue at all (*Litoria*).

These results lead us to conclude that passive properties of the tongues, along with inertial effects of head and jaw movements, may be more important than contractile properties in producing the diversity of feeding behavior observed in frogs. In addition, our results on HG (combined with EMG data

from other sources) lead us to propose that HG is a synergist of GG in tongue protraction (see below). This may be the case in all frogs, but its contribution to accelerating the tongue may be most significant in inertial elongators.

*Comparison of forces*

We found no differences in the maximum isometric forces between the protractors (GG) in the three frog species (Table 2). Maximal muscle forces appear more than adequate to overcome the inertia of the tongue from rest in all three species (tongue mass varies from 1.5–3% of maximal GG tetanic force). Since all three species have tongues of equivalent mass and since protraction involves accelerating the tongue only, it is not surprising that the GG forces are equivalent. However, the tongues of these frogs differ about 31-fold in the acceleration they experience during protraction. These data show that differences in acceleration are not accounted for by differences in the ratio of maximal protraction force to tongue mass and may result from differences in intrinsic shortening velocity of the tongue muscles.

We measured tetanic relaxation times in order to estimate relative abilities of these frogs to produce repeated protractions. Marsh ('90) has argued that TT ½R is a good indicator of biochemical deactivation rates (i.e., cessation of cross-bridge activity), which are strongly correlated with the rates at which contraction can be reactivated. The faster the deactivation rate, the greater can be the frequency of repeated contractions. As our data show (Table 2), GG in *Bufo* has the fastest TT ½Rs. This accords well with descriptions in the literature that *Bufo* typically feeds on small, clustered insects like ants by repetitive tongue protractions (Zug and Zug, '79; Toft, '81; Emerson, '85). The exceptionally high acceleration seen during tongue protraction in *Bufo* may indicate that their muscles have a higher maximum shortening velocity ($V_{max}$), but since overall acceleration will also depend on mechanical factors (inertia, elastic storage, etc.), future studies that incorporate direct measurement of $V_{max}$ and force–velocity relationships will be valuable in clarifying the role of these properties in tongue function.

The difference in tongue acceleration among species could also result in part from differential activation of muscle force, e.g., the inertial elongator might recruit all of its motor units at once during protraction to overcome inertia from rest as quickly as possible; the more slowly moving mechanical puller might recruit less than maximum numbers of motor units in any given protraction. The basic ability of mechanical pullers and hydrostatic elongators to correct trajectory and alter tongue movements on-line (Nishikawa, '99) is consistent with this notion. Anderson et al. ('98) recently found that the number of motor neurons serving the tongue muscles is roughly twice as great in *Hemisus marmoratum* (slow, hydrostatic elongator) as in *Bufo*, suggesting greater ability for precise control of motor unit recruitment in *Hemisus*. Future studies of muscle fiber diversity and motor unit properties should add greatly to our understanding of how differential motor unit recruitment may affect tongue movements in these frogs.

The GG muscles did not differ markedly in the pattern of force development at different stimulus frequencies (Fig. 4) or tetanic train durations (Fig. 5). These results support a conclusion that the basic activation dynamics are similar between species for GG, i.e., nearly the same amounts of force are produced with the same stimulation input. This suggests that the distribution of muscle fiber types is similar among species, since activation rates depend on the intrinsic biochemical properties of the muscle fibers, in particular the myosin isoforms (for review, see Peters, '89). Differences, however, in contraction and twitch half-relaxation times (*Litoria*<*Bufo* <*Dyscophus*) as well as lower fatigability of GG in *Litoria* (Fig. 7) show that at least some aspects of muscle fiber physiology differ among species. These could be metabolic differences, such as in oxidative capacity that affect fatigability. The differences in contraction and relaxation speeds in GG apparently produce only small differences in the dynamics of whole muscle activation at different stimulus frequencies or durations.

What is the significance for our interpretation of GG function of the hydrostatic design of the tongue in *Dyscophus*? Under isometric conditions, most measurements were made at the tongue length where maximum force occurs; a relatively long length in GG (182% of $L_o$; Table 2). If the transverse fibers of the central compartment of GG attach to parallel sheets of connective tissue that are approximately equally stretched, then the transverse fibers would necessarily be shortened as the muscle is elongated. Thus, as the

sarcomeres of the longitudinal GG fibers are stretched to maximum actomyosin overlap, the fibers in the transverse compartment may be shortened and, therefore, have reduced force capability. So, at maximum natural length, reduction in whole-muscle GG force due to antagonism from the central compartment is probably small in all of our force-based tests (max. Tw, TT, force–frequency, force–duration, and fatigue).

The largest differences in force properties among frog species were found in the HG muscles. Both *Dyscophus* and *Litoria* produced maximal tetanic forces that were 1.5–2 times greater than in *Bufo*, and the maximum tetanic force of the HG of *Bufo* did not differ significantly from its GG force. In general, one expects that HG should be relatively larger than GG simply because it has to move the mass of both the tongue and prey during retraction. One also expects that the force produced by the retractor muscle should correlate with the feeding habits of the species in question. Most frogs are generalist feeders, taking a wide variety of prey (Toft, '81). There are no field descriptions of feeding in both *Litoria* and *Dyscophus*. Both took large waxworms and crickets in the lab, suggesting that they are among the generalists, not limited to small prey. Thus, the relatively large force capability of HG in both species should give them a wide range of prey options, and is probably typical of generalist feeders. *Bufo*, on the other hand, is known to specialize in feeding on relatively small prey (Zug and Zug, '79; Toft, '81; Emerson, '85), so that a relatively small HG muscle should be adequate for retrieval of prey.

In order to achieve higher muscle shortening velocities and fling the tongue out of the mouth most rapidly, one expects that in an inertial feeding frog like *Bufo* the tongue mass should be minimized relative to the size of the animal. Our data show this small ratio of tongue mass to body mass (Table 1). It is interesting to note, however, that *Bufo* does not sacrifice force in the protractor; only the retractor is relatively smaller in size, as estimated by the amount of force produced. If *Bufo* is representative of other inertial feeders, our data suggest that in the evolution of the inertial mode of feeding GG may remain relatively large in order to provide adequate force and shortening velocity to power rapid tongue protraction, while HG is reduced. It may be the case that, in order to minimize tongue mass, the most rapidly accelerating inertial feeders differentially decrease the size of HG and, as a result, may become limited to feeding on relatively small prey.

The activation dynamics of HG also differed more markedly among species with variation in frequency (Fig. 4) and duration (Fig. 5) of stimulation. The species with slower contraction times (*Dyscophus* and *Bufo*) in HG produced the largest percent of maximum force with the lowest stimulus input. These differences suggest that *Dyscophus* and *Bufo* HGs have larger populations of relatively slow muscle fibers and motor units with lower recruitment thresholds than in *Litoria*. In contrast to results in mammals in which slow motor units are typically small and nonfatigable (McDonagh et al., '80; Burke, '81), slower-contracting frog motor units are relatively large and fatigable (Peters, '94). Peters ('94) also found that these units produced greater force at low stimulation frequencies than the faster motor units. It is likely that the small HG in *Bufo* with slowest CT is composed almost exclusively of a relatively small number of large motor units. *Dyscophus* and *Litoria* are more likely to have mixed populations of large, slow units and small, fast units. Again, differential recruitment in a mixed-fiber muscle would allow a broader range of functions and may be typical of the generalist feeders.

### *Length–Tension*

The length–tension curves for GG in the three frog species (Fig. 3) do not differ significantly at any point over their natural range of length change. For HG, the tension values over the natural lengths of *Litoria* and *Dyscophus* are significantly greater than for GG, and are identical to each other. Clearly the outlier is HG in *Bufo*. The overall results are consistent with the notion that force in GG is less than HG due to the fact that GG moves the tongue mass only, whereas HG must also move the prey during retraction. Again, HG in *Bufo* is small as a likely mechanism to reduce overall tongue mass.

What is particularly interesting about the length–tension curves is that 1) all of the GGs reach maximum force at relatively long lengths, and 2) that HG in *Bufo* reaches maximum force at a length that is significantly shorter than in the other species. One normally expects muscles to reach their maximum forces at or near lengths where they must initiate movement (classic resting length) (e.g., Stephens et al., '75; Peters and Rick, '77; Goslow, '85;). For the GG muscle,

in initiating protraction this should be near $L_o$ (resting tongue length); for HG, peak force should occur near its maximum extended length, from which it would initiate retraction. Instead, our results show that the GGs all reach peak or near peak force over a range from ≈150–180% of $L_o$. As expected, HGs in *Litoria* and *Dyscophus* reach peak force near their maximum lengths (between 150–170% of $L_{HG}$). In contrast, *Bufo* HG is near peak force from $L_{HG}$ to 130% of $L_{HG}$.

How are we to understand these length–tension relationships in the context of natural tongue movement? Most skeletal muscles do not elongate by more than 30% from their resting lengths during natural movements (Gans and Bock, '65; Stephens et al., '75). One could argue that because of the extreme elongation of the tongue (by 50–100% of $L_o$), if optimum sarcomere length in GG occurred at $L_o$, it would be impossible to increase sarcomere length enough to achieve this overall tongue elongation. As a result, GG sarcomeres are relatively short at $L_o$. This argument might hold true for feeding in *Dyscophus* and *Bufo*, but in *Litoria* the tongue rarely elongates beyond $L_o$ in feeding. At least for *Dyscophus* and *Bufo*, the architecture of the tongue muscles may be designed more to allow elongation than to optimize the efficiency of force production. Thus, it appears that either the amount of force needed for tongue protraction is small relative to its maximum force so that GG can initiate movement while low on its length–tension curve, or something assists GG during protraction, or both. That something might simply be the inertia of mouth opening. However, our data suggest that, in addition, HG may act as a synergist to GG in protraction.

It has long been known that stimulation of GG alone does not produce complete tongue protraction (e.g., Gans and Gorniak, '82a). Several mechanisms have been proposed to assist GG in protraction, including movement of the hyoid (Emerson, '77) and stiffening of the tongue from contraction of GG plus muscles near the tongue, such as the submentalis (i.e., the ballista model; see Gans and Gorniak, '82b). These have not been fully supported by experimental studies (Nishikawa and Gans, '96). The gross anatomy of the tongue, however, suggests that HG could pull the tongue pad forward as it straightens (Fig. 1), providing the assistance that GG may require for full protraction.

What might the roles of HG be as a synergist of GG? It could 1) break the adhesion between the ventral surface of the tongue and the floor of the mouth, 2) stiffen the tongue while shortening by changing its viscoelastic properties, i.e., coactivation of HG with GG would increase pressure at the base of the tongue, in effect, giving GG something to pull against, and, 3) most directly, HG could protract the tongue tip as it straightens. In our isolated preparation, stimulation of the HG muscles alone, while the tongue was in its resting position, produced forward movement of the tongue tip. The anatomy suggests that HG would be most effective as a synergist early in tongue protraction (Fig. 1). Thus, HG would have to be active near the tongue's resting length ($L_{HG}$). Since HG forces in *Litoria* and *Dyscophus* are so much larger than their GG forces, even contraction at these short lengths would produce significant force to assist GG in protraction. However, HG in *Bufo* is reduced. If its length–tension curve were not shifted so that it could produce near maximum force closer to resting length, it might not be able to produce enough force to act as an effective synergist. As mentioned above, in order to achieve such rapid accelerations and velocities in *Bufo*, it may be that virtually all motor units in GG are activated at once. *Bufo* in particular may also require simultaneous coactivation of HG and GG to produce rapid tongue protraction.

Evidence for coactivation of HG with GG is clear, at least for *Bufo*. EMG studies (Gans and Gorniak, '82a; Matsushima et al., '85) commonly show a period of low-level activity in HG prior to tongue movement. This activity has been attributed to tongue positioning and possible shape changes in the tongue pad (Matsushima et al., '85). When the large EMG burst occurs in GG, signaling the beginning of protraction, HG is often silent. The HG burst to initiate retraction takes place approximately 30–50 msec following the beginning of the GG burst. Analysis of EMG data has typically centered on these bursts. If coactivation of HG with GG is important, the low-level EMG activity in HG prior to the GG burst should be reexamined. Our contractile data also provide a caution in the interpretation of EMG data. As noted above, EMG shows that HG often turns off prior to the EMG burst in GG. However, the exceptionally long twitch half-relaxation times of HG (lasting over 100 msec in *Bufo*, Table 2)

suggest that significant amounts of the HG force from the early low-level activity may still be present throughout the GG burst.

If this hypothesis is true, the frog tongue represents a unique structure in which, because of the odd wrapping of the HG fibers around the pulley-like hyoid, this muscle produces antagonistic actions about the same joint. The model of tongue protraction that we have just presented depends on coordinated timing of force production in the two tongue muscles to protract the tongue. This may explain a general mechanism for frog tongue protraction, or it may turn out that synergy of HG with GG is primarily important only in inertial elongators. There is some evidence that HG may not act as a synergist in all frogs. In a denervation study, Tso et al. ('95) found that HG is necessary for normal tongue protraction in *Bufo* and *Phrynomerus* (hydrostatic), but not in *Bombina* (mechanical puller). Thus, the proposed role of HG as a synergist in tongue protraction needs to be tested in a wider variety of frog species using EMG, denervation, and other techniques.

As far as we know, retraction of the tongue has not been examined in great detail either. Given the unique structure of the tongue muscles (Fig. 1), it is possible that when GG is fully elongated at the end of protraction, it could assist in tongue retraction. The fact that GG reaches maximum isometric force at extended lengths suggests that this is a viable hypothesis to test.

### *Inertia and passive forces*

The most interesting finding from our studies is that very few differences in isometric contractile properties are evident, especially in GG, that would explain the differences in tongue use among the three species. Especially noteworthy are the relatively minor differences in isometric contraction speeds found among species in which tongue velocity and acceleration vary markedly (Tables 1, 2). Clearly, factors other than intrinsic contraction times must be responsible for these behavioral differences.

One factor that may be important is the contribution of head and jaw movements to tongue acceleration through inertial effects (e.g., Deban and Nishikawa, '92; Nishikawa and Gans, '96; Nishikawa, '99). Overall tongue velocity and acceleration depend on the inertial effects on the tongue due to these movements. In all three of our species, the measured accelerations of the head and jaws were large enough (i.e., >acceleration of gravity) to have significant inertial effects on tongue acceleration. The coordination of these inertial effects with muscle contractions should be especially important in controlling speed and direction in fast-moving tongues, but they undoubtedly are involved in tongue movements of most frogs.

In addition, our data suggest that differences in protraction speed may be due in part to passive properties of the tongues. Our data (Fig. 6) show that passive resistance to elongation of the tongue is greatest in *Litoria*, which does not typically elongate its tongue beyond $L_o$. Passive resistance was least in *Bufo* and *Dyscophus* which both elongate their tongues dramatically (to 180–200% of jaw length). High passive tension is presumably due to larger amounts of connective tissue in *Litoria* with greater numbers of collagen fibers directed parallel to the long axis of the tongue. Webster ('96) found this to be true in another mechanical puller, *Hyla*. In *Bufo*, Webster ('96) found that the amount of connective tissue is much less than in *Hyla*, and in a hydrostatic feeder (*Hemisus marmoratum*), Nishikawa et al. ('99) found that most of the collagen fibers were directed perpendicular to the long axis of the tongue. This would produce resistance to increasing the diameter of the tongue, but not to its longitudinal elongation (Kier and Smith, '85). Both shortening speeds of the muscles and the resistance to tongue elongation could be decreased by lower passive tension in the tongue, increasing the speed and/or the extent of tongue protraction. In animals like *Bufo* and *Dyscophus*, which can develop high tongue velocities, decreased passive resistance to elongation should allow the tongue to move further in a given amount of time (i.e., increase tongue protraction speed). But *Hemisus* also has low resistance to elongation, not for speed, but to allow great elongation and flexibility of movement. Thus, a decrease in passive stiffness of the tongue may have evolved in both inertial and hydrostatic tongues for a variety of functions related to increased ability to elongate.

In summary, when we began this study we expected that predictable differences in isometric contractile properties would be found in the tongue muscles of frog species with different feeding styles. To our surprise, few differences in contractile properties were found, and the most divergent properties were in the retractor muscles, not the pro-

tractors. We propose that coactivation of HG with GG may be important in producing normal tongue protraction, especially in the fastest elongators. Inertial effects from jaw, head, and body movements, and the striking differences in passive resistance to elongation, emerge as the factors which may contribute most to diverse tongue function in frogs. The tongue muscles of frogs differ primarily in their ability to elongate, reflected both in sarcomere design and passive properties of connective tissues. These factors, more than the contractile properties yet known, may account for the diversity we see in tongue dynamics during feeding behavior.

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