Functional implications of supercontracting muscle in the chameleon tongue retractors

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Summary

Chameleons capture prey items using a ballistic tongue projection mechanism that is unique among lizards. During prey capture, the tongue can be projected up to two full body lengths and may extend up to 600 % of its resting length. Being ambush predators, chameleons eat infrequently and take relatively large prey. The extreme tongue elongation (sixfold) and the need to be able to retract fairly heavy prey at any given distance from the mouth are likely to place constraints on the tongue retractor muscles. The data examined here show that *in vivo* retractor force production is almost constant for a wide range of projection distances. An examination of muscle physiology and of the ultrastructure of the tongue retractor muscle shows that this is the result (i) of active hyoid retraction, (ii) of large muscle filament overlap at maximal tongue extension and (iii) of the supercontractile properties of the tongue retractor muscles. We suggest that the chameleon tongue retractor muscles may have evolved supercontractile properties to enable a substantial force to be produced over a wide range of tongue projection distances. This enables chameleons successfully to retract even large prey from a variety of distances in their complex three-dimensional habitat.

Key words: Chameleonidae, *Chamaeleo oustaleti*, super-contracting striated muscle, prey capture, force, tongue retractor, ultrastructure.

Introduction

Chameleons belong to the most primitive lizard clade (Iguania), members of which characteristically use their tongue to capture prey items (Schwenk and Throckmorton, 1989; Schwenk, 2000). Chameleons diverge from the primitive prey-capture mode by projecting their tongue ballistically up to twice their body length to capture prey (Wainwright et al., 1991). During tongue projection, the accelerator muscle, which surrounds the entoglossal process, contracts and generates the force to shoot the tongue off the hyoid (Wainwright and Bennett, 1992b; Van Leeuwen, 1997). After prey contact, the tongue with adhering prey is pulled back into the mouth by the tongue retractor muscles (Wainwright and Bennett, 1992a). It has been hypothesised that the highly specialised ballistic tongue projection mechanism evolved in response to the ambush foraging mode of prey capture used by chameleons. Indeed, chameleons show a number of morphological and behavioural characters [cryptic coloration, slow locomotion and muscle physiology, zygodactylous feet and prehensile tail; for an overview, see Schwenk (Schwenk, 2000)] that are thought to be related to their highly specialised sit-and-wait foraging mode. This extreme specialisation is probably the result of the three-dimensional complexity of their preferred habitat (i.e. shrubs and trees).

As sit-and-wait foragers usually eat infrequently and tend to capture relatively large prey (Andrews, 1979; Pough et al., 2001), it is important that chameleons are extremely accurate in capturing their prey and bringing it back to the mouth. This constraint on the visual and tongue projection systems has resulted in a specialised depth perception mechanism (Harkness, 1977; Ott and Schaeffel, 1995), leading to an extremely accurate prey-capture mechanism (Bell, 1990). As chameleons are known to take large prey (Broadley, 1973; Schleich et al., 1996), the tongue retraction mechanism is probably constrained as well. Not only should chameleons be able to retract the tongue with adhering large prey, but they should be able to do so over a wide range of distances. This might be especially problematic as chameleons are known to project their tongue to distances of up to two body lengths (Wainwright et al., 1991; Schwenk, 2000; Herrel et al., 2000). This implies that the tongue retractor should be able to generate large forces over at least a sixfold change in length (Rice, 1973). Given the length/tension properties of typical vertebrate muscles [with only a short plateau where force production is near maximal (Rome and Lindstedt, 1997; Burkholder and Lieber, 2001)], this seems unlikely. However, on the basis of behavioural observations of chameleons capturing large prey, such as lizards, positioned at a range of distances, we hypothesise that chameleons are able to produce large forces

over a wide range of prey distances and that this may be related to the supercontracting properties of the tongue retractor muscle (Rice, 1973).

The first aim of this paper is to examine how tongue retraction forces in chameleons vary with prey distance. In addition, we examine the physiology and structure of the tongue retractor muscle to investigate whether the supercontracting properties of the tongue retractor might facilitate the generation of continuously large forces over a wide range of distances.

Materials and methods

Force measurements

We recorded *in vivo* tongue retraction forces for an adult female *Chamaeleo oustaleti* Mocquard (snout–vent length 127.4 mm) and two adult female *Chamaeleo calyptratus* Duméril (snout–vent length 127.73 and 126.73 mm) by attaching a cricket in an alligator clip to an isometric force transducer (Kistler type 9203). The chameleon was trained to shoot its tongue at the cricket and, upon tongue retraction, forces were recorded by the transducer. For each individual, at least 50 trials were performed, and the maximal recorded tongue retraction force was plotted against tongue projection distance.

To confirm these results *in vitro*, we investigated the length/tension properties of the tongue retractor muscles in four live, anaesthetised adult *Chamaeleo calyptratus* (three males, one female). In this experiment, the animals were deeply anaesthetised with Ketamine (100–200 mg kg^{-1} body mass), and bipolar stainless-steel electrodes were implanted bilaterally into the tongue retractor muscles. The animal was kept under deep anaesthesia by administering additional Ketamine (half the original dose) every 2–3 h.

In a preliminary experiment, the tongue was clamped into a clip attached to a force transducer (Kistler type 9203). In all other experiments, the animal was mounted upside down in a purpose-built holder, the hyoid was immobilised in the resting condition, and the tongue pad was sutured to a muscle lever (Cambridge Technology model 6650 force lever connected to an Aurora Scientific series 305B lever system controller). Initially, the muscle was twitch-stimulated (Grass S48 stimulator connected to a Grass SIU5 stimulus isolation unit), and stimulation voltage was increased until maximal force output was obtained (at 25 V). In all subsequent experiments, muscles were stimulated at 30 V to ensure maximal muscle recruitment.

For two individuals, the muscle length was varied and the passive tension was recorded. At each length, the muscles were twitch-stimulated (30 V, 2 ms pulse duration) and the tongue retraction forces were recorded. For at least three twitches in two individuals, muscle twitch kinetics were recorded (maximal isometric tension, time to peak tension, half-relaxation time). For two other animals, the muscle was kept at resting length and stimulated with tetanic trains of 300 ms (2 ms pulse duration) of increasing frequency. The fusion
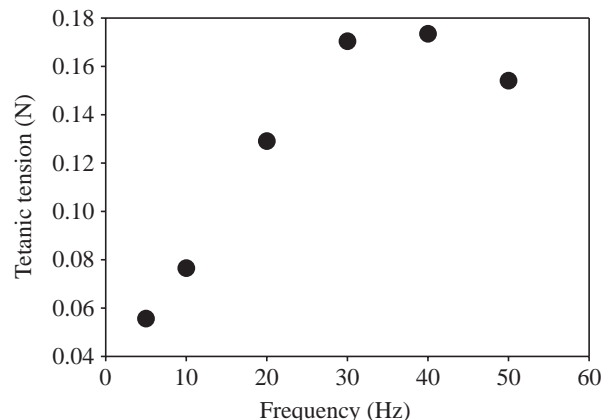

Fig. 1. Frequency/tension relationship of the m. hyoglossus in *Chamaeleo calyptratus* at resting length. The muscle showed a fused tetanus at 40 Hz, producing a maximal force of 0.173 N.

frequency (40 Hz) (Fig. 1) and tension at fusion were determined. Next, tongue length was varied, the passive tension recorded, the muscle stimulated with 300 ms tetanic trains at 40 Hz and the active tension recorded. Throughout the experiment, the temperature of the animal was kept at 32 °C by a heat lamp and continuously monitored with a YSI tele-thermometer and thermocouple. After all recordings, the animals were killed by injecting a lethal dose of Ketamine (twice the anaesthetic dose).

Morphology of the hyolingual system

Three preserved specimens of *Chamaeleo jacksonii* (snout–vent length, SVL, 72.8±3 mm, mean ± S.D.), one preserved *C. oustaleti* (SVL 135.7 mm) and one preserved *C. calyptratus* (SVL 187.4 mm) were dissected and stained to characterize the hyolingual muscles (Bock and Shear, 1972). Drawings were made from all stages of the dissection using a dissecting microscope (Nikon SMZ-10) provided with *camera lucida* (Herrel et al., 2001). In all these animals, we measured the length of the m. hyoglossus before and after the connective tissue surrounding the muscle had been cut and the muscle unfolded.

For light microscopy, the entire head of one preserved *Chamaeleo jacksonii* (SVL 69.4 mm) and the hyolingual system of three additional preserved *C. jacksonii* (SVL 74.6±3.1 mm) and one preserved *C. oustaleti* (SVL 145.8 mm) were prepared for paraffin histology using standard techniques (Humason, 1979). Serial 10 μm sections were made (transverse, sagittal and frontal) and stained with Masson's trichrome [see also Herrel et al. (Herrel et al., 2001)]. In addition, the tongues of a preserved *C. oustaleti* (SVL 174.2 mm) and a preserved *C. jacksonii* (SVL 78.5 mm) were sectioned sagittally, and selected sections were stained with Verhoeff's elastin stain (Bancroft and Stevens, 1977).

Transmission electron microscopy

For transmission electron microscopy, the m. hyoglossus of an adult *Chamaeleo melleri* (SVL 220.3 mm) was removed

unilaterally under deep anaesthesia (Ketamine, $200\,\mathrm{mg\,kg^{-1}}$) and cut in half. Tissue samples were removed from the middle third of the muscle. The animal was killed with an overdose of Ketamine after removal of the muscle samples. Tissue samples were fixed in 6.25 % glutaraldehyde in $0.1\,\mathrm{mol\,l^{-1}}$ sodium cacodylate buffer (pH 7.4) for 24 h. One half of the muscle was fixed in its resting position (but unfolded), and the other half was extended maximally (to approximately 180 % of its unfolded length) and tied onto a wooden stick using surgical wire. At least three small rectangular blocks of tissue, taken from the middle part of the muscle, were cut from both samples, thoroughly washed in $0.1\,\mathrm{mol\,l^{-1}}$ sodium cacodylate buffer and postfixed for 2 h in 1 % osmium tetroxide in $0.1\,\mathrm{mol\,l^{-1}}$ sodium cacodylate buffer. After postfixation, samples were rinsed thoroughly first in sodium cacodylate buffer and then in $0.05\,\mathrm{mol\,l^{-1}}$ maleic acid in distilled water (pH 5.2). Subsequently, samples were stained '*en bloc*' with 0.5 % uranyl acetate in $0.05\,\mathrm{mol\,l^{-1}}$ maleic acid buffer (pH 5.2), washed with $0.05\,\mathrm{mol\,l^{-1}}$ maleic acid buffer (pH 5.2) and dehydrated through a graded series of ethanols. The samples were then cleared in propylene oxide and embedded in resin (EMBed 812). Longitudinal, oblique and cross sections were cut, stained with uranyl acetate and examined with a JEOL 1200 Ex II transmission electron microscope at 60 kV. Pictures were taken of both resting and extended samples ($10\,000\times$ magnification) at different locations for several sections within each sample. The sarcomere length, filament lengths (thick and thin) and I-band thickness were measured for 30 sarcomeres using digital calipers (Mitutoyo model CD-20DC).

Values in the text are given as means $\pm$ S.D.

Results
In vivo *retraction forces*

In vivo tongue retraction forces in both *C. oustaleti* (0.59 ± 0.09 N; $N=15$) and in the two *C. calyptratus* (0.66 ± 0.08 N; $N=30$) were high over a wide range of distances and only decreased markedly at distances of less than 5 cm (Fig. 2).

Stimulation experiment

At resting length, the m. hyoglossus reached a peak tension of 0.027 ± 0.003 N in 42 ± 5.3 ms (averaged over two individuals). Half-relaxation times were 35 ± 1.15 ms. The length/tension diagrams established for single twitches (Fig. 3) show a continuously high (80 % of maximum twitch force) force production for muscle lengths of 700–1000 % of the muscle resting length. Forces are highest at 1300 % of muscle resting length (0.27 N and 0.46 N). Twitch force decreases slightly at higher extensions, but remains at or above 80 % of the maximal twitch force in both animals (Fig. 3).

Tetanic stimulations resulted in higher forces (up to 0.63 N), but showed a similar pattern (Fig. 4A). Although the absolute force was considerably lower in the preliminary experiment (Fig. 4B), this was not surprising given that only
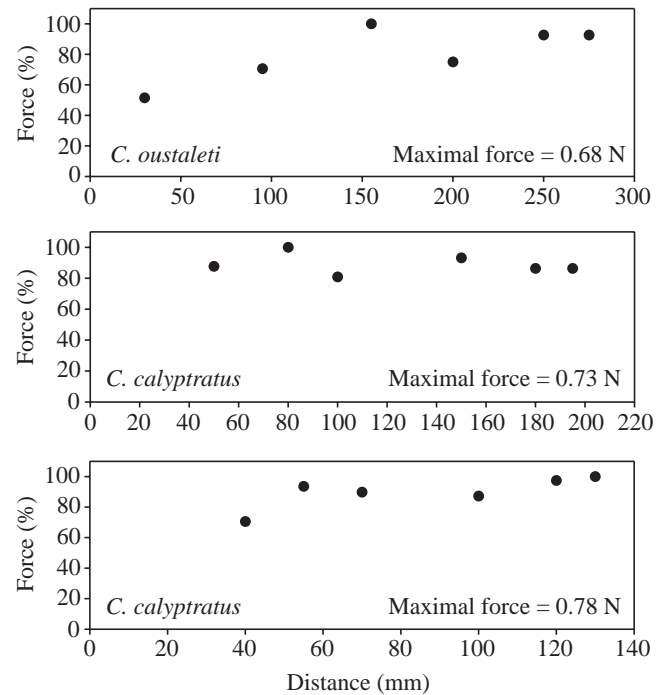

Fig. 2. *In vivo* total tongue retraction forces for an adult female *Chamaeleo oustaleti* and two adult female *C. calyptratus*. At least 50 trials were performed per individual, and the largest forces recorded for each distance were plotted against tongue length. All chameleons were able to produce large retraction forces over a wide range of distances.
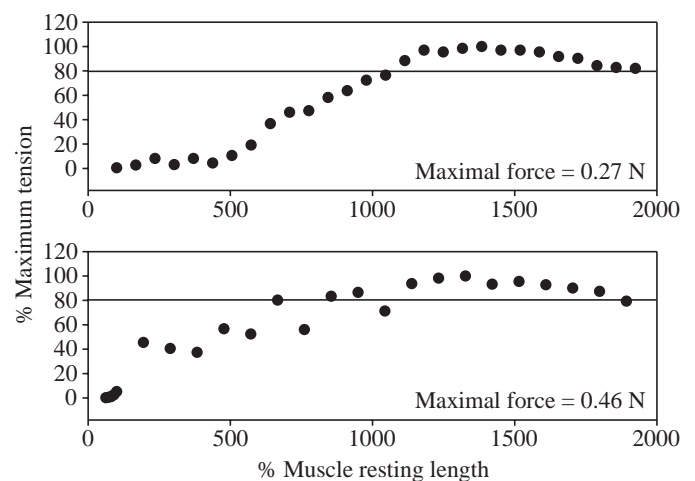

Fig. 3. Length/tension diagrams for the m. hyoglossus in *Chamaeleo calyptratus*. In these experiments, the tongue of the chameleon was attached to a force lever, its length changed, the muscle twitch-stimulated and the forces recorded. As in the *in vivo* experiments, the forces stay high for a wide range of tongue extensions. The more rapid decline of force at shorter lengths is probably due to the absence of an active hyoid retraction (note that the hyoid was immobilised in these experiments).

part of the tongue retractor muscle was stimulated in this experiment (owing to the position of the electrodes). The maximal force obtained during the stimulation of the other
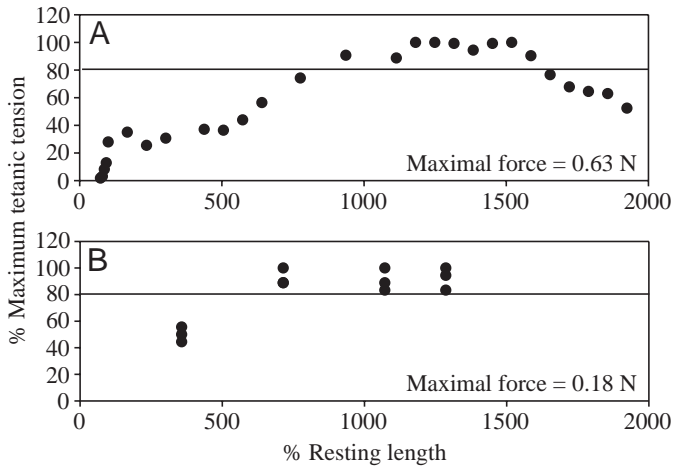
Fig. 4. Length/tension diagrams for the m. hyoglossus in *Chamaeleo calyptratus*. (A) The results of an experiment similar to that shown in Fig. 3, but with the muscle tetanically (40 Hz) stimulated. (B) The results of a preliminary experiment in which the tongue was attached to a force transducer and stimulated through two bipolar electrodes inserted into the anterior part of the muscle. Here, forces were recorded several times for four discrete tongue extensions.

animal has, however, a similar magnitude to the forces observed *in vivo*. In the tetanic stimulation experiments, forces also rose to 80 % of maximal tetanic force at approximately 700 % of muscle resting length. Forces remained high up to 1500 % of resting length and decreased thereafter to levels somewhat lower (50 % of maximum force) than those observed during the twitch stimulations (Fig. 4A).

Morphology

We briefly describe the morphology of the m. hyoglossus and the hyolingual apparatus. For a detailed description of the morphology of the hyolingual apparatus, see Bell (Bell, 1989), Schwenk (Schwenk, 2000) and Herrel et al. (Herrel et al., 2001). The chameleon hyolingual apparatus consists of a fleshy, muscled tongue attached to the hyoid. The tongue pad is connected to the accelerator muscle that sits on the entoglossal process of the hyoid. The tongue retractor, the m. hyoglossus, originates at the medial aspect of the second hyoid cornua over its entire length (Fig. 5A). Near its origin (proximal part) the muscle is rather bulky, but as it runs ventrad along the second hyoid cornua, the muscle belly narrows, passes under the articulation of the first cornua with the basihyoid and passes forward. Once past the first cornua (distal part), the muscle is folded upon itself in three dimensions (Fig. 5A), until it reaches the posterior side of the m. accelerator. The m. hyoglossus continues to run alongside the m. accelerator (under the strong layer of connective tissue surrounding the m. accelerator) for approximately one-quarter of the length of the latter and inserts firmly onto its lateral aspect. The extremely small overall muscle length (*C. calyptratus*: 12.35±2.40 mm; $N=4$) is largely due to the complex folding that takes place in its distal part. When unfolded, the muscle extends to 368±43 % of its folded length ($N=5$).
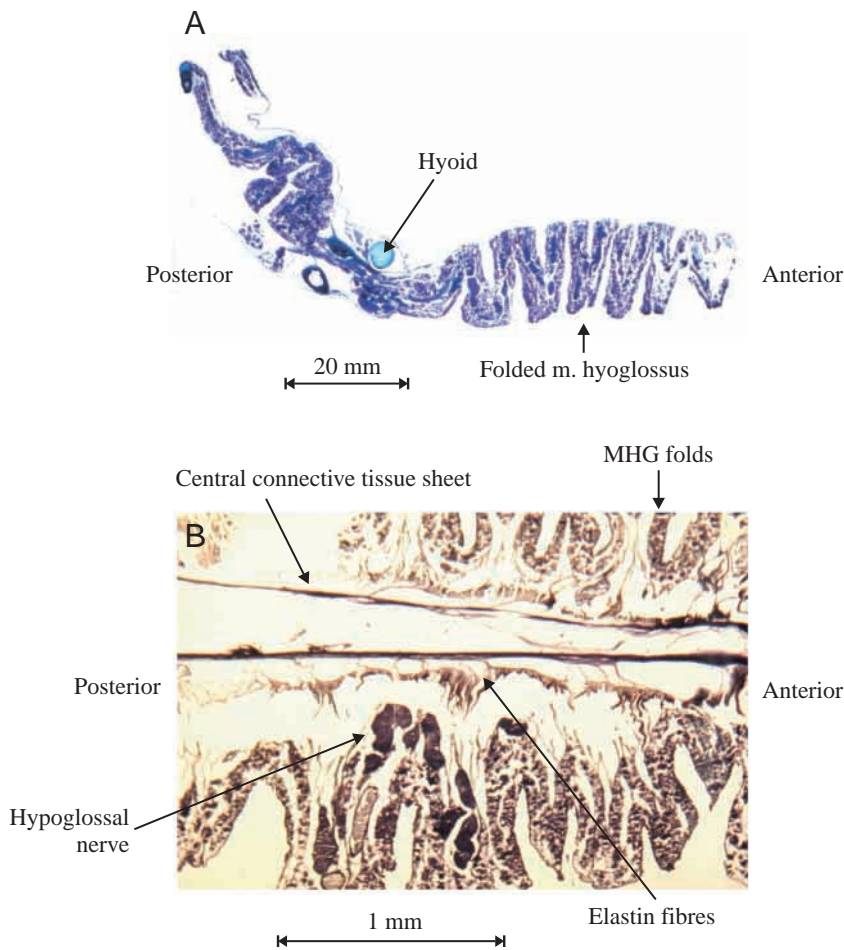

Fig. 5. (A) Sagittal (vertical) section through the hyolingual apparatus of a *Chamaeleo oustaleti* (trichrome stain). Whereas the proximal part of the m. hyoglossus (near its origin) runs approximately straight alongside the second hyoid cornua (not visible in this plane of section), the distal part of the tongue retractor muscle (m. hyoglossus) is folded in a complex manner in three dimensions. Anteriorly, the m. hyoglossus inserts onto the connective tissue sheet surrounding the m. accelerator (not shown here). (B) Frontal (horizontal) section through the hyolingual apparatus in a *Chamaeleo jacksonii* (Verhoeff's elastin stain). The folded tongue retractor and hypoglossal nerve are clearly visible. Centrally, the connective tissue sheet sends off branches of elastin fibres running into the folds of the m. hyoglossus. Here too, the folding of the retracted m. hyoglossus can be observed. MHG, m. hyoglossus.

Rest   Extended

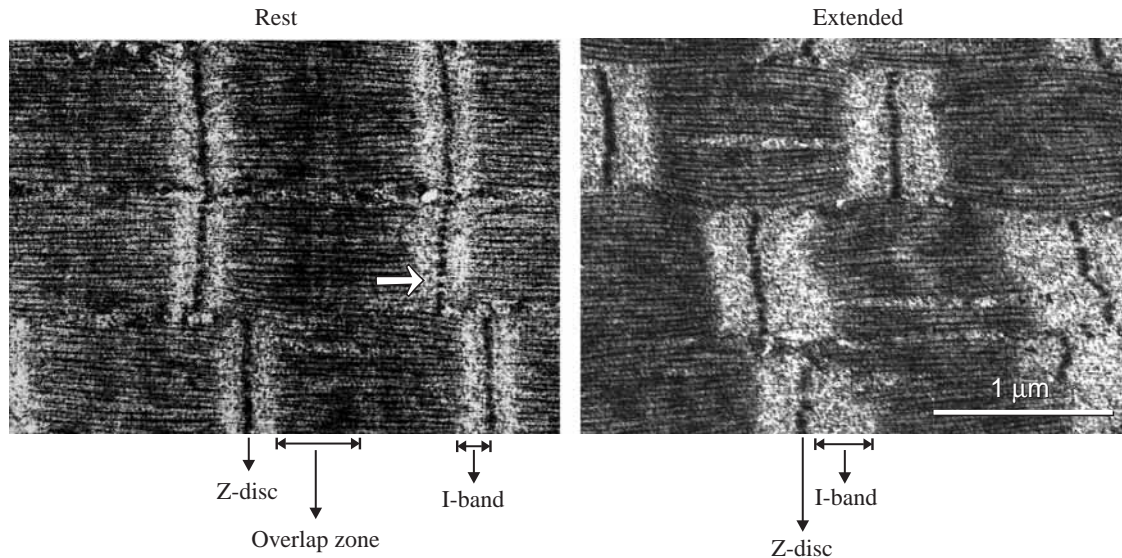Z-disc   I-band   I-band

Overlap zone   Z-disc

Fig. 6. Transmission electron micrographs (longitudinal section) through the tongue retractor muscle (m. hyoglossus) in a relaxed (left) and a maximally extended (right) state. Note the perforations in the Z-disks (arrowhead) characteristic of supercontracting muscle. Also note the relatively short I-bands and the large overlap between the thick and thin filaments. The force produced by a muscle is proportional to the number of cross bridges engaged and, thus, to the overlap between the thick and thin filaments.

Inside, and connected to, the m. hyoglossus sits an extremely well-developed connective tissue sheet that connects the hyoid to the accelerator muscle (Zoond, 1933; Bell, 1989). An analysis of elastin-stained sections of the m. hyoglossus shows the presence of elastin in the connective tissue branching off from this main internal connective tissue sheet into the folds of the m. hyoglossus (see Fig. 5B). Surrounding the m. hyoglossus is a strong external connective tissue sheet connecting the hyoid to the m. accelerator and the tongue pad. Its fibres are oriented transversely to the long axis of the muscle; see also Herrel et al. (Herrel et al., 2001).

Muscle ultrastructure

An examination of the muscle ultrastructure using transmission electron microscopy showed the typical perforations in the Z-disks that indicate that the muscle is indeed of the supercontracting type (Fig. 6). Sarcomere lengths were considerably shorter than those reported for other vertebrates [rest, $1.26\pm0.024\,\mu m$; extended, $1.60\pm0.140\,\mu m$, means ± S.D., $N=30$; compare with data in Van Leeuwen (Van Leeuwen, 1992), Nishikawa et al. (Nishikawa et al., 1999), Burkholder and Lieber (Burkholder and Lieber, 2001)]. These short sarcomere lengths seem to be the result of short thick filaments ($1.00\pm0.049\,\mu m$ in chameleon m. hyoglossus *versus* $1.6\,\mu m$ typically reported for vertebrate muscle) and thin filaments [$0.618\pm0.049\,\mu m$ for the chameleon m. hyoglossus *versus* $0.95\,\mu m$ typically reported for vertebrate muscle (Van Leeuwen, 1992; Nishikawa et al., 1999); note, however, that myosin filament lengths vary widely in invertebrate muscle (Full, 1997)]. Given the novelty of these measurements, they should be checked in other species of chameleon. The I-band thickness was small in both the resting ($0.13\pm0.012\,\mu m$) and extended ($0.31\pm0.072\,\mu m$) samples. By comparing the sarcomere and

filament lengths, it becomes clear that filament overlap is large both at rest (77 % of each actin filament in overlap) and when extended (51 % of each actin filament in overlap).

Discussion

The results from the force measurements show that chameleons are capable of producing high tongue retraction forces over a large range of tongue extension distances (Fig. 2). The only previous measurements of tongue retraction forces (Dischner, 1958) showed somewhat lower forces for a small *C. montium* (43 g *versus* 74.5±5.1 g, $N=3$ for the animals in this study). The results from the stimulation experiment largely confirmed the *in vivo* performance measurements and showed that chameleons are indeed able to exert high and fairly constant forces over a wide range of tongue extension distances (Fig. 2). The differences between the *in vivo* and *in vitro* data are mainly in the more rapid decrease in force at shorter distances. This might be the result of an active contribution of hyoid retraction to overall tongue retraction forces *in vivo*. Although hyoid movements were prevented in the *in vitro* experiments, *in vivo* a posterior movement of the hyoid will effectively aid in pulling back the tongue and prey. Given the amount of hyoid displacement observed *in vivo* (Wainwright and Bennett, 1992a; Meyers and Nishikawa, 2000), this would keep the total force output of the system high until distances of approximately one-third of a body length are reached.

In vivo, it is only at distances of less than approximately one-third of the body length of a chameleon that a decrease in performance is observed (Fig. 2). One interesting behavioural observation that might correlate with this decrease in performance is that most chameleons tend to refuse to shoot their tongues at prey positioned at distances of less than one-

third of their body length and, in many such cases, will move away from the prey before attempting to capture it (Burrage, 1975; Schwenk, 2000) (A. Herrel and J. J. Meyers, personal observations). Although the tongue retractor forces are presumably always high enough to retract the mass of the prey into the mouth, pulling back prey that are holding onto branches and coping with the inertial effects of large prey falling in mid-air aided by gravitational forces might require near-maximal force production.

As basic muscle mechanics show that a trade-off between sarcomere length and force production is unavoidable (Gordon et al., 1966), we investigated the ultrastructure of the chameleon tongue retractor muscle. A comparison of two samples of the tongue retractor muscle (rest *versus* extended) shows that, even at maximal extension of the muscle (as determined by *in vivo* measurements and by manual extension of the tongue in anaesthetised specimens), the overlap between the thin and thick filaments remains fairly large (50 %, Fig. 6). This implies that the amount of force that can be exerted by the muscle is large, even when extended to the limits observed during prey capture. The maximal extension capacity of the muscle *in vivo* seems to be limited by the thick connective tissue sheet connecting the m. accelerator and the tongue pad to the entoglossal process (see Fig. 5) (Herrel et al., 2001). Upon retraction, normal cross-bridge cycling is possible until the muscle is contracted to its unfolded resting length. At that time, thin filaments start to overlap and thick filaments will abut onto the Z-disks. In normal vertebrate muscle, this would result in a drastic reduction of force output (dropping rapidly to zero force) (Gordon et al., 1966), but the perforations in the Z-disks in the chameleon tongue retractor muscle allow the thick filaments to slide through, and cross-bridge cycling can continue (Hoyle et al., 1965; Osborne, 1967). The changes in the spatial organisation of the filaments probably cause the decrease in force production observed at distances of less than a body length (see Fig. 3, Fig. 4) (Huxley, 1965; Osborne, 1967). The ultrastructural modifications of the tongue retractor muscles in chameleons thus enable them to capture and successfully retract prey into the mouth over a variety of distances at high performance levels. This is made possible by the arrangement of the sarcomeres at maximal extension (showing large overlap), which is determined by passive mechanisms (connective tissue) and by the perforations in the Z-disks that allow continued cross-bridge cycling over large length changes. At shorter muscle lengths, hyoid retraction presumably plays a major role in retracting the tongue with adhering prey.

The chameleon m. hyoglossus is extremely long, so it has to be folded in a complex way upon tongue retraction to fit within the space available in the oral cavity (see Fig. 5). This is potentially problematic as any erroneous folding might impede subsequent tongue projection and could potentially damage the hypoglossal nerve running alongside it. The multiple elastin connections between the internal connective tissue sheet and the tongue retractor muscle (Fig. 5) (Herrel et al., 2001) presumably ensure correct folding of the muscle by exerting small guiding forces. As muscles are thought to function as muscular hydrostats (constant-volume cylinders), the intramuscular pressures are probably large upon maximal contraction (during which the whole-muscle length is actually shorter than the resting length). Without external forces, any disturbance of the maximally retracted tongue system could result in random folding. Upon relaxation of muscular tension, the internal pressure will cause the tongue retractor to expand until forces are in equilibrium. Here, the elastin connections can provide the required external guiding forces to ensure correct folding of the system. Similar functions of elastin sheets associated with long complexly folded structures (such as ligaments or muscles) have been proposed previously (e.g. the anterior maxillo-mandibular ligament in the rainbow trout) (Aerts and Verraes, 1987). The elastin sheet surrounding the hypohyal-hypobranchial 1 ligaments in cichlid fish (Anker, 1989) is probably one of the best-documented examples.

Chameleons are known to eat large prey including other vertebrates (Broadley, 1973; Schleich et al., 1996) (M. Cuadrado personal communication), so the ability to retract the tongue forcefully over a wide range of distances is likely to be very important. The unique structural properties of the tongue retractor, as well as the prehensile tongue (Herrel et al., 2000), might thus have evolved to accommodate the capture of large prey. Quantitative data on diet in general and more specifically on the size of prey are scarce, but qualitative reports on diet in several species indicate the presence of numerous large prey [*C. chameleon*, lizards, wasps, mantids (Schleich et al., 1996); *C. namaquensis*, lizards, snakes, beetles (Branch, 1998); *C. melleri*, birds (Broadley, 1973)]. However, this aspect of the chameleon diet needs to be investigated quantitatively to test the above hypotheses.

Supercontractile properties have not been reported for other vertebrates that use a ballistic prey-capture mechanism with extreme tongue elongation (and thus need to contract their tongues by more than 50 %). Instead, some frogs and plethodontid salamanders fold their extremely long tongue retractor muscle upon retraction in the same way as chameleons do (Nishikawa et al., 1999; Deban et al., 1997). However, as these animals typically eat small prey, the constraint on constant high force production is likely to be reduced. In contrast to a previously proposed hypothesis emphasising length changes (Rice, 1973), we hypothesise that the supercontracting properties of the chameleon tongue retractors evolved to enable continuously high force to be produced over large length changes. Supercontraction might therefore be present in other systems in which the production of a constant near-maximal force is required over large length changes, as suggested by Osborne (Osborne, 1967).

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