

CONTRACTILE PROPERTIES OF MUSCLES USED IN SOUND PRODUCTION AND LOCOMOTION IN TWO SPECIES OF GRAY TREE FROG

RICHARD L. MARSH*

Department of Biology, 414 Mugar, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA

*e-mail: rmarsh@lynx.dac.neu.edu

Accepted 25 August; published on WWW 28 October 1999

Summary

The sound-producing muscles of frogs and toads are interesting because they have been selected to produce high-power outputs at high frequencies. The two North American species of gray tree frog, *Hyla chrysoscelis* and *Hyla versicolor*, are a diploid–tetraploid species pair. They are morphologically identical, but differ in the structure of their advertisement calls. *H. chrysoscelis* produces very loud pulsed calls by contracting its calling muscles at approximately 40 Hz at 20 °C, whereas, *H. versicolor* operates the homologous muscles at approximately 20 Hz at this temperature. This study examined the matching of the intrinsic contractile properties of the calling muscles to their frequency of use. I measured the isotonic and isometric contractile properties of two calling muscles, the laryngeal dilator, which presumably has a role in modulating call structure, and the external oblique, which is one of the muscles that provides the mechanical power for calling. I also examined the properties of the sartorius as a representative locomotor muscle. The calling muscles differ greatly in twitch kinetics between the two species. The calling muscles of *H. chrysoscelis* reach peak tension

in a twitch after approximately 15 ms, compared with 25 ms for the same muscles in *H. versicolor*. The muscles also differ significantly in isotonic properties in the direction predicted from their calling frequencies. However, the maximum shortening velocities of the calling muscles of *H. versicolor* are only slightly lower than those of the comparable muscles of *H. chrysoscelis*. The calling muscles have similar maximum shortening velocities to the sartorius, but have much flatter force–velocity curves, which may be an adaptation to their role in cyclical power output. I conclude that twitch properties have been modified more by selection than have intrinsic shortening velocities. This difference corresponds to the differing roles of shortening velocity and twitch kinetics in determining power output at differing frequencies.

Key words: muscle mechanics, skeletal muscle, force–velocity curve, twitch kinetics, sound production, external oblique muscle, laryngeal dilator muscle, sartorius muscle, tree frog, *Hyla versicolor*, *Hyla chrysoscelis*.

Introduction

Natural selection has matched the properties of skeletal muscles to their *in vivo* functions. The extent of this matching and its limits has been the subject of considerable research effort (Josephson, 1975; Goldspink, 1977; Bennett, 1984; Johnston, 1991; Rome and Lindstedt, 1997). Further improvements in our understanding of this matching are in part dependent on improving knowledge of *in vivo* muscle function (Marsh et al., 1992; Girgenrath and Marsh, 1997). Particularly important are investigations of homologous muscles that have evolved significant differences in function (Josephson and Young, 1987). Sound-producing muscles have provided convenient models of muscles that must function at high frequency (Josephson and Young, 1985; Josephson, 1985; Conley and Lindstedt, 1996; Rome et al., 1996). In addition to operating at high frequencies, these muscles must produce a high power output to provide the energy for transduction to sound. The present study examines the intrinsic properties of the sound-producing muscles in two closely related species of

North American gray tree frog, *Hyla chrysoscelis* and *Hyla versicolor*.

H. versicolor is a tetraploid species that almost certainly evolved from the morphologically identical diploid *H. chrysoscelis*, although perhaps in more than one tetraploidy event (Ralin, 1977; Ralin et al., 1983; Ptacek et al., 1994). These frogs are sympatric over a substantial portion of their ranges (Ptacek et al., 1994). The two species differ in the structure of their calls (Ralin, 1977; Gerhardt, 1978; Girgenrath and Marsh, 1997), and it is a reasonable hypothesis that the calls diverged as a result of intense selection against triploid hybrids that would result from interbreeding. The calls of both species are trills consisting of a series of similar pulses. The calls differ in their pulse repetition frequency, which is approximately 40 Hz at 20 °C in *H. chrysoscelis* and 20 Hz in *H. versicolor* at the same temperature. The much lower pulse frequency in *H. versicolor* is in line with the hypothesis that the initial tetraploidy event decreased pulse frequency in *H.*

versicolor and further differentiation occurred by natural selection (Gerhardt, 1994). Mate-choice experiments in the laboratory and in the field have shown that females prefer the pulse frequency and pulse shape of conspecific males and also prefer loud calls.

The energy for the sound production in these animals comes largely from the trunk muscles, the external and internal obliques. Simultaneous electromyography and high-speed video recording has shown that these muscles contract at a frequency that matches the sound pulse frequency (Girgenrath and Marsh, 1997). Sound is produced in each pulse as air is forced from the lungs to the vocal sac. As the trunk muscles relax, air is returned to the lungs by elastic recoil of the stretched vocal sac. Thus, virtually all the work needed to produce sound comes from the trunk muscles. During shortening, some of this work goes directly to produce sound and some is stored as elastic strain energy in the vocal sac. This stored energy is used to move the air back into the lungs, re-expanding the trunk. In addition, limited evidence from electromyography suggests that the laryngeal muscles also contract and relax with each pulse (Schmidt, 1965). This suggestion is consistent with the proposed role of these muscles in actively modulating the pulse structure of the call (Martin, 1972; Martin and Gans, 1972). Unlike the trunk muscles, we do not know the strain cycle of the laryngeal muscles during calling.

Given the difference in pulse frequency between these species, one would predict *a priori* that the sound-producing muscles in *H. chrysoscelis* would have faster twitch kinetics (Josephson, 1981; Josephson and Young, 1987; Marsh, 1990; Rome et al., 1996). These twitch kinetics are a measure of the faster deactivation required by the shorter time per pulse. In addition, one would predict that the sound-producing muscles should have a high intrinsic shortening velocity to allow for power production at high frequencies (Josephson, 1993) and that the intrinsic shortening velocity of the muscles in *H. chrysoscelis* should perhaps be higher than that for the muscles of *H. versicolor*. This latter prediction is not entirely straightforward because, during sound production, the muscles of *H. chrysoscelis* also shorten less than do those of *H. versicolor* (Girgenrath and Marsh, 1997).

McLister et al. (1995) examined the contractile properties of one of the laryngeal muscles (tensor chordarum=posterior constrictor) in these two species and compared these data with those obtained for the sartorius as a representative limb muscle. Their twitch data agree with the predictions above in that the laryngeal muscle in *H. chrysoscelis* was found to have considerably faster twitch kinetics than the homologous muscle in *H. versicolor*. However, quantitatively, the difference was much less than the difference in contractile frequencies during calling. Surprisingly, however, their data on the laryngeal muscle appear to indicate that this muscle has a lower intrinsic shortening velocity than the sartorius muscle in both species. Also, they found that both the sartorius and the laryngeal muscles in *H. chrysoscelis* actually had a lower maximal shortening velocity (V_{\max}) than did the homologous muscles in *H. versicolor*.

The present study re-examines the intrinsic properties of the calling muscles in the two species of gray tree frog and provides the necessary background for a more complex analysis of the contractile performance of these muscles (Girgenrath and Marsh, 1999). I provide data on both a laryngeal muscle and the external oblique, one of the trunk muscles that powers sound production. Examining the external oblique is important because it is possible that the laryngeal muscles function with very small strains and thus do not need to shorten as fast as the major power-producing muscles. I also provide data on the sartorius muscle of both species for comparison.

Materials and methods

Animals

Hyla versicolor LeConte were collected in the vicinity of Boston, MA, USA, and *Hyla chrysoscelis* Cope were obtained from a commercial supplier (Charles Sullivan) who collected them in Wilson County, TN, USA. The animals overlapped in body mass, although the *H. chrysoscelis* were smaller on average. The mean (\pm S.E.M.) body mass and snout-vent length for *H. chrysoscelis* were 5.24 ± 0.35 g and 39.0 ± 1.06 mm ($N=8$), respectively. Corresponding values for *H. versicolor* were 7.49 ± 0.48 g and 45.2 ± 1.45 mm ($N=12$). The animals were maintained at a temperature of approximately 25 °C in moist aquaria and fed a diet of crickets supplemented with vitamins and calcium carbonate. Animals were studied within 10 days of collection. Except where indicated, the data reported were obtained during the time of the year at which the animals were calling in their natural populations (between late May and early July).

Muscle preparations

The sartorius muscle was dissected whole with the proximal end still attached to the pelvis. The distal end was tied just beyond the end of the fibers to a lightweight silver chain that formed the connection to the ergometer lever. The pelvis was clamped to the bottom of the muscle chamber. The sartorius preparations from *H. chrysoscelis* and *H. versicolor* had mean (\pm S.E.M.) masses of 7.58 ± 0.86 mg and 13.21 ± 1.24 mg ($N=8$) and mean lengths of 15.46 ± 0.42 mm ($N=9$) and 18.46 ± 0.59 mm, respectively.

The external oblique is a sheet-like muscle extending obliquely in a ventral and posterior direction from the vertebral spines to near the midline on the ventral surface. This muscle is closely underlain by the internal oblique, which runs ventrally and anteriorly. The external oblique preparation consisted of a strip approximately 3 mm wide of muscle cut parallel to the fibers from origin to insertion. The cuts extended through the internal oblique. The resulting preparation had short non-contractile fragments of fibers from the internal oblique adhering to the inner surface of the muscle. Preliminary experiments indicated that trying to remove these fragments before the contractile measurements often resulted in damage to the external oblique. Therefore, I waited until

after the measurements and then removed these fragments before weighing the strip of external oblique. The muscle strip was placed in the chamber by tying the dorsal end to a post on the bottom of the chamber and the ventral end to a lightweight silver chain connected to the ergometer lever. The mean masses of the external oblique in these preparations were 37.44 ± 6.75 mg ($N=7$) and 39.41 ± 3.55 mg ($N=8$) and the mean lengths were 19.0 ± 1.0 mm and 24.6 ± 0.7 mm, in *H. chrysoscelis* and *H. versicolor*, respectively. These masses represent only a small fraction (approximately 5%) of the total masses of these muscles in the animals (Marsh and Taigen, 1987).

Three sets of muscles are arranged around the larynx of tree frogs (Duellman and Trueb, 1986). The laryngeal muscles offer several challenges in measuring contractile properties. First, they are small. Second, in common with the external and internal obliques, they are quite delicate because they apparently lack significant amounts of connective tissue. Third, they originate and attach to cartilaginous elements that are difficult to attach rigidly to the contractile apparatus. For several reasons, I chose to work on the laryngeal dilator. First, it is relatively superficial, and thus requires little dissection to isolate it from its origin (on the hyoid cartilage) to its insertion (on the arytenoid cartilage). Second, it is parallel-fibered, which makes analysis of its force–velocity properties in terms of intrinsic properties straightforward. In contrast, the muscle studied by McLister et al. (1995), the tensor chordarum (also known as the posterior sphincter), has many fibers that are shorter than the muscle length (R. L. Marsh, personal observation). Third, previous data on the isometric properties of the laryngeal dilator in other frogs indicate that its twitch speed varies with the pulse frequency of the calls produced (Manz, 1975).

The laryngeal dilator muscle was dissected free along with the arytenoid cartilage and part of the hyoid cartilage. This preparation was mounted in the chamber by clamping the arytenoid cartilage to the bottom of the chamber. The cartilage was clamped as close as possible to the attachment of the muscle fibers and as firmly as the strength of the cartilage would allow. The portion of the hyoid cartilage from which the muscle originated was stiffened by passing a size 00 insect pin longitudinally through it. A yoke was passed around this pin, and a lightweight silver chain was connected between this yoke and the lever arm of the ergometer. The laryngeal dilator was not significantly different in size between the two species, having a mean mass and length of 7.33 ± 0.86 mg and 4.5 ± 0.2 mm ($N=6$) and 9.37 ± 1.33 mg and 4.7 ± 0.17 mm ($N=6$) in *H. chrysoscelis* and *H. versicolor*, respectively.

Contractile measurements

During the measurements, the muscles were suspended vertically in a chamber and bathed with recirculating Ringer's solution (115 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.0 mmol l^{-1} MgSO₄, 20 mmol l^{-1} imidazole, 1.8 mmol l^{-1} CaCl₂, 11 mmol l^{-1} glucose, pH 7.9) maintained at 20 °C. Field stimulation was provided by platinum plate electrodes driven

by a power amplifier slaved to a Grass S44 square-wave stimulator. Supramaximal square-wave stimuli of 0.5 ms duration were used. The muscles were attached to the arm of a Cambridge Technology (model 300) ergometer. This instrument had a step response time (0–100%) of 1.8 ms, a force resolution of 50 mg and a length resolution of 3 μm. The maximum compliance under the conditions of this study was 7 μm. Force and position signals were preamplified by a custom-made direct current amplifier and were recorded by either a 14-bit A/D converter running in an Apple IIe computer or a 12-bit A/D converter running in a Macintosh computer. Sampling frequency was 2000 Hz for all measurements.

Immediately after being placed in the chamber, the muscle was adjusted to approximately optimal length using twitches. The muscle was then allowed to recover for 45–60 min. After the recovery period, length was adjusted to the length (L_0) that yielded maximal force (P_0) in tetanic contractions. At this length, the kinetics of post-tetanic twitches were recorded. Subsequently, shortening was recorded in 10–12 isotonic contractions with forces ranging from approximately $0.95P_0$ to $0.02P_0$. Maximum isometric force was measured at the beginning, middle and end of this series, and P_0 at the time of the isotonic contractions was estimated by linear extrapolation. The decline in force was typically less than 15% of the starting value.

After the measurements, the muscle was removed from the chamber, and non-contractile fiber fragments, bone and cartilage were dissected away before determining the muscle mass. Cross-sectional area was calculated by dividing muscle volume (assuming a density of 1 g cm^{-3}) by length.

Statistical analyses

Force–velocity data were fitted with the exponential–linear equation of Marsh and Bennett (1986):

$$V = \frac{B(1 - P/P_0)}{(A + P/P_0)} + C(1 - P/P_0),$$

where V is shortening velocity in $L_0 \text{ s}^{-1}$, P is force in N cm^{-2} , B and C are constants with dimensions of $L_0 \text{ s}^{-1}$ and A is a dimensionless constant. The non-linear curve-fitting procedures in the MacOS application Igor from WaveMetrics were used to fit the curves. These data were handled in two ways. First, a curve was fitted to the composite data from all preparations of a given muscle. This curve provides a good descriptor of the average properties of the muscle. The constants of this equation are provided by the application along with the estimates of the standard errors of each parameter. The problem with these curves, however, is the uncertain nature of the statistics associated with non-linear curve-fitting (Draper and Smith, 1981). Also, no equivalent of analysis of covariance exists for comparing the curves. Therefore, to compare the force–velocity data of different muscles, the data from each preparation were fitted individually to generate individual estimates of the maximum velocity of shortening at zero force (V_{max}), the velocity of shortening at $0.4P_0$ ($V_{0.4P_0}$) and the power ratio (R_P).

The R_P is a measure of the curvature of the force–velocity relationship and is equal to the maximum isotonic power divided by the product of V_{\max} and P_0 after conversion to appropriate units (Marsh and Bennett, 1986). The velocity of shortening at $0.4P_0$ was chosen for comparison in addition to V_{\max} because it is close to the point on the curve where peak isotonic power is generated, and this point can be estimated with less error than V_{\max} . Because V_{\max} is estimated by extrapolation of the steepest part of the curve, it is subject to more variation than predicted values within the range of data collected.

Values generated from the force–velocity analysis and the isometric measurements were compared using two-factor analysis of variance (ANOVA), with muscle and species as the factors. Pair-wise comparisons were made using the Bonferroni–Dunn *post-hoc* procedure. This test allows for multiple comparisons by increasing the critical difference between means that is required to demonstrate a significant difference. Differences were accepted as significant when $P < 0.05$.

Values are presented as means \pm S.E.M.

Results

Isometric properties

The kinetics of post-tetanic twitches (Fig. 1B,C) showed significant variation between species and among the different muscles studied (ANOVA, $P < 0.0001$). Overall, the twitch was faster in the calling muscles of *H. chrysocelis* than in those of *H. versicolor*, whereas the sartorius muscles of the two species were similar in their isometric kinetics. The time to peak force in the twitch ($t_{P,tw}$) and the time to 50% relaxation ($t_{50\%R}$) were both significantly shorter in the external oblique and laryngeal dilator muscles of *Hyla chrysocelis* than in the same muscles in *H. versicolor* ($P < 0.0001$, Bonferroni–Dunn procedure) and in the sartorius of *H. chrysocelis* ($P < 0.01$). Overall, the external oblique and laryngeal dilator muscles of *H. versicolor* take 1.81 and 1.70 times as long, respectively, to reach peak force and to relax to 50% of this value as do the same muscles in *H. chrysocelis*. The sartorius muscles of the two species were not significantly different ($P > 0.9$) in twitch kinetics.

As indicated in Fig. 1A, tetanic force (P_0) varied significantly among the muscles studied (two-factor ANOVA, $P < 0.0001$), but not between the species when homologous muscles were compared ($P = 0.78$). The mean P_0 of the external oblique of both species taken together was $8.51 \pm 0.45 \text{ N cm}^{-2}$, which was only 37% of the mean P_0 for the sartorius of $23.1 \pm 0.907 \text{ N cm}^{-2}$. The P_0 of the laryngeal dilators was also relatively low, averaging $6.51 \pm 0.54 \text{ N cm}^{-2}$ or 28% of the value for the sartorius muscles. The difference between the external obliques and the laryngeal dilators was not statistically significant.

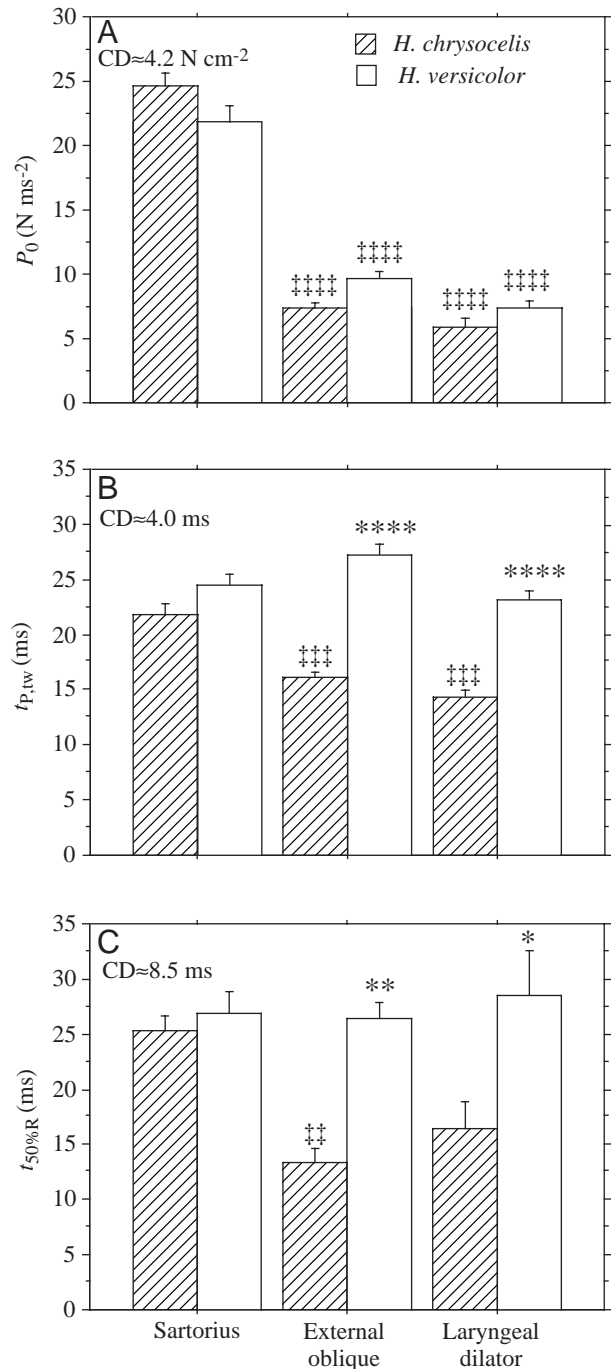
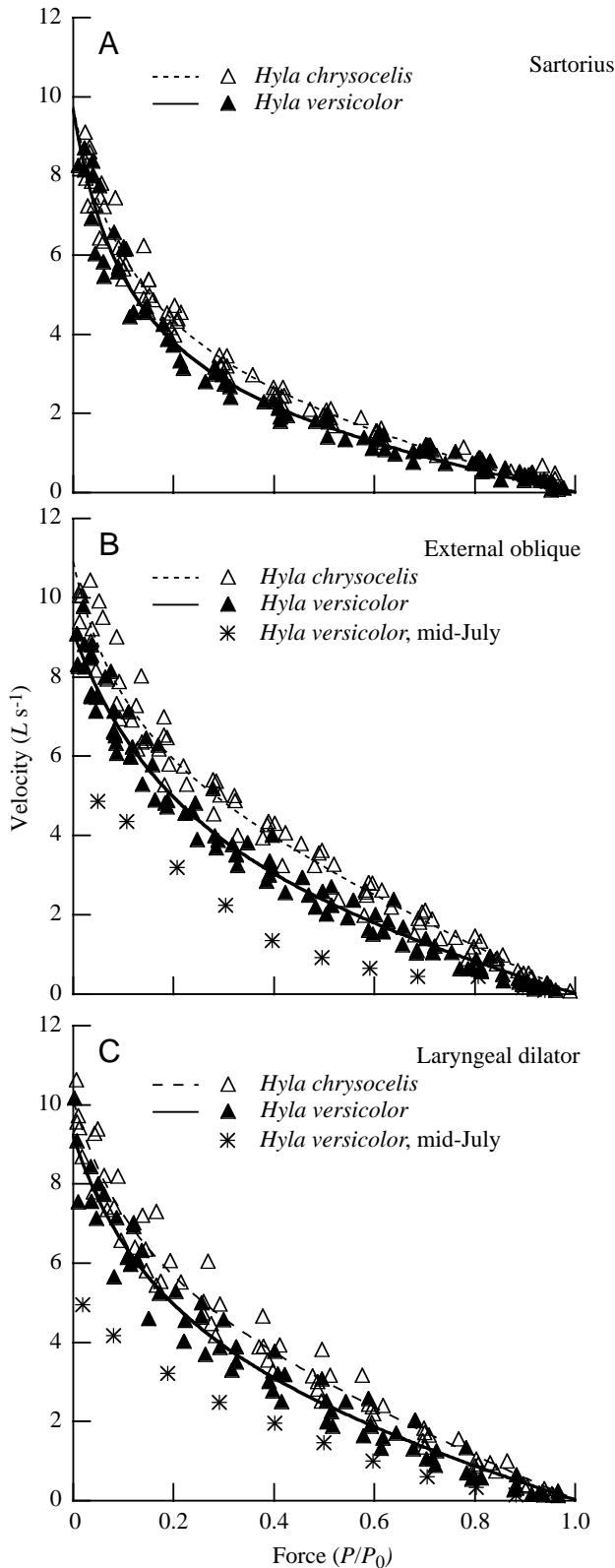


Fig. 1. Comparison of isometric properties among several muscles in *Hyla versicolor* and *H. chrysocelis*. (A) Maximum isometric force (P_0). (B) Time to peak force in a twitch ($t_{P,tw}$). (C) Time from peak twitch force to 50% relaxation ($t_{50\%R}$). Twitch values are for post-tetanic twitches. Mean values for *H. versicolor* are shown in open columns and those for *H. chrysocelis* are shown in hatched columns. Sample sizes for the various muscles are given in Table 1. Error bars indicate the S.E.M. The critical difference (CD) given in each panel indicates the minimum difference required to obtain a significant ($P < 0.05$) difference using the Bonferroni–Dunn procedure (see Materials and methods). Double daggers (‡) indicate statistically significant differences between the calling muscles and the sartorius muscle of the same species; †† $P < 0.01$, ††† $P < 0.001$, †††† $P < 0.0001$. Asterisks (*) indicate statistically significant differences between the two species for an individual muscle; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

Isotonic properties

All muscles, with the exception of one preparation, were found to have maximum shortening velocities that were close to $10 L_0 s^{-1}$ at $20^\circ C$. The single animal whose external oblique and laryngeal dilator had dramatically lower V_{max} values was



collected in the first week of July and studied on 16 July (Fig. 2B,C). This date is approximately 2 weeks after calling ended in the population from which the frog was collected. The data from this single animal raise the intriguing possibility that the properties of the calling muscles change with season. In the present study, the data from the muscles of this animal were excluded from further analysis. The composite data (Fig. 2) indicate that the calling muscles of *H. chrysocelis* have slightly greater V_{max} values than those of *H. versicolor*. Using data derived from curves fitted to individual preparations (Table 1; Fig. 3), two-way ANOVA indicated a significant effect of species ($P < 0.0047$) but not of muscle ($P = 0.40$) on V_{max} . Using the Bonferroni–Dunn procedure, only the difference in the external obliques was found to be statistically significant between the two species ($P < 0.05$).

The values of R_p , which is a measure of the degree of curvature, were found by two-way ANOVA to be significantly different among muscles ($P < 0.0001$) and between species ($P = 0.0003$). The curves of the calling muscles are considerably flatter than those of the sartorius muscles (Figs 2, 3C). Pairwise comparisons indicated that the external oblique and laryngeal dilator of both species had significantly higher R_p values (flatter curves) than the sartorius muscles of the same species ($P < 0.003$). No pairwise comparisons across species were found to be significant.

The values of $V_{0.4P_0}$, which reflect the combined effects of differences in V_{max} and R_p , were higher in the muscles of *H. chrysocelis* than in those of *H. versicolor*, and this species difference was significant as judged by two-way ANOVA ($P < 0.0001$). In pairwise comparisons, statistically significant differences were found between the external oblique ($P < 0.01$) and laryngeal dilator ($P < 0.05$) muscles of the two species (Fig. 3B). In the sartorius muscles, the difference was not statistically significant.

Discussion

The calling muscles of the two tree frog species examined here are specialized in a number of ways to suit their function in powering sound production. Previous work has documented the extraordinary aerobic capacities of these muscles that allow them to function in sound production for several hours at a time (Taigen and Wells, 1985; Taigen et al., 1985; Marsh and Taigen, 1987; Ressel, 1996). The high oxygen demand of these muscles during calling leads to whole-animal metabolic rates that exceed those found during forced exercise. In addition to these unusual metabolic properties, the calling muscles must have contractile properties matching the mechanical power

Fig. 2. Composite force–velocity data for tree frog muscles; sartorius (A), external oblique (B) and laryngeal dilator (C). Data for *Hyla versicolor* are shown as filled symbols and solid lines and those for *H. chrysocelis* as open symbols and dashed lines. Curves were fitted to a modified hyperbola using non-linear curve-fitting techniques (see Materials and methods). Parameters for the curves are given in Table 1. Force P is expressed as a fraction of maximal isometric force P_0 .

Table 1. Isotonic data at 20 °C for muscles of gray tree frogs

Species	Muscle	<i>N</i>	V_{\max} ($L_0 s^{-1}$)	R_P	<i>A</i>	<i>B</i> ($L_0 s^{-1}$)	<i>C</i> ($L_0 s^{-1}$)
<i>Hyla chrysoscelis</i>	Sartorius	8	9.74	0.108	0.128±0.002	0.908±0.017	2.664±0.031
	External oblique	7	10.54	0.153	0.140±0.005	0.740±0.028	5.252±0.050
	Laryngeal dilator	6	10.12	0.150	0.157±0.006	0.847±0.040	4.718±0.066
<i>Hyla versicolor</i>	Sartorius	9	9.67	0.090	0.114±0.003	0.888±0.020	1.907±0.039
	External oblique	8	9.13	0.133	0.257±0.006	1.724±0.051	2.426±0.059
	Laryngeal dilator	6	9.19	0.135	0.212±0.008	1.312±0.063	3.007±0.084

Summary statistics were generated by fitting the hyperbolic-linear equation (see text) to composite data for each muscle.

Fitting a single curve through the composite data provides good overall description of the data for each muscle, but cannot be used to compare the curves statistically (see Materials and methods). Comparisons of various predicted values were made after fitting curves to the data from individual muscles (see Fig. 3).

V_{\max} indicates the maximum velocity of shortening at zero force predicted from the fitted curve.

R_P indicates the power ratio, calculated by dividing the peak power predicted from the fitted curve by the product of V_{\max} and maximum isometric force P_0 . This ratio is a measure of the flatness of the curve; a straight line from V_{\max} to P_0 would have an R_P of 0.25.

A, *B* and *C* are the constants from the hyperbolic-linear equation (see Materials and methods).

Predicted constants are given ± S.E.M.

output required for species-specific vocalizations. As with many sexually selected characters, the initial selective pressures that led to the evolution of specific call structures in different anurans are not entirely clear (Martin, 1972; Gerhardt, 1994). However, female mate choice currently provides a strong selective force that maintains important characteristics of the call (Gerhardt, 1994). Specifically, for the two species examined here, call structure provides the cues that help prevent interbreeding, which would lead to sterile triploid hybrids.

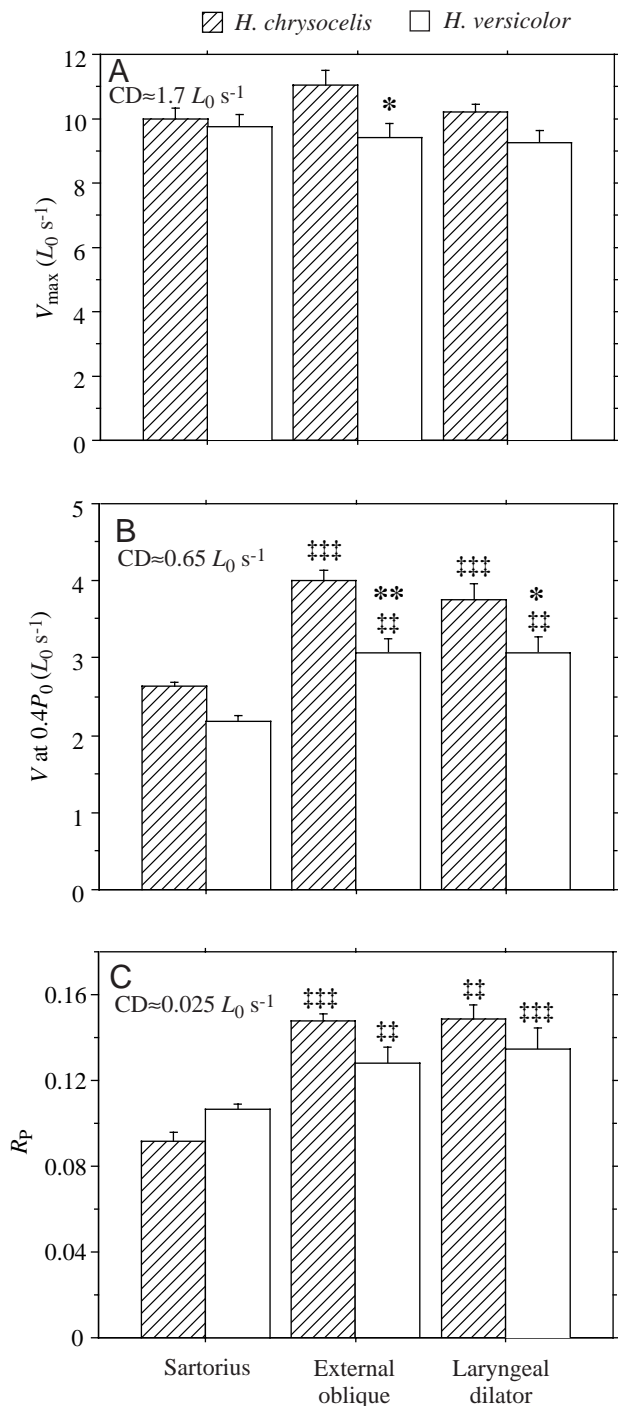
In the case of a number of species of hylid frogs, as well as other anuran species, the pulse rate within a trilled call is one of the characteristics used by females in mate choice (Gerhardt, 1994). The gray tree frogs examined in the present study produce pulses by active contraction of the trunk muscles (Girgenrath and Marsh, 1997), and it is important that the calling muscles be able to produce power effectively at these frequencies (Girgenrath and Marsh, 1999). It seems likely that selection should operate to minimize the volume of muscle recruited to produce a given volume of sound, i.e. to maximize power output at the appropriate contractile frequency. Even if the energy consumption is not large on an organismal scale, maintaining energy balance within individual muscle fibers will be favored if efficiency is maximized. In the case of tree frogs, the energetic cost of calling is in fact extremely high at the organismal level. However, selection for operation at high contractile frequencies may very well lead to limits on power output and efficiency due to functional trade-offs (Josephson, 1985; Josephson and Stevenson, 1991; Conley and Lindstedt, 1996; Rome and Lindstedt, 1998). Such limits could arise because of limits in selecting for muscles with faster shortening velocities or result from selection for the shorter twitch times that are required for power output at high frequencies. A distinction needs to be drawn between the properties of muscles that are subject to selection and those that result from selection. The latter properties may reflect limits or trade-offs

in the selective process and not characteristics that have been selected for directly.

I have shown here that the two sibling species of North American gray tree frog, *H. versicolor* and *H. chrysoscelis*, have calling muscles (external oblique and laryngeal dilator) with contractile properties that differ across species as predicted *a priori* from differences in contractile frequency *in vivo*. The species differences in contractile properties are specific to the muscles used in calling. No such differences exist for the sartorius, a locomotor muscle. The calling muscles differ between species in both twitch properties and isotonic properties, but the differences in twitch properties are greater.

Muscles that are used for cyclical power production need to be activated and deactivated during approximately the time available for shortening *in vivo*. On the basis of the measured pulse frequencies, the total time available for one complete contractile cycle in *H. versicolor* is approximately twice that found in *H. chrysoscelis* (Ralin, 1977; Gerhardt, 1978; Girgenrath and Marsh, 1997). A more detailed examination of the *in vivo* use of the trunk muscles indicates that the time available for shortening in *H. versicolor* compared with *H. chrysoscelis* is even greater than would be predicted from the pulse frequencies (Girgenrath and Marsh, 1997). The trunk muscles in *H. versicolor* shorten for a longer portion of the cycle (75% versus 60% of the cycle time). Thus, the shortening time in *H. versicolor* is 2.5 times longer than that found in *H. chrysoscelis*.

The operating frequency of muscle that produces cyclical work cannot be predicted directly from isometric twitch times because the lengthening and shortening that occur during cyclical work influence the rates of activation and deactivation (Askew and Marsh, 1998). These influences make it possible for a muscle to cycle faster than would be predicted from isometric twitch times alone. However, one might still predict that the difference in twitch times would be proportional to the time available for shortening if the effects of the length cycle



are similar among the muscles compared. My measurements indicate that the twitch in *H. versicolor* takes approximately 1.75 times as long in *H. versicolor* as it does in *H. chrysocelis*. This ratio is larger than the value of 1.5 found by McLister et al. (1995) for the tensor chordarum of the same two species. Even so, the difference in twitch times between the species is not as great as the difference in time available for shortening. McLister et al. (1995) speculate that the lack of concordance between the time available for contraction and time taken for a twitch indicates that the muscles of *H. versicolor* are not as well adapted to the *in vivo* pulse frequency as they could be.

Fig. 3. Comparison of isotonic properties among several muscles in *Hyla versicolor* and *H. chrysocelis*. (A) Maximum velocity of shortening (V_{max}). (B) Velocity of shortening at $0.4P_0$, where P_0 is maximum isometric force. (C) Ratio of peak isotonic power divided by the product of P_0 times V_{max} (R_p). Mean values for *H. versicolor* are shown in open columns and those for *H. chrysocelis* are shown in hatched columns. Sample sizes for the various muscles are given in Table 1. Error bars indicate the S.E.M. The critical difference (CD) given in each panel indicates the minimum difference required to obtain a significant ($P < 0.05$) difference using the Bonferroni–Dunn procedure (see Materials and methods). Double daggers (†) indicate statistically significant differences between the calling muscles and the sartorius of the same species; †† $P < 0.01$, ††† $P < 0.001$. Asterisks (*) indicate statistically significant differences between the two species for an individual muscle; * $P < 0.05$, ** $P < 0.01$.

However, it is difficult to sustain this conclusion because the evolution of twitch time is probably driven by a series of functional trade-offs. Longer twitches allow for longer force production with presumably greater economy because of reduced rates of Ca^{2+} pumping. However, depending on the mechanics of the system, rapid relaxation at the end of a sustained contraction may also be required. The muscles of *H. versicolor* can produce force effectively during the period of shortening, despite a twitch that seems too fast compared with that in *H. chrysocelis*, because they are activated by two stimuli per cycle compared with one stimulus in *H. chrysocelis* (Girgenrath and Marsh, 1997, 1999). Is it more advantageous for power output and/or efficiency to activate these muscles with two stimuli and have a shorter twitch or to have a single stimulus and a longer twitch? This question is not readily answerable without the ability to manipulate the properties of the muscles experimentally. Also, selection may favor particular twitch characteristics if they contribute to the shape of the sound pulse produced, because a species-specific pulse shape is preferred by female tree frogs (Gerhardt and Doherty, 1988).

The muscles used in calling also have force–velocity curves that appear well suited to the production of mechanical power. As expected from the pulse frequency of their calls, the calling muscles of *H. chrysocelis* are somewhat faster than the homologous muscles in *H. versicolor*. The V_{max} values of the laryngeal dilator and external oblique are faster in *H. chrysocelis* than in the calling muscles of *H. versicolor* (Figs 2, 3), but the differences are quite small. However, the force–velocity curves were also somewhat flatter in *H. chrysocelis*, which leads to a larger difference in velocity over the middle of the force–velocity curve. This difference is potentially biologically significant because power output is highest over this range of velocities.

The force–velocity curves of the calling muscles in both species are significantly different from the curves for the corresponding sartorius muscles. The calling and locomotor muscles have similar V_{max} values, but the curves of the calling muscles are significantly flatter. The sartorius muscles have R_p values of approximately 0.1, a value typical of other locomotor muscles in small anurans (Marsh, 1994). The calling muscles,

which have R_P values of 0.13–0.15, are similar in curve shape to certain insect muscles that also function in a similar manner (Josephson, 1984). The flatter force–velocity curves of the calling muscles result in considerably higher velocities at intermediate forces and allow high power outputs from these muscles during cyclical contractions despite rather low force production (Girgenrath and Marsh, 1999).

Despite the basic similarity between the twitch kinetics reported here for the laryngeal dilator and sartorius and the data of McLister et al. (1995) for the tensor chordarum and the sartorius of the same species, the force–velocity data from these two studies are quite different. The data of McLister et al. (1995) for the tensor chordarum, a laryngeal muscle, indicate that this muscle is significantly slower than the sartorius. This result seems at odds with the proposed high-frequency operation of the larynx during calling. I found V_{\max} values at 20 °C for the calling muscles that were nearly twice those found at 25 °C by McLister et al. (1995). My data indicate that these muscles have V_{\max} values similar to those of the sartorius muscle, for which I also report considerably higher V_{\max} values than those measured by McLister et al. (1995). Although the possibility exists that the different laryngeal muscles studied have substantially different force–velocity curves, this possibility seems less likely given the similarity in twitch kinetics. The lower V_{\max} values reported by McLister et al. (1995) for the tensor chordarum could, in part, be accounted for by significant architectural differences in the muscles (see Materials and methods). In addition, McLister et al. (1995) kept their animals in captivity for up to a year, and the isotonic properties of the calling muscles may change on a seasonal basis (Fig. 2; M. Girgenrath and R. L. Marsh, unpublished observations). However, these effects cannot readily explain the differences in the isotonic curves for the sartorius muscles between the two studies. The values reported here for the V_{\max} of the sartorius of approximately $10 L_0 s^{-1}$ at 20 °C accord well with data for this muscle in other small frogs (Marsh, 1994).

In conclusion, pulse frequencies in the calls of the North American gray tree frogs *Hyla chrysoscelis* and *Hyla versicolor* have apparently diverged since the origin(s) of *H. versicolor* as a tetraploid of *H. chrysoscelis*, and this difference in frequency has probably been maintained by female mate selection (Gerhardt, 1994). The present study examined differences in intrinsic properties of the muscles used in calling between these two species. The isometric twitch in *H. versicolor* takes 1.75 times longer than the twitch in *H. chrysoscelis*, indicating a major difference in the kinetics of deactivation between the two species. For the trunk muscles, the difference in twitch times is smaller than the difference in the time available for shortening *in vivo*, which is approximately 2.5 times longer in *H. versicolor* than in *H. chrysoscelis*. *In vivo*, *H. versicolor* prolongs activation and matches force production to the time available for shortening by having two stimuli per cycle (Girgenrath and Marsh, 1997, 1999). The differences in intrinsic shortening velocity between the species are smaller than the differences in twitch kinetics

and are thus much smaller than the difference in pulse frequencies between the calls of the two species. Clearly, the performance during the call is determined mainly by changes in other variables, e.g. deactivation rates and alterations in the length trajectory of the muscles (Girgenrath and Marsh, 1997, 1999), rather than by changes in intrinsic velocity. Indeed, this system is an example of what is probably a general rule in muscle-driven systems. The frequency of the movement must of necessity be matched to a certain minimum deactivation rate (maximum twitch time) to allow lengthening to occur with minimal resistance at the end of the shortening phase (Marsh, 1990; Josephson, 1993). Conversely, having a deactivation rate that is too high wastes energy in Ca^{2+} pumping during the power stroke. However, frequency may vary considerably without equivalent changes in V_{\max} , depending on the detailed kinematics of the movement (Josephson, 1984; Girgenrath and Marsh, 1997, 1999). In the present case, changes in the length trajectory in *H. versicolor* compared with that in *H. chrysoscelis* serve to maintain a near-optimal shortening velocity despite only a slightly lower V_{\max} (Girgenrath and Marsh, 1997, 1999). Work on the scaling of intrinsic properties with body size also indicates that V_{\max} changes less than does the kinetics of deactivation or contractile frequency (Marsh, 1988, 1994; James et al., 1998). The reasons that a particular matching of frequency and V_{\max} has evolved are not entirely clear. Predicting this relationship is complicated by the fact that having a higher V_{\max} increases power output regardless of operating frequency (Weis-Fogh and Alexander, 1977; Askew and Marsh, 1997).

I would like to thank T. L. Taigen for stimulating my initial interest in the calling muscles of tree frogs and for providing some of the *H. versicolor* used in this study. This work was supported by grant AR39318 from the NIH.

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