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COMPARATIVE CONTRACTILE DYNAMICS OF CALLING AND LOCOMOTOR MUSCLES IN THREE HYLID FROGS

JAMES D. MCLISTER*, E. D. STEVENS AND JAMES P. BOGART
Department of Zoology, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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Summary

Isometric twitch and tetanus parameters, force–velocity curves, maximum shortening velocity (V_{max}) and percentage relaxation between stimuli (%*R*) across a range of stimulus frequencies were determined for a muscle used during call production (the tensor chordarum) and a locomotor muscle (the sartorius) for three species of hylid frogs, *Hyla chrysoscelis*, *H. versicolor* and *H. cinerea*. The call of *H. chrysoscelis* has a note repetition rate (NRR) approximately twice as fast as the call of *H. versicolor* (28.3, 42.5 and 56.8 notes s^{-1} for *H. chrysoscelis* and 14.8, 21.1 and 27.4 notes s^{-1} for *H. versicolor* at 15, 20 and 25 °C, respectively). *Hyla cinerea* calls at a very slow NRR (approximately 3 notes s^{-1} at 25 °C). *Hyla versicolor* evolved from *H. chrysoscelis via* autopolyploidy, so the mating call of *H. chrysoscelis* is presumably the ancestral mating call of *H. versicolor*.

For the tensor chordarum of *H. chrysoscelis*, *H. versicolor* and *H. cinerea* at 25 °C, mean twitch duration (19.2, 30.0 and 52.9 ms, respectively), maximum isometric tension (P_0; 55.0, 94.4 and 180.5 kN m^{-2}, respectively), tetanic half-relaxation time (17.2, 28.7 and 60.6 ms, respectively) and V_{max} (4.7, 5.2 and 2.1 lengths s^{-1}, respectively) differed significantly (P<0.05) among all three species. The average time of tetanic contraction to half-P_0 did not differ significantly between *H. chrysoscelis* (14.5 ms) and *H. versicolor* (15.8 ms) but was significantly longer for *H. cinerea* (52.6 ms).

At 25 °C, V_{max} differed significantly among the sartorius muscles of *H. chrysoscelis*, *H. versicolor* and *H. cinerea* (5.2, 7.0 and 9.8 lengths s^{-1}, respectively) but mean twitch duration (29.5, 32.2 and 38.7 ms, respectively), P_0 (252.2, 240.7 and 285.1 kN m^{-2}, respectively) and tetanic half-relaxation time (56.3, 59.5 and 60.7 ms, respectively) did not differ significantly. The average time of contraction to half-P_0 did not differ significantly between *H. chrysoscelis* (23.7 ms) and *H. versicolor* (22.9 ms) but was significantly shorter for *H. cinerea* (15.6 ms).

The only consistent contractile differences found in this study between the calling muscle and locomotor muscle of *H. chrysoscelis*, *H. versicolor* and *H. cinerea* were that the calling muscles generated less tension and their force–velocity relationship was much more linear. These differences may be attributable to ultrastructural differences between calling and locomotor muscles.

The relationship between %*R* and stimulus frequency clearly shows that the tensor chordarum of *H. chrysoscelis* is capable of functioning at higher contractile frequencies (i.e. NRRs) than the tensor chordarum of *H. versicolor* which, in turn, can function at higher contractile frequencies than the tensor chordarum of *H. cinerea*. However, the calling muscle of *H. versicolor* appears to be much faster than it needs to be. Possibly, neurological changes regulating NRR have evolved faster than physiological changes to the muscle.

The principal modification to the tensor chordarum of *H. versicolor* in response to the evolution of a slower NRR is a reduction in deactivation rate.

Key words: treefrog, *Hyla chrysoscelis*, *Hyla versicolor*, *Hyla cinerea*, muscle, tensor chordarum, sartorius, call mechanics, contractile dynamics, evolution, polyploidy.

Introduction

The muscles most important for call production in frogs are the trunk and laryngeal muscles (Schmidt, 1965, 1972; Martin and Gans, 1972; Marsh and Taigen, 1987). Although little is known about the contractile properties of calling muscles, it is known that they differ ultrastructurally and biochemically from other anuran skeletal muscles. During call production, oxygen consumption and power output are very high for an extended period (Pough and Gatten, 1984; Taigen and Wells, 1985; Taigen *et al.* 1985; Given and McKay, 1990). Not surprisingly, most of the observed differences between calling and non-

*Present address: Department of Ecology and Evolutionary Biology, University of California, Irvine, CA 92717, USA.

calling skeletal muscles are associated with the different energy requirements of these muscles. Anuran calling muscles consist entirely of fast oxidative glycolytic (FOG) fibres (Marsh and Taigen, 1987) and have much higher concentrations of the enzymes associated with oxidative metabolism (Given and McKay, 1990; Marsh and Taigen, 1987). Calling muscles also tend to contain more mitochondria, more extensive sarcoplasmic reticulum (SR), smaller myofibrillar cross-sectional areas, greater amounts of endogenous glycogen granules and more endogenous lipid droplets than locomotor muscles (Schneider, 1977; Marsh and Taigen, 1987; McLister, 1992). Given these general ultrastructural and biochemical differences, calling muscles may also differ from locomotor muscles in contractile dynamics. Differences in the amount of SR and the cross-sectional area of the myofibrils, for example, may be associated with differences in twitch dynamics (Reger, 1978; Akster *et al.* 1985; Josephson and Young, 1987). Also, the viscosity effects of large quantities of SR, mitochondria and lipid droplets may influence the force–velocity relationship of muscles (Josephson, 1984).

Of broader interest, the comparative contractile dynamics of calling muscles may provide insight into the evolution of muscle function. Anuran mating calls are species-specific and act as reproductive isolating mechanisms (Blair, 1958; Duellman and Trueb, 1986). In accordance with this function, call production is a very stereotyped, repetitive behaviour and muscles specialised for call production will have a very strict set of contractile demands placed upon them. The mating calls of related species, no matter how diverse, necessarily evolved and diverged from the mating call of a common ancestor, and homologous calling muscles, in their ancestral state, would have had identical contractile dynamics. Any present contractile differences, therefore, must have evolved in response to, or in conjunction with, call divergence. Because it is possible to quantify both the contractile differences among anuran calling muscles and the specific selective regimes under which those contractile differences evolved (i.e. the divergent contractile demands imposed upon calling muscles by divergent call parameters), anuran calling muscles are a suitable natural system for studying the evolution of contractile dynamics.

An ideal species pair for comparing the contractile dynamics of call production is *Hyla chrysoscelis* Cope and *H. versicolor* LeConte. These two species are morphologically and ecologically indistinguishable from one another but differ in chromosome number and mating call (Ralin, 1977). *Hyla chrysoscelis* is a diploid species with 24 chromosomes whereas *H. versicolor*, a tetraploid species, has 48 chromosomes (Bogart and Wasserman, 1972). The principal difference between the mating call of these two species is note repetition rate (NRR). The NRR of both species increases linearly as temperature increases but, when calling at the same temperature, the NRR of *H. chrysoscelis* is approximately twice as fast as the NRR of *H. versicolor* (Jaslow and Vogt, 1977; Ralin, 1977; Gerhardt, 1978; Bogart and Jaslow, 1979). The morphological and ecological similarity, a high degree of genetic similarity and the twofold difference in chromosome number strongly suggest that *H. versicolor* evolved from *H. chrysoscelis via* the process of autopolyploidy (Bogart and Wasserman, 1972; Ralin, 1977). Thus, for the calling muscles of *H. chrysoscelis* and *H. versicolor*, there is a plausible evolutionary relationship (the calling muscles of *H. chrysoscelis* represent the ancestral condition of the calling muscles of *H. versicolor*) and a well-defined divergence of muscle contractile requirements (the calling muscles of *H. versicolor* generate notes at half the rate of those of *H. chrysoscelis*).

This study compares the contractile properties of a laryngeal muscle and a leg muscle from *H. chrysoscelis*, *H. versicolor* and a more distantly related species, *H. cinerea* (Schneider), which has a much slower NRR than either *H. chrysoscelis* or *H. versicolor* (see Fig. 1C). The principal objectives were to determine whether any general contractile differences exist between calling and locomotor muscles, to determine how the contractile dynamics of calling muscles differ in relation to NRR, and to determine the nature and extent of the changes in contractile dynamics that have occurred in the calling muscle of *H. versicolor* in response the evolutionary reduction in NRR. For *H. chrysoscelis* and *H. versicolor*, any differences between the calling muscles could feasibly be attributable either to call or to ploidy differences. The effects of polyploidy on tissue development, physiology and enzyme regulation vary depending upon the enzymes (Becak and Pueyo, 1971; Sezaki *et al.* 1983; Warner and Edwards, 1989), tissues (Pollack and Koves, 1977; Sperry, 1988) or species being studied (Cassini and Caton, 1986; Werner and Edwards, 1993). Therefore, the possibility that polyploidy affects the contractile properties of anuran muscle could not be ruled out *a priori*, and comparisons between the non-calling locomotor muscles of *H. chrysoscelis* and *H. versicolor* were used to provide a control against this possibility. Because *H. chrysoscelis* and *H. versicolor* are so similar in morphology and ecology, there is no apparent adaptive reason to expect a contractile difference between homologous leg muscles.

Call mechanics and terminology

The term ‘note repetition rate’ is not typically used to describe the mating calls of *H. chrysoscelis*, *H. versicolor* or *H. cinerea* [the equivalent terms more frequently used for this call parameter are ‘pulse rate’ or ‘pulse repetition rate’ for *H. chrysoscelis* and *H. versicolor* (e.g. Gerhardt and Doherty, 1988) and ‘call rate’ for *H. cinerea* (Gerhardt, 1987)]. Some of the standard terms for the acoustic parameters of frog calls, however, become inappropriate when the mechanics of call production are taken into consideration; call parameters that are acoustically similar may be derived from different mechanical processes. For example, the smallest temporal unit of a frog’s call, typically referred to as a ‘pulse’ (Duellman and Trueb, 1986, page 89), consists of a single burst of sound, and the mating calls of *H. chrysoscelis*, *H. versicolor* and *H. cinerea* all consist of a series of pulses (the resolution of the

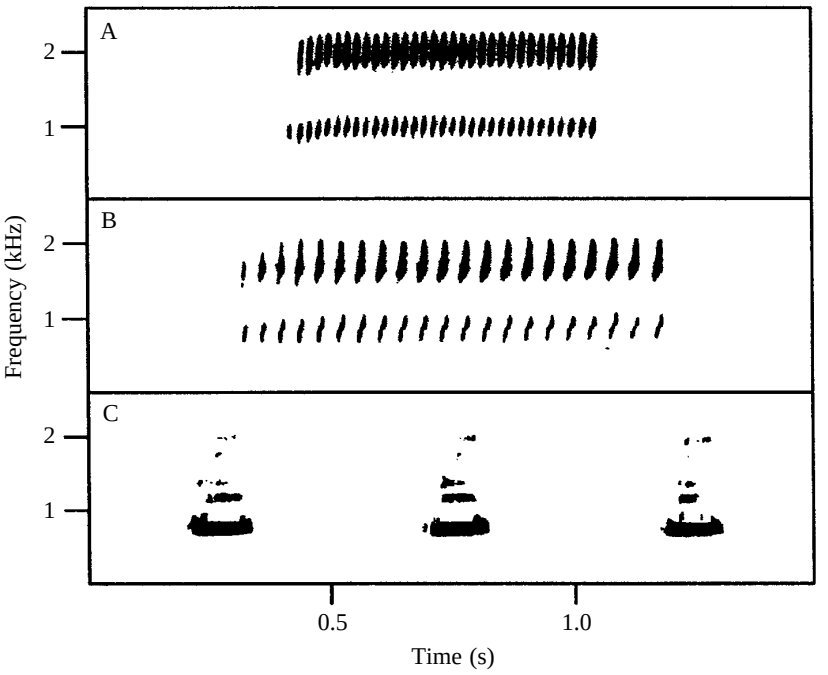

Fig. 1. Sonograms of the mating calls of (A) *Hyla chrysoscelis*, (B) *H. versicolor* and (C) *H. cinerea*. These sonograms show the different note repetition rates (NRRs) of each species. The calls of *H. chrysoscelis* and *H. versicolor* were recorded on 11 June 1992 from the same pond in Hubbard Co., Minnesota, USA (air temperature 18 °C; water temperature 23 °C). *Hyla cinerea* was recorded on 27 July 1992 in the laboratory (air temperature 23 °C). Recordings were made with a Uher 400 report tape recorder (tape speed 9.5 cm s^{-1}) and a Uher M 517 microphone, and sonograms were produced on a Kay Elemetrics Vibralizer 7030A sound spectrograph.

sonogram in Fig. 1C is not high enough to distinguish these pulses; see Allan and Simmons, 1994). Although pulse production may be directly linked to muscle contraction in some species, in other species a series of pulses occurs as a passive result of air flowing through the larynx (i.e. without direct muscle activity; see Martin, 1972). Therefore, the rate of pulse production is not necessarily indicative of the *in vivo* contractile frequency of the calling muscles and, in some cases, may actually be many times faster.

For this study, it was important to identify units of sound in the calls of the three species that are mechanically similar and are directly dependent upon the contraction of calling muscles. An important mechanical component of call production that is driven by direct muscle activity (Martin and Gans, 1972; Sarbadhikary and Marsh, 1994) is the cyclical flow of air, back and forth through the larynx, between the lungs and the vocal sacs. In this paper, the term 'note' refers to the unit of sound, consisting of one or more pulses, produced during a single airflow cycle. The number of notes per call (i.e. the number of times that air flows back and forth between the lungs and the vocal sacs) can be determined by observing the vocal sacs of calling frogs; in *H. cinerea*, the vocal sac undergoes a single inflation during each call, but in *H. chrysoscelis* and *H. versicolor* the vocal sacs increase and decrease in volume many times per call in conjunction with each pulse (Sarbadhikary and Marsh, 1994; J. D. McLister, personal observation). Therefore, the 'pulse repetition rate' of *H. chrysoscelis* and *H. versicolor* is, mechanically, more comparable to the 'call rate' of *H. cinerea* than it is to the 'pulse repetition rate' of *H. cinerea*. Using the term 'note repetition rate', rather than 'pulse repetition rate' or 'call rate', ensures that the same mechanical call parameter is being referred to in all three species.

Materials and methods

Animals

The frogs used in this study were males collected during the breeding season (April to June) in 1991 and 1992. Mating calls were used to identify *H. chrysoscelis* and *H. versicolor* in the field; *H. cinerea* was distinguished from the other two species on the basis of call and morphological characters. Individuals of *H. chrysoscelis* were collected at five sites, *H. versicolor* were collected at six sites, and *H. cinerea* were collected from one site (see Table 1 for locality information). The ranges of body sizes (snout–vent length) of the collected specimens were 34–50 mm (*N*=15) for *H. chrysoscelis*, 34–54 mm (*N*=27) for

Table 1. *Localities where specimens were collected*

Locality	Latitude	Longitude	Species
Bastrop Co., Texas, USA	30°02′N	97°12′W	*Hyla cinerea*
Hubbard Co., Minnesota, USA	47°14′N	95°09′W	*H. chrysoscelis*, *H. versicolor*
Jackson Co., Michigan, USA	42°17′N	84°21′W	*H. chrysoscelis*, *H. versicolor*
Parry Sound District, Ontario, Canada	45°11′N	79°40′W	*H. versicolor*
Phelps Co., Missouri, USA	37°38′N	91°58′W	*H. chrysoscelis*
Rainy River District, Ontario, Canada	48°48′N	94°33′W	*H. versicolor*
Sainte Anne Rural Municipality, Manitoba, Canada	49°40′N	96°39′W	*H. chrysoscelis*, *H. versicolor*
Travis Co. Texas, USA	30°13′N	97°53′W	*H. chrysoscelis*
Wellington Co., Ontario, Canada	43°33′N	80°15′W	*H. versicolor*

H. versicolor and 42–53 mm (N=4) for *H. cinerea*. All experiments involving comparisons between *H. chrysoscelis* and *H. versicolor* included specimens from several sites and always included representatives of both species from sympatric populations. The treefrogs were maintained at room temperature in the laboratory for periods ranging from less than 1 week to almost 1 year (average 132 days for *H. chrysoscelis*, 126 days for *H. versicolor* and 60 days for *H. cinerea*) and were fed a diet of crickets and meal worms.

Chromosome counts were used in the laboratory to verify the identification of *H. chrysoscelis* and *H. versicolor*. 2 h prior to being killed, each frog (all three species) received a 0.1 ml injection of aqueous colchicine solution (1 mg ml^{-1}) into its dorso-lymphatic sacs and was then placed in an incubator for 2 h at 30 °C. Frogs were killed by pithing or over-anaesthetisation in 7 % aqueous tricane methane sulphonate (MS222). The eyes were dissected from each frog and placed in distilled water for 2 h. Chromosomes were obtained by scraping epithelial tissue from the cornea and squashing this tissue in 70 % acetic acid between a glass slide and a coverslip.

Muscle preparation

Immediately after the tissues had been removed for chromosome analysis, the larynx was dissected from each specimen and one of the laryngeal muscles was removed. The laryngeal muscle used in this study was the tensor chordarum [Wilder's (1896) nomenclature]. From many of the frogs, a sartorius muscle was also removed with a piece of the ilium still attached. Both muscles were placed in cold (5 °C), oxygenated saline (115 mmol l^{-1} NaCl, 1 mmol l^{-1} KH_2PO_4, 1 mmol l^{-1} $MgSO_4$, 20 mmol l^{-1} $NaHCO_3$, 1.26 mmol l^{-1} $CaCl_2$) until use.

Although the tensor chordarum is more commonly referred to as the 'constrictor laryngeus posterior' (after Trewavas, 1933) or the 'sphinctor laryngis posterior' (after Gaupp, 1896), these names are misleading because they imply that contraction of this muscle causes the larynx to close. Preliminary studies indicate that, in hylid larynges, this muscle does not affect the position of the laryngeal cartilages when it contracts (McLister, 1992). 'Tensor chordarum' is a more appropriate name since the contraction of this muscle increases the tension on the vocal cords and shifts them medially from their lateral position within the larynx to a position where the flow of air causes the vocal cords to vibrate (Wilder, 1896; Trewavas, 1933: page 514; Martin, 1972; McLister, 1992). Electromyographic evidence suggests that, in *H. versicolor*, this muscle contracts during the production of each note (Schmidt, 1965).

A long tendon at the anterior end of the tensor chordarum and a short ligament at the posterior end provided points to which a short length of thin silk thread (size 6-0) could be tied. The anterior end of the muscle was tied *via* the piece of thread to the arm of a Cambridge Technology dual-mode ergometer/servomotor while the piece of thread tied to the posterior end of the muscle was held firmly in a clamp. The preparation for the sartorius differed in that only one end of the muscle, the end attached to the ergometer, was tied with a silk thread. The other end of the sartorius was held in place by clamping the piece of ilium to which the muscle was attached. The resting length of each muscle (L_0) could be adjusted by shifting the relative position of the clamp and the ergometer arm.

Once secured in the apparatus, the muscle was submerged in saline between two platinum plate electrodes. The saline was bubbled with a 100:1 mixture of oxygen and carbon dioxide and a waterbath was used to regulate the temperature of the saline. The pH of the aerated saline was temperature-dependent and varied from 7.8 at 15 °C to 8.0 at 30 °C. Before beginning any set of experiments, both L_0 and the electrode stimulus voltage were adjusted to levels which maximised twitch force. Force and muscle length during each experimental protocol were recorded using a digital storage oscilloscope. Afterwards, each muscle was removed from the apparatus, blotted dry and weighed to the nearest 0.01 mg. The cross-sectional area of each muscle (in cm^2) was then estimated by dividing the muscle mass by the product of L_0 and muscle density (1.05 g cm^{-2}).

Measuring muscle contractile dynamics

Experimental protocols were designed to measure isometric twitch and isometric tetanus parameters, the relationship between force and shortening velocity, and the capacity of the muscles to relax between stimuli delivered at various frequencies (percentage relaxation *versus* stimulus frequency). Except where otherwise stated, all measurements of muscle contractile performance were made at 25 °C. For all protocols, stimulus pulse-width was 0.4 ms.

Isometric twitch

For measuring isometric twitch parameters, each muscle was subjected to ten separate twitch stimuli. The values of each parameter for each muscle were taken as the average value measured from the ten isometric twitches. The measured twitch parameters were maximum isometric twitch force (P_t) and five time variables: latent period, time to half-P_t, time to P_t, half-relaxation time and twitch duration. The latent period was measured as the time interval between the stimulus and the start of tension development. The time to half-P_t and the time to P_t were measured as the interval between the stimulus and the time required to reach half-P_t and P_t, respectively. Half-relaxation time was measured as the interval between the time at which P_t was reached and the time at which 50 % relaxation was reached. Twitch duration was measured as the interval between the start of tension development and the time at which 50 % relaxation was reached (i.e. the sum of the time to P_t and the half-relaxation time minus the latent period). For the tensor chordarum muscles of *H. chrysoscelis* and *H. versicolor*, twitch parameters were measured at 15 and 20 °C as well as at 25 °C.

A protected least significant difference (LSD) analysis (Steel and Torrie, 1980) was used to test for differences in twitch parameter when all three species were being compared (at

25 °C), but, where only *H. chrysoscelis* and *H. versicolor* were compared (at 20 °C and 15 °C), *t*-tests were used.

Isometric tetanus

Maximum isometric force (P_0) and two time variables, the time to half-P_0 and tetanus half-relaxation time, were measured for each muscle for five different exposures to tetanic stimuli. The tetanic stimulus consisted of a train of biphasic pulses delivered at a frequency of 200 Hz for 400 ms. The time to half-P_0 was measured as the time from the first stimulus pulse to the time at which tension reached half-P_0. The tetanus half-relaxation time was measured from the time of the last pulse of the tetanus stimulus to the time at which tension returned to half-P_0. There was always a minimum rest interval of 2 min prior to each tetanus stimulus. The tetanus parameters for each muscle were taken as the mean value measured from the five tetanus events. Protected LSD analysis was used to test for significant differences among species.

Force–velocity relationships

The isotonic quick-release method described by Jewell and Wilkie (1958) was used to measure the shortening velocities of sartorius and tensor chordarum muscles against a series of loads. At a time shortly after maximum tension (P_0) had been reached, the force on the muscle was suddenly reduced to a fixed, predetermined load and the muscle contracted at a relatively constant rate. The shortening velocity (muscle lengths per second, $L\,s^{-1}$) of each muscle was calculated for a series of loads ranging from $0.05P_0$ to $0.78P_0$ (typically, the force–velocity relationship changes when relative load is greater than $0.78P_0$; Edman *et al.* 1976).

Sigmaplot (version 1.02, copyright 1986–1994, Jandel Corporation) was used to fit the data generated from each muscle to Hill's (1938) hyperbolic equation:

$$(V + b)(P + a) = (P_0 + a)b\,,$$

where V is shortening velocity ($L\,s^{-1}$), P is relative load, a is a force constant and b is a velocity constant. Since P was relative load, P_0 in this equation takes the value of 1. The best-fitting Hill equation determined for each muscle was used to estimate the maximum shortening velocity (V_{max}) (i.e. the value of V when P=0). All force–velocity data were collected at 25 °C.

Percentage relaxation versus *stimulus frequency*

If muscle activity is responsible for the production of each individual note in the mating call of the three species examined, then the muscles involved in note production must be able to function at frequencies equal to the NRR of a particular species. To assess the capacity of the muscles to function over a range of different possible NRRs, the isometric contractile dynamics of the muscles were monitored over a range of different stimulus frequencies. Nine different stimulus trains were tested, each consisting of a series of nine twitch stimuli delivered at a specific frequency (i.e. each stimulus train represented the neurological stimulus required to generate a single nine-note call with a specific NRR). The different stimulus trains were applied to each muscle in the sequence 10, 20, 30, 40, 60, 50, 35, 25 and 15 Hz, with two repetitions of each frequency. A 1 min rest period separated the application of each stimulus train.

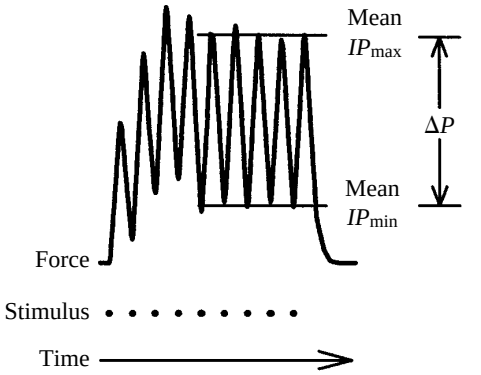

Fig. 2. A typical isometric force curve generated by a muscle exposed to a nine-pulse stimulus train. Shown on the curve are the mean interpulse maximum tension (IP_{max}), the mean interpulse minimum tension (IP_{min}) and the tension fluctuation (ΔP) for the last five stimulus pulses. These parameters were used to calculate the average percentage relaxation between successive stimuli (%R; see text).

An example of the isometric force evoked by a single stimulus train is shown in Fig. 2. The highest and lowest tensions exerted by each muscle between subsequent stimulus pulses were termed the interpulse maximum tension (IP_{max}) and the interpulse minimum tension (IP_{min}) respectively. The difference between the average IP_{max} and average IP_{min} was termed the tension fluctuation (ΔP). The percentage relaxation between two successive stimulus pulses (%R) for each muscle at each stimulus frequency was calculated as:

$$\%R = 100(\Delta P/\text{average } IP_{max})\,.$$

Because the tension curve for each stimulus train did not usually establish a stable pattern until after the third or fourth stimulus pulse, only the IP_{max} and IP_{min} values associated with the last five stimulus pulses in each train were used in the calculations.

Determination of average note repetition rates

Linear regression models describing the effect of temperature on NRRs for *H. chrysoscelis* and *H. versicolor* have been calculated by Bogart and Jaslow (1979), Gayou (1984), Gerhardt (1978), Jaslow and Vogt (1977), Ralin (1977) and Zweifel (1970). The average expected NRR values in notes per second for *H. chrysoscelis* and *H. versicolor* used in this study are the averages of the expected NRRs calculated from these regression models for 15, 20 and 25 °C (Table 2). For *H. chrysoscelis*, the average expected NRRs at 15, 20 and 25 °C are 28.3, 42.5 and 56.8 notes s^{-1}, respectively; for *H. versicolor*, the average expected NRRs at 15, 20 and 25 °C are 14.8, 21.1 and 27.4 notes s^{-1}, respectively. The range 15–25 °C approximates the range of temperatures at which *H. chrysoscelis* and *H. versicolor* commonly call (Gerhardt and Doherty, 1988).

Table 2. *Published regression equations for note repetition rate (NRR) of* Hyla chrysoscelis *and* H. versicolor *and the expected mean NRRs based upon each equation at 15, 20 and 25 °C*

Regression equation	Source	Expected mean NRR (notes s^{-1}) 15 °C	20 °C	25 °C
Hyla chrysoscelis				
**y*=3.78*x*–33.93	Bogart and Jaslow (1979)	22.77	41.67	60.57
**y*=3.05*x*–15.80	Gerhardt (1978)	29.95	45.20	60.45
**y*=2.59*x*–2.90	Jaslow and Vogt (1977)	35.95	48.90	61.85
**y*=2.36*x*–3.40	Ralin (1977)	32.00	43.80	55.60
y=2.88*x*–21.75	Ralin (1977)	21.45	35.85	50.25
y=2.41*x*–8.46	Zweifel (1970)	27.69	39.74	51.79
Mean		28.3	42.5	56.8
S.D.		5.5	4.5	4.9
Hyla versicolor				
**y*=1.32*x*–7.50	Bogart and Jaslow (1979)	12.30	18.90	25.50
**y*=1.22*x*–2.25	Gayou (1984)	16.05	22.15	28.25
**y*=1.15*x*–2.30	Gerhardt (1978)	14.95	20.70	26.45
**y*=1.30*x*–4.95	Jaslow and Vogt (1977)	14.55	21.05	27.55
**y*=1.22*x*–4.30	Ralin (1977)	14.00	20.10	26.20
y=1.19*x*–0.51	Ralin (1977)	17.34	23.29	29.24
y=1.43*x*–7.16	Zweifel (1970)	14.29	21.44	28.59
Mean		14.8	21.1	27.4
S.D.		1.6	1.4	1.4

y, NRR; *x*, temperature in °C; * indicates equations calculated for populations within the geographic range of the populations listed in Table 1.

Results

Isometric twitch

The mean values of the isometric twitch parameters for both muscles of each species are presented in Table 3. With the exception of latent period, all twitch parameters were significantly different between the tensor chordarum of *H. chrysoscelis* and the tensor chordarum of *H. versicolor* at all three temperatures. Although NRR differs between these two species by a factor of two, twitch duration of the tensor chordarum differed between these two species by a factor of 1.5.

The tensor chordarum of *H. cinerea* differed from the tensor chordarum of the other two species for every twitch parameter except P_t/P_0, which was not significantly different from P_t/P_0 for the tensor chordarum of *H. chrysoscelis*. There were no significant differences between the sartorius muscles of *H. chrysoscelis* and *H. versicolor*. The sartorius of *H. cinerea* differed from the sartorius of the other two species only in P_t/P_0.

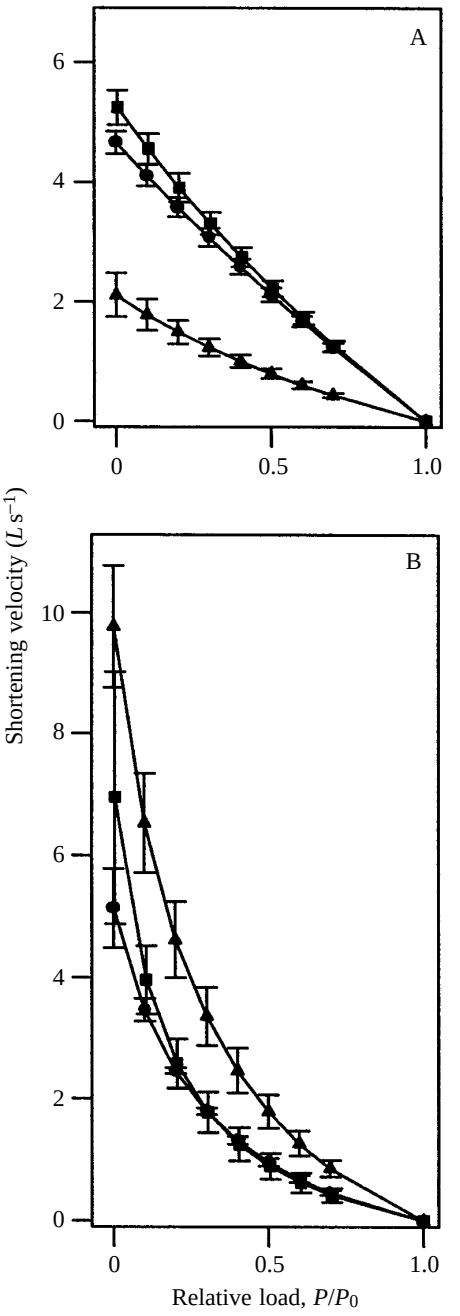

Fig. 3. Force–velocity plots for (A) the tensor chordarum and (B) the sartorius of *Hyla chrysoscelis* (circles), *H. versicolor* (squares) and *H. cinerea* (triangles) at 25 °C. Sample sizes for the tensor chordarum were 4 for *H. chrysoscelis*, 11 for *H. versicolor* and 4 for *H. cinerea*. Sample sizes for the sartorius were 5 for *H. chrysoscelis*, 4 for *H. versicolor* and 3 for *H. cinerea*. The vertical bars represent 95 % confidence intervals. *L*, muscle length.

Isometric tetanus

Mean values of the isometric tetanus parameters are presented in Table 4. For the tensor chordarum, the time to half-P_0 was not significantly different for *H. chrysoscelis* and *H. versicolor*, but tetanus half-relaxation time was significantly longer in *H. versicolor*. Both of these parameters were longer

Table 3. *Means and 95 % confidence intervals for the isometric twitch parameters of the tensor chordarum and sartorius muscles of* Hyla chrysoscelis, H. versicolor *and* H. cinerea

Species	(*N*)	Latent period (ms)	Time to half-P_t (ms)	Time to P_t (ms)	Twitch half-relaxation time (ms)	Twitch duration (ms)	P_t/P_0
Tensor chordarum							
15 °C							
H. chrysoscelis	(8)	4.5±0.6	13.1±3.5†	26.2±4.7†	20.2±4.3†	41.9±7.2†	0.45±0.15†
H. versicolor	(8)	4.5±0.7	16.8±2.5*	36.4±2.7*	32.2±5.6*	64.1±6.7*	0.63±0.07*
20 °C							
H. chrysoscelis	(10)	3.3±0.5	8.8±1.3†	18.1±2.1†	12.5±2.6†	27.2±4.0†	0.41±0.13†
H. versicolor	(11)	3.2±0.4	12.4±1.4*	25.3±2.2*	19.4±3.0*	41.6±4.2*	0.58±0.07*
25 °C							
H. chrysoscelis	(10)	2.6±0.3	7.0±1.1†	13.7±2.0†	8.1±1.3†	19.2±2.2†	0.34±0.13†
H. versicolor	(18)	2.5±0.3	9.3±0.5*	19.2±0.8*	13.3±1.2*	30.0±1.6*	0.48±0.05*
H. cinerea	(3)	5.8±3.4*,†	16.7±5.4*,†	37.2±1.9*,†	21.5±12.7*,†	52.9±14.3*,†	0.18±0.25†
Sartorius							
25 °C							
H. chrysoscelis	(8)	3.0±0.3	8.8±0.5	18.2±2.1	14.3±2.9	29.5±4.7	0.19±0.06
H. versicolor	(12)	2.8±0.4	9.2±0.9	19.4±2.5	15.7±3.6	32.3±6.0	0.19±0.08
H. cinerea	(3)	2.5±0.8	9.2±0.4	20.8±1.2	20.4±1.8	38.7±3.1	0.38±0.13*,†

P_t, maximum twitch tension; P_0, maximum tetanic tension.

*Mean is significantly different from *H. chrysoscelis* (P=0.05).

†Mean is significantly different from *H. versicolor* (P=0.05).

Note that the variances determined for each species were used to calculate the 95 % confidence limits, whereas statistical tests used the pooled variance of all three species. Therefore, the comparatively large confidence limits calculated for *H. cinerea* merely reflect the small sample size for this species.

for the tensor chordarum of *H. cinerea* than they were for either of the other two species. The maximum tension for this muscle differed among all three species; the faster the muscle, the lower the value of P_0.

There were no significant differences between the sartorius muscles of *H. chrysoscelis* and *H. versicolor*. The sartorius of *H. cinerea* differed only in the time to half-P_0, which was significantly faster than that of either *H. chrysoscelis* or *H. versicolor*.

Force–velocity curves

Force–velocity plots for the calling and locomotor muscles of *H. chrysoscelis*, *H. versicolor* and *H. cinerea* are shown in Fig. 3. The muscle with the fastest average V_{max} in this study was the sartorius of *H. cinerea* ($9.8\,L\,s^{-1}$) and the slowest was the tensor chordarum of *H. cinerea* ($2.1\,L\,s^{-1}$). The V_{max} values for the tensor chordarum of *H. chrysoscelis* and *H. versicolor* were $4.7\,L\,s^{-1}$ and $5.2\,L\,s^{-1}$, respectively. The V_{max} values for the sartorius muscle of *H. chrysoscelis* and *H. versicolor* were $5.2\,L\,s^{-1}$ and $7.0\,L\,s^{-1}$, respectively. For both muscles, the average V_{max} was significantly higher for *H. versicolor* than for *H. chrysoscelis*.

For all three treefrog species, the force–velocity plots for the tensor chordarum muscles were less curved than the sartorius force–velocity plots. One way to compare the degree of curvature among force–velocity plots is to consider the value of the ratio a/P_0 (Josephson, 1984). As a/P_0 increases, the force–velocity plot approaches linearity. The values of a (since

Table 4. *Means and 95 % confidence intervals for the isometric tetanus parameters of the tensor chordarum and sartorius muscles of* Hyla chrysoscelis, H. versicolor *and* H. cinerea *at 25 °C*

Species	(*N*)	Time to half-P_0 (ms)	Tetanus half-relaxation time (ms)	P_0 (kN m^{-2})
Tensor chordarum				
H. chrysoscelis	(10)	14.5±2.4	17.2±2.6*	55.0±19.9†
H. versicolor	(16)	15.8±1.4	28.7±2.3†	94.4±16.7*
H. cinerea	(3)	52.6±19.9*,†	60.6±3.6*,†	180.5±117.8*,†
Sartorius				
H. chrysoscelis	(6)	23.7±3.6	56.3±10.6	252.2±38.2
H. versicolor	(8)	22.9±3.0	59.5±5.8	240.7±36.5
H. cinerea	(3)	15.6±2.8*,†	60.7±14.3	285.1±69.0

*Mean is significantly different from *H. chrysoscelis* (P=0.05).

†Mean is significantly different from *H. versicolor* (P=0.05).

Note that the variances determined for each species were used to calculate the 95 % confidence limits, whereas statistical tests used the pooled variance of all three species. Therefore, the large confidence limits calculated for *H. cinerea* result from the small sample size for this species.

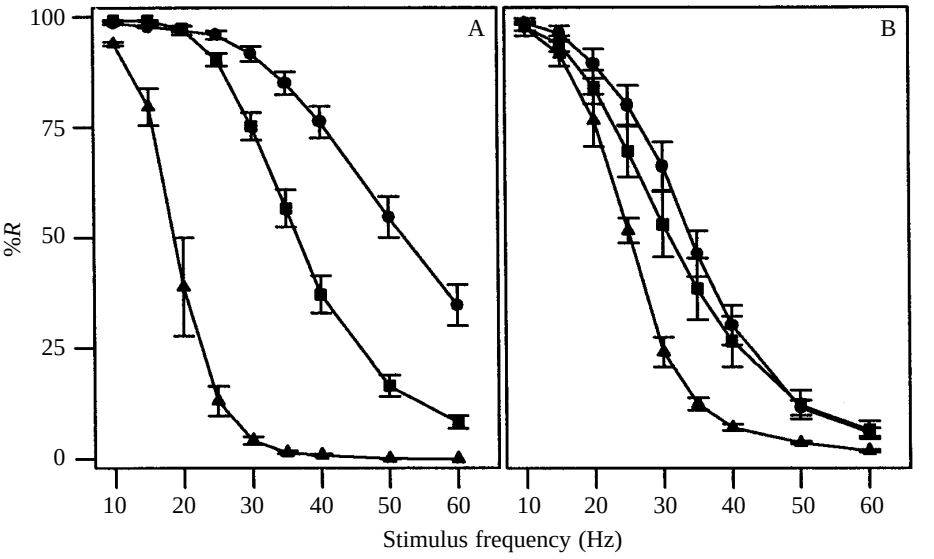

Fig. 4. Percentage relaxation between stimuli (%*R*) plotted against stimulus frequency for (A) the tensor chordarum and (B) the sartorius of *Hyla chrysoscelis* (circles), *H. versicolor* (squares) and *H. cinerea* (triangles) at 25 °C. Sample sizes for the tensor chordarum were 14 for *H. chrysoscelis*, 16 for *H. versicolor* and 3 for *H. cinerea*. Sample sizes for the sartorius were 10 for *H. chrysoscelis*, 11 for *H. versicolor* and 3 for *H. cinerea*. The faster the muscle, the higher the %*R* values are at any particular stimulus frequency. The vertical bars represent 95 % confidence intervals.

relative loads were used in our calculations, $a/P_0=a$ and a is dimensionless) which best fit the force–velocity data for the tensor chordarum and sartorius, respectively, were 5.15 and 0.30 for *H. chrysoscelis*, 2.80 and 0.17 for *H. versicolor*, and 1.48 and 0.29 for *H. cinerea*; the values of a for the calling muscles were approximately an order of magnitude larger than the values of a for the locomotor muscles.

Percentage relaxation versus *stimulus frequency*

The curves illustrated in Figs 4 and 5 show the relationship between %*R* and stimulus frequency for the tensor chordarum and sartorius muscles of all three species. At lower stimulus frequencies, all muscles relaxed nearly 100 % between stimuli. As stimulus frequency increased, however, percentage relaxation between stimuli decreased. At higher stimulus frequencies, %*R* was considerably greater for the tensor chordarum of *H. chrysoscelis* than it was for either *H. versicolor* or *H. cinerea*. The tensor chordarum of *H. cinerea* underwent very little relaxation between stimuli delivered at frequencies greater than 20 Hz and, at stimulus frequencies in excess of 50 Hz, zero relaxation occurred (i.e. the muscle was completely fused).

If a muscle is to function at a particular frequency, it must be able to undergo some degree of contraction and relaxation when stimulated at that frequency. The %*R* curves clearly show that the calling muscle of *H. chrysoscelis* is capable of functioning at higher contractile frequencies than the calling muscles of *H. versicolor*, which, in turn, can function at higher contractile frequencies than the calling muscles of *H. cinerea* (Fig. 4). (Note that, because the NRR of *H. cinerea* does not equal its pulse repetition rate, it is possible that the *in vivo* contractile frequency of some of the calling muscles of this species corresponds to the pulse repetition rate rather than to the NRR. For the tensor chordarum of *H. cinerea*, however, the *in vivo* contractile frequency must correspond with NRR because stimulation frequencies equal to the pulse repetition rate would result in tetanus.)

While the curves for %*R versus* stimulus frequency differed greatly for the tensor chordarum muscles of the three species, the curves for the sartorius muscles (Fig. 4B) differed very little. At higher frequencies, %*R* was smaller for the sartorius of *H. cinerea* than it was for the other two species, but this difference was small relative to the differences among the tensor chordarum muscles. Only at stimulus frequencies of 25 and 30 Hz did *t*-tests reveal a significant difference between the mean %*R* of *H. chrysoscelis* and *H. versicolor*; otherwise the curves for the sartorius of these two species did not differ. While this difference suggests that ploidy may affect the contractile performance of anuran muscle, the differences between the sartorius muscles of these two species is very small relative to the differences between the tensor chordarum muscles.

Fig. 5 presents the mean %*R* curves for the tensor chordarum muscles of *H. chrysoscelis* and *H. versicolor* at

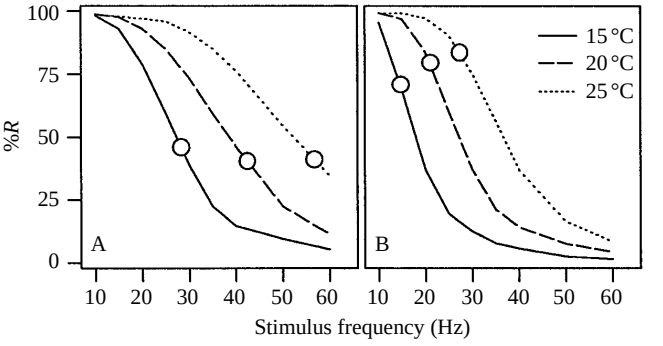

Fig. 5. Percentage relaxation between stimuli (%*R*) plotted against stimulus frequency for the tensor chordarum of (A) *Hyla chrysoscelis* and (B) *H. versicolor* at three different temperatures. The open circles represent the %*R* value on each curve corresponding to the expected note repetition rate (i.e. $\%R_{NRR}$) of each species at each temperature. As temperature decreases, the muscles become slower and the %*R* values corresponding to any particular stimulus frequency can be seen to decrease.

different temperatures (25, 20 and 15 °C). Open circles in Fig. 5 mark the point on each curve where stimulus frequency equals the average NRR for each species at each temperature as determined by the above equations. The %R value corresponding to each NRR (%R_{NRR}) provides a measure of tensor chordarum performance at frequencies equal to NRR. Fig. 5 shows that the %R_{NRR} values for *H. chrysoscelis* were consistently close to 40 % at all three temperatures (46.0 % at 15 °, 40.4 % at 20 °C and 41.1 % at 25 °C) while the %R_{NRR} values for *H. versicolor* were higher than those of *H. chrysoscelis* and increased markedly with temperature (70.7 % at 15 °, 79.4 % at 20 °C and 83.4 % at 25 °C). Relaxation rates of muscles under isometric conditions are generally slower than relaxation rates when the muscles are allowed to shorten (Josephson and Stokes, 1989) so the measured values of %R_{NRR} probably underestimate the *in vivo* values of %R_{NRR} for both species.

Discussion

Calling and non-calling muscles

The contractile dynamics of the sartorius and other locomotor muscles have been examined for many species of frogs (e.g. Putnam and Bennett, 1983; Renaud and Stevens, 1983; see Gans and de Gueldre, 1992, for a review). In contrast, very little has been published concerning the contractile properties of anuran calling muscles. Manz (1975) determined the fusion frequencies for the laryngeal muscles of the European treefrog *H. arborea* over a range of temperatures. Wells and Taigen (1992) discuss some unpublished data regarding an unspecified calling muscle in *H. versicolor*, for which the fusion frequency at 20 °C was estimated to be 100 Hz, a value close to the predicted fusion frequency of the tensor chordarum of this species at 20 °C (see Fig. 5).

Despite general ultrastructural and biochemical differences between calling muscles and locomotor muscles, we found few consistent contractile differences between them. The contractile properties of the tensor chordarum were much more variable among species than the contractile properties of the sartorius, but this variability should be expected, given that the mating call is more variable among species than is locomotor behaviour. There was also a tendency for calling muscles to have lower P_0 values, which may have been due, in part, to the abundance of non-contractile elements within the fibres of the calling muscles. Increasing the amount of SR, mitochondria and lipid droplets reduces the proportion of contractile structures (myofibrils) per unit cross-sectional area which, in turn, results in lower P_0 values per unit cross-sectional area. Transmission electron micrographs of tensor chordarum muscles, however, reveal that the amounts of SR and mitochondria are similar for *H. chrysoscelis* and *H. versicolor* and that the volume occupied by lipid droplets may actually be greater in *H. versicolor* (McLister, 1992). Therefore, the low P_0 values observed for the tensor chordarum muscles cannot be entirely explained by the abundance of non-contractile elements, otherwise the P_0 for the tensor chordarum of *H. versicolor* would be similar to, or less than, that for the tensor chordarum of *H. chrysoscelis*.

The force–velocity plots differed between calling and locomotor muscles. Mathematical models indicate that increasing the viscosity within a muscle reduces the curvature of that muscle's force–velocity plot (Josephson, 1984). Josephson (1984) speculated that the linear appearance of force–velocity plots for certain insect muscles may be due to the viscous drag generated by the large amounts of SR and mitochondria within those muscles. The calling muscles of *H. chrysoscelis* and *H. versicolor* have an extensive SR, and the calling muscles of all three species have many more mitochondria and lipid droplets than the non-calling muscles (Marsh and Taigen, 1987; McLister, 1992) so viscosity effects may explain the very linear force–velocity curves obtained for the tensor chordarum. The force–velocity plot derived for the tensor chordarum of *H. cinerea* was slightly more curved than those for the tensor chordarum of the other two species. However, the tensor chordarum of *H. cinerea* has less intracellular lipid and a less extensive SR (McLister, 1992) so viscosity effects would be less pronounced in this species.

One curious difference between the calling muscles of *H. chrysoscelis* and *H. versicolor* is the difference in V_{max}. It is improbable that a higher V_{max} for the calling muscle of *H. versicolor* would have evolved in response to a slower NRR. Because the V_{max} values for both the tensor chordarum and the sartorius are significantly higher in the tetraploid species from values in the corresponding muscles of the diploid species, this small increase in V_{max} may be attributable to some unknown effect of polyploidy on muscle development. Nevertheless, the high degree of similarity between the sartorius muscles of these two species suggests that any direct effects that polyploidy may have on muscle performance are of minor consequence.

Contractile differences and call differences

Generally, the contractile dynamics of the tensor chordarum are consistent with the interspecific differences in NRR; the faster the NRR of a species, the shorter the twitch duration of the tensor chordarum. However, contractile frequency is not the only performance constraint under which the contractile dynamics of a muscle must evolve. The contractile requirements of the tensor chordarum during each note, for example, are probably very different between *H. cinerea* and the other two species. In *H. chrysoscelis* and *H. versicolor*, the twitch duration of the tensor chordarum is comparable to the duration of each note whereas, in *H. cinerea*, the twitch duration of this muscle (52.9 ms at 25 °C) is much shorter than the note duration (approximately 100 ms; see Fig. 1C). Therefore, a more prolonged stimulus per note must be required for the tensor chordarum muscles of *H. cinerea* to maintain tension on the vocal cords throughout the entire note. Laryngeal morphology also differs between *H. cinerea* and the other two species (McLister, 1992), which may impose additional differences in the contractile requirements of the tensor chordarum of *H. cinerea*.

Between *H. chrysoscelis* and *H. versicolor*, the contractile dynamics of the calling muscles differed less than was expected. Despite the twofold difference between the NRRs of these two species, the twitch duration of the tensor chordarum of *H. chrysoscelis* was not twice as fast. The different $\%R_{NRR}$ values, as shown in Fig. 5, reflect a shift in the relationship between the twitch dynamics of the tensor chordarum and note production that has occurred as *H. versicolor* evolved from *H. chrysoscelis*. However, laryngeal morphology does not differ between these two species (McLister, 1992), and the reduction of NRR in *H. chrysoscelis* as temperature decreases does not, evidently, require any change in $\%R_{NRR}$ (Fig. 5). Therefore, it is unlikely that the evolutionary reduction of NRR in the call of *H. versicolor* would have required a shift in the $\%R_{NRR}$ of the tensor chordarum.

The NRR of *H. versicolor* should represent the fastest contractile frequency at which a calling muscle of this species will ever need to function. In female hylids, calling muscles are not very well developed (Eichelberg and Schneider, 1974) and, since females do not call, it can be inferred that calling muscles are unimportant for activities other than call production. Even if calling muscles are used for other activities, it is unlikely that any activity a treefrog could perform would require a contractile frequency anywhere near as fast as the NRR of *H. versicolor*. Yet, if 40 % relaxation between stimuli is sufficient to generate a series of distinct notes, as it is for *H. chrysoscelis* (Fig. 5), then the tensor chordarum of *H. versicolor* should be physiologically capable of performing at a much faster NRR than it does [on the basis of the $\%R$ curves in Fig. 5, the theoretical maximum NRR values (NRR values corresponding to $\%R$ values of 40 %) for *H. versicolor* are 19.5 notes s^{-1} at 15 °C, 29.4 notes s^{-1} at 20 °C and 39.2 notes s^{-1} at 25 °C]. Is the tensor chordarum of *H. versicolor* 'over-designed' or does some aspect of note production in *H. versicolor* require a higher $\%R_{NRR}$?

Apart from the NRR, there are a few other, less obvious, acoustic differences between the mating calls of *H. chrysoscelis* and *H. versicolor*. *Hyla chrysoscelis* sings at a higher pitch (or carrier frequency) than *H. versicolor* (Blair, 1958; Bogart and Jaslow, 1979; Mable and Bogart, 1991; see also Fig. 1), and the amplitude modulation (AM) at the beginning of each note differs greatly between these two species (Gerhardt and Doherty, 1988). While it is known that factors unassociated with tensor chordarum activity (e.g. laryngeal morphology, pulmonary air pressure) do influence the pitch and AM of frog calls (Martin, 1972), the call mechanics of hylid frogs are insufficiently understood to discount the importance of tensor chordarum activity in determining pitch and AM parameters. More must be known concerning the specifics of laryngeal muscle activity during note production before it can definitely be concluded whether or not the tensor chordarum of *H. versicolor* is 'over-designed'. However, if this muscle is indeed faster than is physiologically necessary, the evolutionary history of *H. versicolor* does provide an explanation of how such an 'over-designed' muscle could have come about.

Evolution of contractile dynamics in Hyla versicolor

Tetraploid individuals arise from diploid parents at a very low frequency as a result of the union of two unreduced gametes. The establishment and persistence of tetraploid species from an ancestral diploid species is unlikely without the rapid evolution of pre-mating reproductive isolation between the two ploidy levels (Thompson and Lumaret, 1992). Under random mating conditions, rare tetraploids are selected against because rare tetraploid individuals mate more frequently with diploids than with other tetraploids and diploid–tetraploid matings produce sterile triploid offspring (Bogart, 1980). The difference in NRR values between *H. chrysoscelis* and *H. versicolor* is a key feature used by females of these two species to identify conspecific males (Gerhardt and Doherty, 1988). It is, therefore, improbable that *H. versicolor* could have evolved as a species if the reduction in NRR had not evolved very quickly and very early in its evolutionary history (Bogart and Wasserman, 1972; Ralin, 1977).

Evolutionary changes in NRR may not necessarily require changes in muscle contractile dynamics. The contractile frequency of a synchronous skeletal muscle is ultimately under neurological control and, while the contractile dynamics of a muscle impose an upper limit to NRR, it should be possible to reduce the contractile rate of a muscle without changing the contractile properties of the muscle (whereas increasing the contractile rate of a muscle may require a change in both the neurological control and the contractile properties of that muscle). The immediate evolution of a slower NRR in *H. versicolor*, therefore, would have required only a neurological change. Once a slower NRR evolved, selection pressures might then have acted to alter the contractile dynamics of the calling muscles to increase the energetic efficiency of call production. Call production is energetically very expensive (Pough and Gatten, 1984; Taigen and Wells, 1985; Taigen *et al.* 1985; Given and McKay, 1990), and the mating success of males is highly dependent upon the number and duration of the calls they produce (Gerhardt, 1991). Any modification to contractile dynamics that increases the energetic efficiency will enable males to produce more calls for the same energetic cost and, therefore, will be favoured by sexual selection.

A muscle that relaxes more extensively than necessary between contractions is energetically inefficient because the deactivation rate is faster than it needs to be and ATP is wasted as a result of excessive Ca^{2+}-ATPase activity (Marsh, 1990). Interestingly, a decrease in deactivation rate appears to be the most important physiological modification to the tensor chordarum of *H. versicolor* in response to a reduced NRR. Deactivation influences some, but not all, of the contractile parameters of a muscle. Under isometric twitch conditions, for example, muscles with slower deactivation rates will have longer twitch durations and will generate greater tension (i.e. the P_t/P_0 ratio will be greater) because the rate of relaxation will be slower and the period between the onset of contraction and the onset of relaxation will be longer (Close, 1965; Hinrichsen and Dulhunty, 1982). The contractile parameters for which the tensor

chordarum of *H. versicolor* was found to be slower than the tensor chordarum of *H. chrysoscelis* (i.e. twitch duration, tetanus half-relaxation time and %*R*) are those that are largely determined by deactivation rate. In contrast, contractile parameters that are determined largely by kinetic processes other than deactivation rate (Marsh, 1990) did not differ between the calling muscles of *H. versicolor* and *H. chrysoscelis* (e.g. latent period, time to half-P_0, most of the force–velocity data) or were actually faster for *H. versicolor* (e.g. V_{max}).

The existence of, an 'over-designed' muscle could come about if a time lag exists between the neurological evolution necessary to reduce the NRR and the physiological evolution necessary to alter the contractile properties of the calling muscles. Whether the tensor chordarum of *H. versicolor* is still in the process of evolving slower contractile dynamics probably depends upon the value of the most energetically efficient $\%R_{NRR}$ and also upon the possibility that parameters, other than NRR, are being selected for which require an energetically more costly $\%R_{NRR}$.

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