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Isometric contractile properties of sexually dimorphic forelimb muscles in the marine toad *Bufo marinus* Linnaeus 1758: functional analysis and implications for amplexus

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Summary

It has been shown in the bullfrog, *Rana catesbeiana* Shaw 1802, that certain forelimb muscles in males have different contractile properties when compared with females, which may result from adaptation for amplexus. We extended this study to a distantly related species, *Bufo marinus* Linnaeus 1758, by testing the isometric contractile properties of three muscles, abductor indicus longus (AIL), and flexor carpi radialis (FCR) (both dimorphic muscles), and extensor carpi ulnaris (ECU) (non-dimorphic control). In males the dimorphic muscles had greater wet mass and cross-sectional area than in the females, and also produced significantly greater isometric force. As in bullfrogs, however, the maximum tetanic force per cm^2 of muscle cross-section did not differ between the sexes. In spite of this similarity in maximum force, the two dimorphic muscles were much less fatigable in the males than in the females. Lower fatigability in males correlated with exceptionally elongated relaxation times that maintained high levels of force between stimulus trains. This sustained force was negligible in the females, suggesting that this feature may allow males to maintain amplexus for prolonged periods. The same sustained force response was observed in the earlier study of *Rana catesbeiana*. Because this response is similar in *Bufo* and *Rana*, muscular properties correlated with amplexus may be shared across anurans by inheritance of this response from a common ancestor.

Key words: muscle, anura, amplexus, *Bufo marinus*, *Rana catesbeiana*.

Introduction

Studies of muscle structure and function have often used frogs as model organisms. They have very large hindlimb muscles that produce the forces for hopping and swimming (e.g. Gillis and Biewener, 2000; Lutz and Rome, 1994; Peters, 1994); however, their forelimbs have not received much attention. The forelimbs are thought to provide postural support and to act as shock absorbers during landing (Peters et al., 1996). A unique aspect of forelimb function in anurans provides an opportunity to look at functional diversity of muscles within a species. Male frogs use their forelimbs to grasp and control the females during the mating embrace, amplexus. As a result, many of their forelimb muscles are larger than those in the females. This sexual size dimorphism is specific to those muscles that control elbow flexion, wrist flexion and thumb extension (Kirby, 1983; Melichna et al., 1972; Oka et al., 1984; Peters and Aulner, 2000; Yekta and Blackburn, 1992).

In an earlier study (Peters and Aulner, 2000), isometric contractile properties (force, speed, fatigability) of several forelimb muscles were compared between male and female bullfrogs, *Rana catesbeiana* Shaw 1802. It was found that the greater mass of the dimorphic muscles in males directly reflected greater force capacity. It was also shown that the male muscles were not simply scaled up in size, but that there were differences in contractile properties between male and female muscles. At maximum stimulation, male muscles produced the same force per cross-section of muscle tissue as did the females; however, at reduced stimulus frequencies, closer to those experienced during natural contractions, some male muscles produced relatively greater force than did the female muscles, indicating that more fibers in male muscles are recruited at lower stimulus input. Dimorphic male muscles were also slower to contract and relax than those of females, but could maintain force for a much longer period without fatiguing. Thus, in bullfrogs the dimorphic male muscles can produce greater force than the female muscles, and can do so without fatiguing.

These experiments raise the question of how general these results are across frog species. Presumably, forelimb muscles in males of all anurans differ from females due to their functions in amplexus. But, are the properties observed in

bullfrogs (high forces at low stimulus rates, slow contraction and relaxation speeds, and high fatigue resistance) typical of all frogs, or do other species achieve amplexus through alternate muscle modifications? The answers to these questions may shed light on how muscle adaptation occurs, and also indicate whether amplexus may be a primitive feature of the entire Order Anura.

We addressed these questions by extending the previous study on bullfrogs (Peters and Aulner, 2000) to compare the isometric contractile properties of forelimb muscles between males and females of another species, *Bufo marinus* Linnaeus 1758. The families Bufonidae and Ranidae are distantly related among the Neobatrachian frogs (Ford and Cannatella, 1993), so if amplexus were present in a common ancestor and passed on to all descendant families one would expect similar muscle properties across the species. However, if amplexus evolved independently in multiple lineages of frogs the properties of forelimb muscles across the species would more likely differ. Thus, the major goal of this study was to observe whether common contractile properties of male muscles for amplexus occur across a diversity of anurans, suggesting that they are sympleisiomorphic features that evolved early in anuran history.

Materials and methods

Adult male and female *Bufo marinus* L. (males: mean body mass=120.8±8.7 g, *N*=18; females: mean body mass= 167.9±16.1 g, *N*=15; ± s.e.m.) were obtained from Glades Herp Supply (Fort Myers, FL, USA). They were housed in the University Vivarium and treated under an approved experimental protocol according to Public Health Service and USDA guidelines. Specimens used in the study were trapped between August and March, so were in non-breeding condition. We could not complete the experiments in a brief time during breeding season and did not wish to complicate the results by trying to maintain breeding hormone levels artificially. Furthermore, the bullfrogs tested in the previous study (Peters and Aulner, 2000) were also non-breeding adults.

Myology

The muscles under study were the abductor indicus longus (AIL), the flexor carpi radialis (FCR), and the extensor carpi ulnaris (ECU) (see Fig. 1 for descriptions). AIL and FCR are known to be sexually dimorphic by size and other characteristics among a variety of anurans (e.g. Mellichna et al., 1972; Oka et al., 1984); ECU is the non-dimorphic control (Peters and Aulner, 2000; Yekta and Blackburn, 1992). The FCR serves as the main ventromedial flexor of the wrist, drawing the wrist into the belly of the female during amplexus. It can also flex the elbow (Fig. 1). The AIL extends the wrist dorsomedially, assisting FCR in holding the female in place. It also abducts the first digit, which during the breeding season has enlarged nuptial pads on the dorsum that hold the female against the male. The ECU extends the wrist joint dorsolaterally (Duellman and Trueb, 1986), which is antagonistic to the movement needed during amplexus.

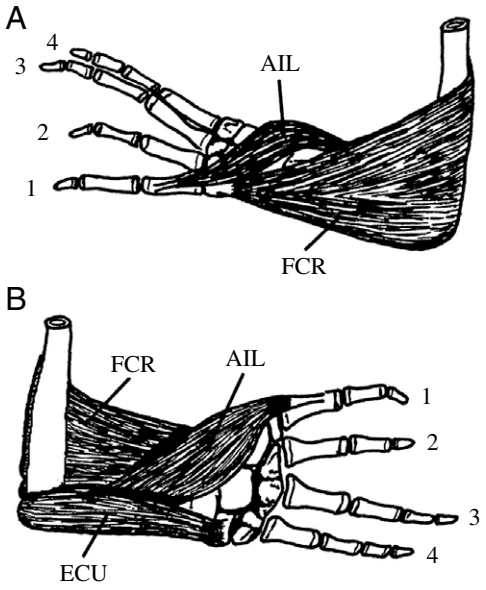

Fig. 1. Muscles of the right forelimb of a male *Bufo marinus* are seen from a medial perspective (A) with hand pronated, and from lateral view (B) with hand supinated. Flexor carpi radialis (FCR) originates along the medial surface of the distal half of the humerus and inserts on the radiale. It is a ventromedial flexor of the wrist, and can also flex the elbow. The abductor indicus longus (AIL) originates laterally from the distal humerus and the radioulnar border and inserts by a narrow tendon onto metacarpal I. It can extend the wrist and abduct the first digit. These are both used in amplexus and are sexually dimorphic by size (see text). The extensor carpi ulnaris (ECU) is a non-dimorphic muscle that originates superficial to AIL on the lateral humeral condyle and inserts on the ulnare. It laterally extends the wrist and is not used in amplexus. (Modified from Peters and Aulner, 2000.)

Contractile properties

In order to measure contractile properties at physiologically relevant lengths, we determined the mean joint angle changes at wrist (anterior angle between forearm and carpometacarpus) and elbow (anterior angle between humerus and forearm) for three limb positions: during quiet standing while forelimbs keep the body erect (L_s) as a reference length, at maximum joint extension (L_e) when the forelimbs leave the ground, and at maximum joint flexion (L_f) at the bottom of a landing. Manipulation of a passive male and female indicated that, as in bullfrogs (Peters and Aulner, 2000), the limb position used in amplexus falls within this range.

Videos (30 frames s^{-1}; Sony C-12 camcorder) of ten sequences from each of 4 males and 4 females were analyzed. Animals were hopped inside a 2 m runway that was 15 cm wide, with a wooden back and Plexiglas™ front that were 30 cm tall. The bottom of the runway was covered with indoor/outdoor carpeting to facilitate normal hopping. Since the arm bones are not held in the same vertical plane, in order to estimate the actual angles between the bones from a two-dimensional image, both lateral and dorsal views were obtained by filming through the Plexiglas™ front of the runway with a

mirror mounted at a 45° angle above the animals. Joint angles were then estimated using the formula:

$$\cos\theta = 1 / [\sqrt{(1+\tan^2\alpha+\tan^2\beta)}] ,$$

where θ is the angle between the bones, α is the angle visualized laterally, and β is the angle visualized from above. For angles where α and β were greater than 90°, the resultant angle was subtracted from 180° to obtain the real angle, θ (Peters et al., 1996).

No significant differences were found in the joint angles used by males and females, so we calculated the combined mean for wrist and elbow joint angles at each position. At standing length (L_s) the mean elbow and wrist angles were 90.6±1.4°, and 121.6±2.6°, respectively. As the arms come off the ground, the elbow and wrist both extend by about 20% to their maximum positions (mean elbow angle at L_e=110.8±3.5°; mean wrist angle at L_e=149.7±3.1°). When the animal lands, the elbow and wrist both flex back to angles that did not differ significantly from those at L_s (mean elbow angle at L_f=89.5±2.4°; mean wrist angle at L_f=126.0±4.3°. These data were then used to estimate the range of muscle lengths during normal movements.

Measurement of contractile properties was done according to established procedures (Peters, 1994; Peters and Aulner, 2000). Frogs were humanely sacrificed by placing them under anesthesia (tricaine methane sulfonate; 200 mg kg^{-1} body mass; subcutaneous injection) and then pithing. This renders the animal brain-dead while leaving the peripheral nerves and muscles intact and responsive for many hours. Body mass was then measured to allow a comparison of forces, taking into account body size differences. To minimize the duration of surgery and experimentation, the contractile properties of only one muscle were measured in each individual. Surgery was performed to expose the experimental muscle and its nerve, leaving them *in situ* within the forelimb. Care was taken to maintain blood supply to the muscle and the forearm was kept moist by wrapping it in gauze and frequently irrigating it with amphibian Ringer's solution. All tests were run at room temperature (22±2°C).

Approximation of the natural range of muscle lengths was estimated by placing a marker tie of suture silk in a stable region of fascia near the muscle origin in line with the muscle length and another on the insertion tendon at the muscle-tendon boundary. The wrist and elbow were placed in turn at the mean angles found at L_s, L_f and L_e and the distances between the two ties were measured to 0.1 mm accuracy using dial calipers. After the experimental procedure, the corresponding muscle on the non-experimental arm was harvested and weighed. Mean muscle cross-sectional area was estimated as muscle mass divided by L_s (Peters and Aulner, 2000).

The animal was mounted in a heavy metal frame by clamping the pelvis and the elbow to immobilize the body and forearm during stimulation. Muscles were cut free at their insertions and attached to an isometric strain gauge (Grass FT03, Astro-Med, Inc., West Warwick, RI, USA; for T_w, resonant frequency=170 Hz, displacement rate=5 mm kg^{-1}; for T_T, resonant frequency=330, displacement rate=1 mm kg^{-1}) using #1 braided surgical silk. This silk was sutured as close to the muscle–tendon boundary as possible and all ties were kept to a length of approximately 2 cm. They were stimulated directly (Grass S88 square wave stimulator) through their nerves using bipolar stainless-steel electrodes. Maximal twitch (single impulse; 0.1 ms duration) and tetanic (670 ms train of impulses; 0.1 ms duration at 80 pulses s^{-1}) contractions were elicited by supramaximal stimuli. These were estimated by finding threshold and stimulating at increasing voltages, until no further increase in force was observed. This level was typically at 2–2.5 times threshold voltage (~5–8 V).

Isometric twitch and tetanic forces were recorded within the natural range of lengths as estimated above. The strain gauge was mounted on a rack and pinion so that lengths could be changed in 1 mm intervals over this length range. Thus, the physiological length at which maximum force was produced could be determined. This length was used for all subsequent tests.

We measured twitch contraction and half-relaxation times, and tetanic half-relaxation time. The twitch contraction time (T_C) was determined (in ms) from the EMG stimulus artefact to the peak of twitch force; the twitch half-relaxation time ($T_{w1/2R}$) was from the peak of twitch force to the point where it fell to half of that peak. The tetanic half-relaxation ($T_{T1/2R}$) times were measured from the offset of electrical stimulation to the point where the tetanic force fell to half of its maximum value (Chadwell et al., 2002; Marsh, 1994).

The ratio of twitch/tetanic force was determined as a first approximation of the amount of force generated by low-level stimulation. Further tests were run that varied either the stimulus rates or durations to test the force generation using different stimuli (Chadwell et al., 2002; Peters and Aulner, 2000). In the force/frequency test, muscles were stimulated for 670 ms at the frequencies of 5, 10, 15, 20, 25, 30, 35, 40, 60 and 80 pulses s^{-1}. In the force/duration test, muscles were stimulated at 30 pulses s^{-1} for durations of 50, 100, 150, 200, 250, 300 and 670 ms. In both of these tests, muscle were rested for 2 min between each stimulus train to minimize effects of fatigue.

A fatigue test was run using intermittent tetanic stimuli (one train of 200 ms duration at 30 pulses s^{-1} once every 2 s) over a period of 4 min. A graphical representation of the time course of fatigue was done by measuring peak force from the baseline for every fifth peak (every 10 s) and averaging in 30-s intervals. These values were then plotted as a percentage of mean maximal force (which always occurred during the first 30-s interval). A fatigue index (FI) was obtained by summing the peak forces for the first 120 s of the fatigue test, dividing by the sum of peak forces over the entire 4 min, and multiplying by 100. Thus, an FI of 50 indicates no fatigue, and increasing fatigue would produce higher FI values.

In the previous study of bullfrogs (Peters and Aulner, 2000), it was noted that during the fatigue test the relaxation time of the dimorphic muscles in males became so elongated that the force did not return to baseline during the 2 s between stimulus trains. This resulted in a percentage of peak force in each train that was

due to force maintained between stimulus trains. This phenomenon, called sustained force, was also found in our results. Sustained force was measured from baseline to the point of greatest relaxation, the trough immediately before the next stimulus train. It was measured every fifth train (every 10 s) and averaged over 30 s intervals throughout the 4 min test. The mean sustained force was then plotted as a percentage of the mean peak force during the same 30 s interval.

Data analysis

The results for each muscle were analyzed and compared between males and females using one-way ANOVA for the non-size-dependent variables (contraction and relaxation times, fatigue indices) and the proportional data (twitch/tetanus ratio; T_T/muscle mass; T_T/muscle cross-section). The proportional data were tested for normality before proceeding with the ANOVA. One-way ANCOVA designs were done using body mass as the covariate for the size-dependent variables (forces, muscle mass, cross-sectional area). The sequential Bonferroni adjustment was used for the multiple comparisons to maintain an experiment-wise error rate of 0.05.

Results

Force properties

The mean values ± s.e.m. for the contractile properties of the three muscles are shown in Table 1. Both muscle mass and cross-sectional area were significantly larger in males for FCR and AIL. For FCR, male muscles averaged about 1.6 times the mass of females and about 2 times the cross-sectional area. For AIL, male muscles averaged nearly 5.6 times the mass and 7 times the cross-sectional area. In ECU, mean masses and cross-sectional areas did not differ between the sexes.

Twitch forces were highly variable and means did not differ between sexes in any of the muscles tested. In the two size-dimorphic muscles, FCR and AIL, males produced significantly larger mean tetanic forces than did females; however, tetanus was not significantly different between sexes for ECU. The male FCR produced approximately 1.5 times the force of the female FCR, and the AIL in males produced about 6.5 times more force than in the females. The mean tetanic force per cross-sectional area and per muscle mass did not differ between the sexes for any of the muscles, therefore tetanic force scaled directly with the greater size of the male muscles.

The twitch/tetanus ratio (T_w/T_T) for each muscle was calculated by dividing the maximum twitch force by the maximum tetanic force. Because of the larger tetanic forces in the males, the mean T_w/T_T tended to be smaller in males but because of the high variability in twitch values, the difference did not reach significance.

Contraction and relaxation times

The twitch contraction times (T_C) and half-relaxation times ($T_{w1/2R}$) for each muscle are shown in Table 1. Only in the non-

Table 1. *Comparison of muscle sizes and contractile properties*

	Muscle					
	AIL		FCR		ECU	
	Male (7)	Female (5)	Male (7)	Female (6)	Male (4)	Female (4)
Twitch						
(g)	15.3±4.7	3.4±0.8	18.9±3.6	19.3±2.8	12.3±2.9	7.1±2.4
(N)	0.15	0.03	0.18	0.19	0.12	0.07
Tetanus						
(g)†	114.6±14.2 *	17.1±2.9	162.9±8.1 *	110.8±11.1	121.5±17.7	98.6±15.2
(N)	1.12	0.17	1.60	1.08	1.19	0.84
(% body mass)†	93.0±11.6 *	11.2±1.8	135.4±9.6 *	53.2±3.8	111.7±10.2	94.6±25.9
Twitch/tetanus ratio	0.13±0.03	0.19±0.03	0.12±0.02	0.18±0.02	0.11±0.02	0.07±0.02
Muscle mass (g)	0.11±0.02 *	0.02±0.002	0.18±0.02 *	0.11±0.01	0.13±0.03	0.11±0.02
Muscle cross-section (cm^2)	0.04±0.01 *	0.006±0.001	0.09±0.01 *	0.05±0.003	0.05±0.01	0.05±0.01
T_T/Muscle cross-section (kg cm^{-2})	2.9±0.25	3.1±0.48	1.8±0.09	2.3±0.2	2.5±0.23	2.2±0.54
T_T/muscle mass (g force g^{-1} muscle)	1161.7±102.1	878.8±108.3	902.4±42.6	995.5±86.1	1011.0±94.4	1020.1±268.5
T_C (ms)	55.9±3.4	54.4±4.2	55.1±4.6	49.5±2.7	54.3±4.0 *	40.0±3.2
$T_{W1/2R}$ (ms)	100.0±18.1	67.8±4.3	70.7±5.1	57.3±6.5	55.5±9.3	48.8±6.0
$T_{T1/2R}$ (ms)	205.8±12.4 *	141.6±7.1	261.0±18.0 *	144.4±8.1	130.0±5.8	121.8±8.0
FI	59.1±1.1 *	68.5±2.7	57.4±0.8 *	61.3±1.3	56.8±1.7	55.6±1.9

T_T, tetanic force; T_C, twitch contraction time; $T_{W1/2R}$, twitch half-relaxation time; $T_{T1/2R}$, tetanic half-relaxation time; FI, fatigue index.

Values are means ± s.e.m.; *N* values are given in parentheses for each sex.

*Significant differences between males and females for a given muscle. To maintain and experiment-wise error rate of 0.05, alpha levels were adjusted for multiple comparisons using the sequential Bonferroni adjustment; k=11.

†Statistical comparisons of force were done using an ANCOVA with body mass as the covariate. Because body mass in females was significantly larger than in males, tetanic force is also shown in percent of body mass to give an easy visual comparison of the magnitude of the differences between the sexes.

dimorphic ECU did differences in the T_C values reach significance, with female contraction times being shorter than in males. Differences in the $T_{w1/2R}$ did not reach significance in any muscle tested, although relaxation times, especially in FCR and AIL, tended to be longer in males.

The tetanic half-relaxation times ($T_{T1/2R}$) were much slower in the male dimorphic muscles than in the females (Table 1). The male AIL was nearly 1.5 times slower than the female AIL (205.8 *vs* 141.6 ms), and the male FCR was 1.8 times slower than the female FCR (261.0 *vs* 144.4 ms). The non-dimorphic ECU, however, did not differ between the sexes in $T_{T1/2R}$ (130.0 in males *vs* 121.8 ms in females).

Force/frequency and force/duration

Fig. 2 plots the force/frequency data for the three muscles over a frequency range of 5–80 pulses s^{-1}. None of the muscles tested showed significant differences between the sexes. However, the two dimorphic muscles developed a higher percentage of their maximum force at lower frequencies than did the non-dimorphic muscle in both sexes. Also, the slopes of the force/frequency curves for AIL and FCR were significantly steeper between 10–30 pulses s^{-1} than in ECU for the combined sexes. Fig. 3 plots the force/duration data for the three muscles over a stimulus duration range of 50–670 ms. The female FCR produced significantly higher force than did males within the range of 50–300 ms. Neither of the other muscles differed significantly in percent force produced at varying frequencies.

Fatigue and sustained force

The fatigue indices for each muscle are shown in Table 1. In FCR and AIL the male muscles had significantly lower fatigue indices than did females, indicating that the males were less fatigable. The fatigue indices for ECU did not differ between the sexes. Fig. 4 shows the time course of the 4 min fatigue test in which force is plotted as a percentage of maximal force produced within the test. In FCR and AIL, the male muscles showed significantly less decline in force than did the females.

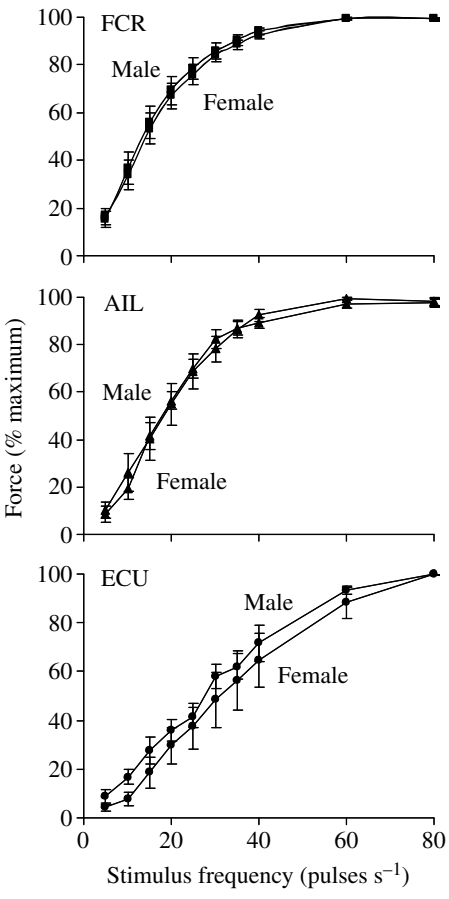

Fig. 2. Force/frequency curves compare the amount of force (as a percentage of maximal force) produced at varying stimulus rates for male and female muscles (FCR, AIL, ECU) over a train duration of 670 ms. Stimulus frequencies ranged from 5 to 80 pulses s^{-1}. No significant differences were found between sexes for a given muscle. Values are means ± s.e.m. Sample sizes for these and subsequent figures comparing male and female muscles are shown in Table 1.

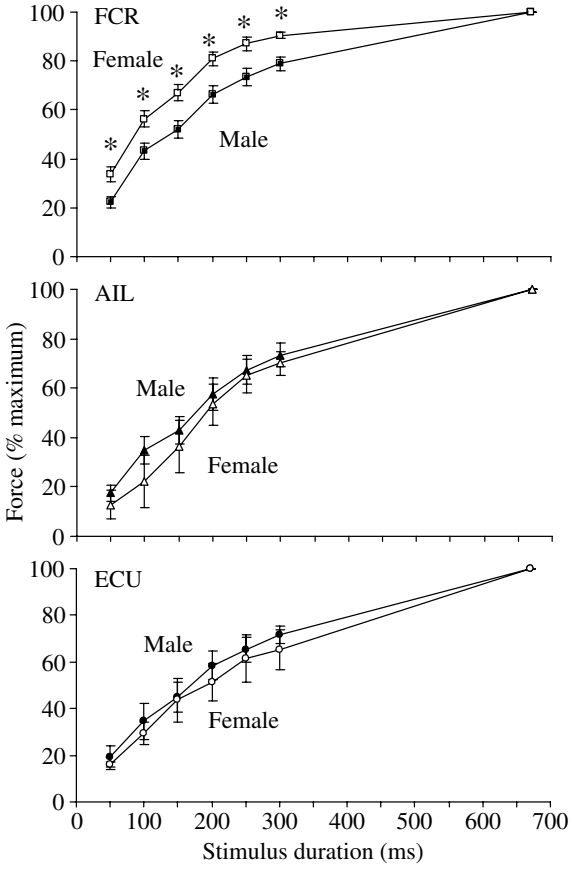

Fig. 3. Force/duration curves compare the amount of force (as a percentage of maximal force) produced by male and female muscles (FCR, AIL, ECU) at different stimulus durations when the stimulus rate was held constant at 30 pulses s^{-1}. Stimulus durations ranged from 50 to 670 ms. The only significant difference (asterisks) occurred in the FCR, in which the female FCR produced more force than the male in the range of 50 to 300 ms (P<0.05, adjusted with sequential Bonferroni; k=7). Values are means ± s.e.m.

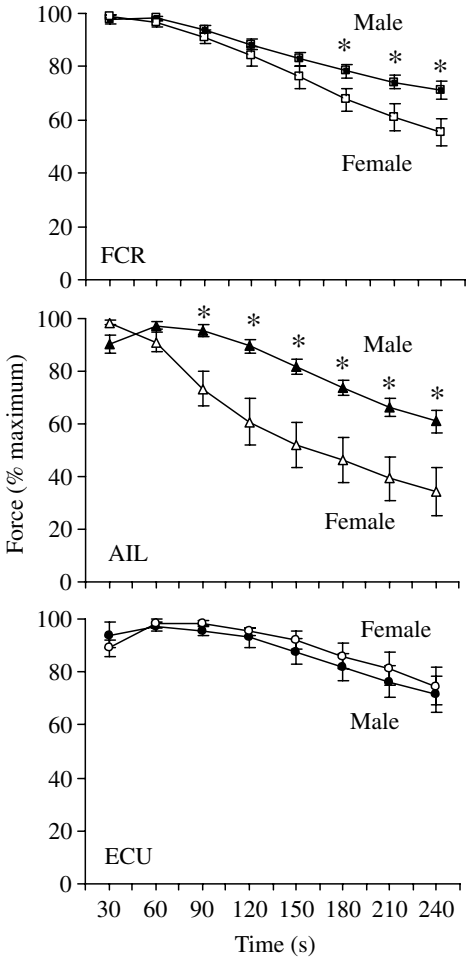

Fig. 4. The time course of fatigue is shown for the three muscles (FCR, AIL, ECU) over 4 min. Force was averaged in 30 s intervals and expressed as a percentage of the maximal mean force. In both FCR and AIL, forces declined more significantly (indicated by asterisks) in females than in males (P<0.05, adjusted with sequential Bonferroni; k=8). Values are means ± s.e.m.

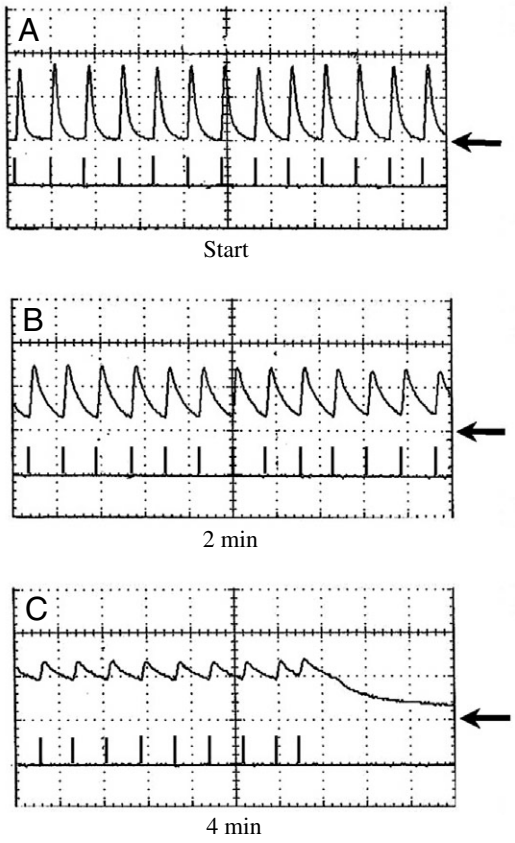

Fig. 5. A representative series of force traces during the 4 min fatigue test is shown for a male FCR. (A) The first 24 s of the intermittent, sub-maximal tetani (1 train/2 s; 200 ms duration, 30 pulses s^{-1}; sweep speed=2.5 s cm^{-1}). Note that the tetanic traces fall back to baseline (arrows) during the 2 s intervals between tetanic trains. By 2 mins into the test (B), the rate of relaxation of the muscle has slowed so that the trace returns only part way to baseline, resulting in sustained force between stimulus trains. By the last 20 s of the fatigue test (C), sustained force comprises approximately 70% of the total force at maximum stimulation. Note that the trace had not returned to baseline within this panel even after more than 8 s following the last stimulus train. In most cases it took from 10–15 s for the force to relax completely to baseline. The tick marks on the lower trace indicate the points of stimulation once every 2 s.

In FCR, males retained an average of 71% of the original force at the end of 4 min, while the females fell to 55% of maximum force. In AIL, males fell to an average of 61% of maximum, but females declined to 34% of maximum. The male and female fatigue curves for ECU were almost identical (Fig. 4).

As noted earlier, during the 4 min fatigue test, there is a progressive prolongation of relaxation time during intermittent stimulation with sub-maximal tetanic trains. Relaxation becomes so delayed that the muscle fails to relax back to baseline even during the 2 s interval between stimulus trains. This delayed relaxation thus results in an increment of force that is sustained between stimulus trains, the sustained force first described in bullfrogs (Peters and Aulner, 2000). Fig. 5 shows the original traces from one of the male *Bufo* during the fatigue test of an FCR. Note that during the first series of tetanic trains the force returns to baseline during the 2 s interval between trains (Fig. 5A). By 2 min into the fatigue test, slowing of relaxation has resulted in an elevation of the trace significantly above baseline (Fig. 5B). By 4 min, the sustained force (measured from baseline to the lowest point of the trough, just before the next stimulus train activates the muscle) comprises an even greater percentage of the total force at peak activation (in this example, approximately 70% of the total force) (Fig. 5C). This sustained force is a major factor that keeps the total force elevated in the males as compared with females, in which the sustained force never exceeded 2% of total force.

Fig. 6 shows a 30 s incremental time course of the mean sustained force plotted as a percentage of mean total force produced in the same time interval. In FCR, the sustained force was significantly higher in males throughout the time course,

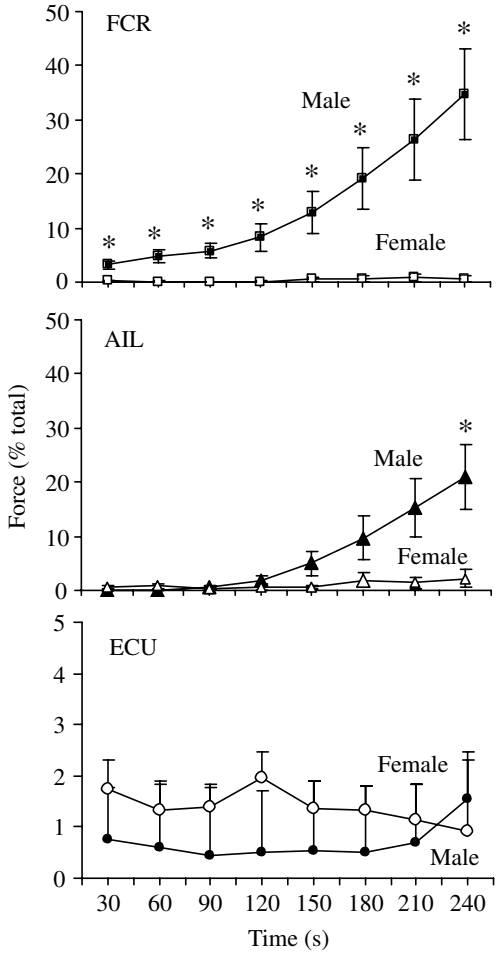

Fig. 6. Sustained force is compared between males and females in the three muscles (FCR, AIL, ECU). Sustained force was averaged over 30 s intervals and expressed as a percentage of the average peak force for the same time interval. In both FCR and AIL, males produced significantly more sustained force than did females; however, in AIL significance was reached only at the 240 s time interval. Male and female ECU both produced equally small amounts of sustained force (note the different force scale). Asterisks indicate significant differences (P<0.05, adjusted with sequential Bonferroni; k=8). Values are means ± s.e.m.

reaching an average maximum at 4 min of about 35% of the total force as compared with only 0.5% in the females. In AIL, the males also produced more sustained force than did females, reaching an average maximum of 21% of total *vs* 2% in females; however, significance was only reached in the 240 s time interval due to large variation in the male response. In ECU, male and female sustained forces did not differ and were barely above baseline, never more than 2% of total force.

Discussion

It has been shown in *Rana catesbeiana* that male forelimb muscles have different properties than homologous female muscles, which correlate with their use in amplexus (Peters and Aulner, 2000). However, it remains to be shown whether properties found in bullfrogs represent general characteristics of the entire order.

Behavioral studies of bullfrogs show that they typically use axial amplexus and maintain it for about 2 h before the female oviposits (Duellman, 1992; Wells, 1977). Data for *Bufo marinus* are more anecdotal, but suggest a comparable axial amplexus lasting for about 2–3 h (Duellman and Trueb, 1986). Phylogenetic studies show that ranids and bufonids are distantly related among anurans (Ford and Cannatella, 1993), so the behavior and the physiological adaptations of forelimb muscles to achieve amplexus are either inherited from a common ancestor (homologous) or, if developed independently, must be convergent.

Muscle force

Total muscle force is a highly plastic feature, so the fact that males in both bullfrogs and marine toads have larger muscles used in amplexus is not strong evidence for a common origin. In several studies, the sexually dimorphic laryngeal muscles of frogs have been shown to have different muscle fiber types in males than in females (Sassoon et al., 1987), and there is an androgen-specific myosin, which differentiates them (Catz et al., 1995). The properties of the myoneural junction and motor neurons to the laryngeal muscles have also been found to differ between males and females (Yamaguchi et al., 2003). These differences appear to develop under the influence of hormonal regulation (Kelley, 1997; Kelley, 2004). A few studies have shown that the size dimorphism in forelimb muscles is also affected by testosterone (Dorlochter et al., 1994; Muller et al., 1969; Regnier and Herrera, 1993a; Regnier and Herrera, 1993b). But less is known about any differences in neuronal or muscle fiber cellular/molecular mechanisms in the limb muscles. While it is likely that the dimorphic muscles of the males have greater numbers of testosterone receptors than in the non-dimorphic muscles, exactly how this structural and functional differentiation occurs is not well studied. It is possible that both the laryngeal muscles and the forelimb muscles used in amplexus form a suite of characters that evolved early in frogs as their reproductive behaviors became established. If this occurs in response to increasing sensitivity to testosterone *via* the same mechanism across muscles used in amplexus (and even the laryngeal muscles), this would provide better evidence for a common adaptation for selective enlargement of sexually dimorphic muscles. Elucidation of the mechanism of selective muscle enlargement in the males is an important goal for future studies.

Contraction and relaxation times

Because our study and the earlier bullfrog study (Peters and Aulner, 2000) examined the contractile properties of whole muscles and not the individual fibers, some aspects of muscle function cannot be easily compared between muscles or species. For instance, the twitch contraction and half-relaxation times in the dimorphic muscles of the bullfrog were significantly longer on average in males (Peters and Aulner,

2000). In *Bufo* we found no significant difference in mean twitch contraction times between the sexes in the dimorphic muscles. It was only in the non-size-dimorphic ECU that the means differed significantly (Table 1). There were no significant differences in the twitch half-relaxation times between the sexes, though the times tended to be longer in the male dimorphic muscles (Table 1), in line with the bullfrog results. Much of this difference may be due to differences in the intrinsic properties of the muscle fibers and their nerves, but longer twitch contraction and half-relaxation times might also reflect architectural features, e.g. number of sarcomeres in series, amount and orientation of connective tissues, etc. The larger male muscles themselves may contribute to slower twitch contraction and relaxation times if their greater mass simply slows the functional response to twitch activation/deactivation. It remains for more detailed study to determine contraction and relaxation times among the individual fibers and correlate these with possible differences in the muscle fiber types present or in architecture.

The differences between sexes for tetanic half-relaxation times are less likely to reflect muscle mass or architectural differences. With a maximal tetanus, all of the muscle fibers were simultaneously and maximally activated for 670 ms. Thus, any elastic resistance during activation should have been overcome. As a result, the longer $T_{T1/2R}$ in males likely reflects differences in deactivation at the cellular/molecular level (Marsh, 1994). This may correlate with the elongation of relaxation which males display in the sustained force response (see below). Though Peters and Aulner did not report these data (Peters and Aulner, 2000), if slower $T_{T1/2R}$ is correlated with elongated relaxation, it is likely that dimorphic muscles of male bullfrogs would also have longer $T_{T1/2R}$ times than in females.

Force frequency and force duration

Peters and Aulner found that in bullfrogs the male AIL produced significantly more force at lower frequencies than did the females (Peters and Aulner, 2000), suggesting that with *in vivo* stimulation the males can produce relatively greater forces than found at the experimental maximum, exaggerating the male/female differences. This difference was also assumed to mean that the physiology of the male muscles was different from the females'. Our results for *Bufo* found no comparable differences in the amount of force generated between the male and female muscles with rate or duration of stimulus. The only significant difference with low level stimulation was a greater force produced by the female FCR relative to that of the male at shorter duration stimulus trains (Fig. 3).

Muscle fiber types

Our results to this point suggest that male and female *Bufo* do not differ as much or in the same ways as did the male and female bullfrogs. Differences in twitch contraction and half-relaxation times, and activation, could be due to the presence of different muscle fiber types and their variable structural and functional properties (Lutz et al., 1998; Rowlerson and Spurway, 1988). However, in a follow-up study, Peters found no difference in the relative proportions of identified muscle fiber types between male and female bullfrogs (Peters, 2001), but there was higher oxidative capacity in the males (as evidenced by higher citrate synthase activity). This suggests a wide range of functional variation within recognized fiber types that may indicate high plasticity of muscle fiber characteristics. Future studies will have to compare, in a more comprehensive way, the muscle fiber types and their structural and functional variation in these muscles.

Fatigue and sustained force

The clearest similarity that we found between our *Bufo* results and the previous study of bullfrogs (Peters and Aulner, 2000), was in the fatigue and sustained force results. Both dimorphic muscles were less fatigable in the males, as shown by their fatigue indices (Table 1) and by the time courses of fatigue over the 4 min test (Fig. 4). Both FCR and AIL in the males of *Bufo* and *Rana* retained an average of ~20–30% more of their initial force than did the females at the end of 4 min.

Peters and Aulner found that in bullfrogs the male muscles retained a high percentage of their initial force between stimulus trains due to an extreme elongation of the relaxation time of the male muscles (Peters and Aulner, 2000). This gross elongation of relaxation developed during the first 1–2 min of the fatigue test in males, and maintained a maximum during the final 2 min. Results for our *Bufo* dimorphic muscles show the same pattern (Fig. 5).

Since our experiments were done on whole muscles with intact circulatory and nerve components, the present results cannot distinguish among several possible mechanisms, which could cause the sustained force response. Higher blood flow in the male muscles could explain their lower fatigability, but the prolonged relaxation is more likely to be due to differences in neural and/or intramuscular factors. Males may have slower turnover of acetylcholine at the myoneural junctions, which could result in a prolonged force response. The prolonged relaxation time could also be caused by a delay in cross-bridge cycling time (Edwards et al., 1975; Westerblad and Lannergren, 1991) or by increased levels of cytosolic Ca^{2+} (Allen et al., 1989; Westerblad and Lannergren, 1990). High cytosolic Ca^{2+} levels would result in an extended time period in which cross-bridge formation could occur, leading to prolonged periods of force production without nerve input, perhaps minimizing the energy required from nerve activity. If the mechanism of sustained force causes the cross-bridges to disengage more slowly than in normal cycling, force might also be maintained with less energy expenditure at the muscle fiber level. Thus, sustained force may result in less energy expended during prolonged contractions, both by the nervous system and at the muscle fiber level.

Males use their forelimbs in similar ways in both amplexus and in the male–male grappling behaviors that typically accompany competition for the females (Howard, 1978; Howard, 1984; Wells, 1977). Both behaviors would select for strength and endurance. We assume that increased strength is

a plastic feature of muscle and can be easily explained by convergence. However, the phenomenon of sustained force appears to be unique to these dimorphic muscles and, as far as we know, unique to anurans. The fact that this feature is present only in males and is found only in those muscles used during amplexus suggests that it is adaptive for amplexus. Because the sustained force response is similar in *Bufo* and *Rana*, these results further suggest that adaptation for amplexus is shared across anurans by inheritance from a common ancestor. The present data do not address the mechanism of sustained force, and so cannot eliminate the possibility of convergence. Much work remains to be done to describe the mechanism of sustained force, but if amplexus is a sympleisiomorphic feature of anurans, the molecular and structural basis for sustained force should be the same across species.

List of symbols

AIL	abductor indicus longus
FCR	flexor carpi radialis
ECU	extensor carpi ulnaris
L_s	standing length
L_e	length at maximum joint extension
L_f	length at maximum joint flexion
FI	fatigue index
T_C	twitch contraction time
$T_{w1/2R}$	twitch half-relaxation time
$T_{T1/2R}$	tetanic half-relaxation
T_T	tetanic force
T_w	twitch force

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