The Journal of Experimental Biology 203, 3639–3654 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB3024

SEXUAL DIMORPHISM IN FORELIMB MUSCLES OF THE BULLFROG, *RANA CATESBEIANA*: A FUNCTIONAL ANALYSIS OF ISOMETRIC CONTRACTILE PROPERTIES

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Accepted 8 September; published on WWW 2 November 2000

Summary

In many species of frog, the forelimb muscles important in amplexus are known to be much larger in males than in females. We studied this dimorphism in three forelimb muscles in the bullfrog [abductor indicus longus (AIL), flexor carpi radialis (FCR) and extensor carpi radialis (ECR)] by testing their isometric contractile properties. One muscle that is not dimorphic, the extensor carpi ulnaris (ECU), was also studied as a control. In addition to being greater in wet mass and in cross-sectional area in the males, our data show that the dimorphic muscles also produce significantly larger isometric forces in males than in females. The tetanic force per cm^2 of muscle cross-sectional area did not differ between the sexes, so that force within a muscle varies directly with muscle size. However, a number of the contractile variables we measured show that male muscles differ functionally from those of females. The male twitch contraction times were significantly longer in the AIL, and the male half-relaxation times were longer in both the AIL and FCR. These two dimorphic muscles were also significantly less fatiguable in males than were the corresponding female muscles. Their higher endurance resulted from the maintenance of high levels of unrelaxed force sustained between trains of stimuli during the fatigue test. This sustained force is much less pronounced in the female muscles, suggesting that high levels of sustained force may be a key functional feature that enables males to maintain amplexus economically for prolonged periods.

Key words: sexual dimorphism, mating, behaviour, amplexus, muscle, contractile properties, bullfrog, *Rana catesbeiana.*

Introduction

Sexual dimorphism provides an opportunity to study structural and functional diversity within a single species. In many anuran amphibians, individual forelimb muscles may be more than 10 times greater in wet mass in males than in females, even though the females are larger in body mass (Howell, 1935; Yekta and Blackburn, 1992). This size dimorphism has been described in a variety of archeobatrachian (*Xenopus laevis*, Dorlochter et al., 1994) and neobatrachian (*Bufo japonicus*, Oka et al., 1984; *Rana temporaria*, Muller et al., 1969; Melichna et al., 1972; *Rana pipiens*, Kirby, 1983; Yekta and Blackburn, 1992) species and is thought to be associated with amplexus (Gaupp, 1896; Howell, 1935; Smith, 1938; Duellman, 1992). In amplexus, males use their forelimbs to clasp females, and they may hold onto a female for hours, days or even weeks until oviposition occurs (Duellman, 1992; Wells, 1977). Larger forelimb muscles in males are assumed to produce enhanced force for clasping the females. In some species, the forelimbs are used to repel other males, either when single males grapple with each other or when a single male tries to remove a male in amplexus from a female.

Studies of forelimb muscle dimorphism in frogs have typically compared male and female muscles either by differences in mass (Howell, 1935; Kirby, 1983; Yekta and Blackburn, 1992) or by differences in muscle fiber types and their sizes (Muller et al., 1969; Melichna et al., 1972; Rubinstein et al., 1983; Oka et al., 1984; Dorlochter et al., 1994). Dimorphic muscles in males are much larger, possessing greater numbers of relatively large muscle fibers than in females. Data on muscle fiber types are variable, with some studies finding that males may have a greater proportion of muscle composed of tonic fibers (Melichna et al., 1972), but others finding no difference in the number of tonic fibers as a proportion of total muscle fibers (Thibert and Nicolet, 1975; Oka et al., 1984). On the basis of current data, there is no taxonomic pattern evident in this variability among fiber types; for example, Melichna et al. (1972) and Thibert and Nicolet (1975) found variability in the proportion of tonic fibers present between males and females of two species of *Rana*.

Endocrine studies have correlated seasonal changes in muscle fiber properties with changing androgen levels (Melichna et al., 1972) and tested whether muscle size and

fiber properties are affected by experimental manipulation of testosterone levels (Muller et al., 1969; Regnier and Herrera, 1993a,b; Dorlochter et al., 1994). These studies have confirmed that male and female forelimb muscles are dimorphic in non-breeding animals as a result of the enhancing effects of testosterone and that dimorphism is exaggerated in the breeding season through androgen-mediated hypertrophy of muscle cell size. In addition to direct effects on muscle, androgens may also exert neurotrophic effects (Pette and Vrbova, 1985; Herrera and Regnier, 1991; Nagaya and Herrera, 1992).

Some data on the contractile properties of forelimb muscles are available (Melichna et al., 1972; Regnier and Herrera, 1993a), but these studies lack detailed functional comparisons of forces and other contractile properties on a diversity of these muscles in naturally occurring limb positions. This study on *Rana catesbeiana* is designed (i) to compare the isometric contractile properties (twitch and tetanic forces, force/frequency relationships, contraction kinetics and fatiguability) of selected forearm muscles between males and females to examine functional aspects of dimorphism, (ii) to construct length/tension curves for each muscle in both sexes to compare force production at lengths that encompass natural behavioral positions, including amplexus, and (iii) to determine whether size comparisons (mass and/or muscle cross-sectional area) are adequate indicators of the real force differences between male and female muscles. The latter goal is important because of the historical use of muscle mass as an indicator of relative force differences and because, with rare or endangered species, for which experimental data cannot be obtained, muscle mass may be the only measurement possible.

Bullfrogs were chosen for study because natural history observations have revealed that the forelimbs are used in a diversity of breeding behaviors, including amplexus, but also in male–male territorial aggression (e.g. Howard, 1978).

Materials and methods

North American bullfrogs (*Rana catesbeiana*, Shaw, 1802) were purchased from Charles D. Sullivan, Co., Inc. (Nashville, TN, USA; 14 males, 12 females) and from Carolina Biological Supply Co. (Burlington, NC, USA; four males, four females). The animals used in this study were collected between September and March, so that data reported here are for non-breeding frogs. Because muscle properties may respond to hormonal differences during breeding (Melichna et al., 1972; Dorlochter et al., 1994), and we could not control for this during the period of our study, we chose to eliminate breeding effects and concentrate on non-breeding animals. We assume that dimorphism would be even more pronounced during the breeding season. Increased testosterone levels at that time would probably enhance the quantitative differences we found, for example in maximum forces produced. However, we are unable to assess the effects of breeding season changes on muscle fiber physiology, so that differences in contraction speeds, fatiguability and responses to a variety of stimuli are unknown. Future studies on the full range of the effects of natural hormones on these contractile properties are necessary before our present data can be fully evaluated.

The animals were kept in a 150 l terrarium under controlled temperature and light conditions (12 h:12 h light:dark; 22±2 °C). They were fed a diet of crickets and exercised regularly throughout the study by placing them in a runway (2.5 m long, 25 cm wide and 50 cm high) with a plywood back and a Plexiglas bottom and front. The bottom was covered with indoor/outdoor carpet to facilitate traction. Frogs were urged to hop for several sequences (usually 4–6 times) until they refused. The procedure was repeated at least three times per week while they were in captivity.

Myology

The forelimb muscles assumed to be involved in amplexus, and which have been found to be dimorphic in size, are those that adduct the forearm, flex the elbow, medially dorsoflex and ventroflex the wrist and abduct the first digit (Yekta and Blackburn, 1992). Four forearm muscles were chosen for this study, the abductor indicus longus (AIL), the flexor carpi radialis (FCR), the extensor carpi radialis (ECR) and the extensor carpi ulnaris (ECU) (Fig. 1). The AIL, FCR and ECR were selected for the high degree of sexual dimorphism in wet mass and protein content found previously in *Rana pipiens* (Yekta and Blackburn, 1992) and *Bufo japonicus* (Oka et al., 1984). The ECU was chosen for the study as a control muscle because it was found to be non-dimorphic (on the basis of mass) in *Rana pipiens* (Yekta and Blackburn, 1992).

The AIL shares a common origin with the extensor digitorum communis longus on the lateral surface of the radioulna. From its lateral origin, it crosses the dorsum of the wrist and inserts by a well-defined tendon on metacarpal I (Fig. 1). The AIL can, therefore, abduct the first digit and/or extend (dorsoflex) the wrist. The FCR is a superficial forearm muscle that originates along the inner border of the distal humerus, above and covering the medial epicondyle. It parallels the radioulna medially and inserts by way of a broad aponeurosis on the ventromedial carpus (Fig. 1). The FCR is the primary medial ventroflexor of the wrist. The ECR is a deep muscle found on the dorsal side of the forearm beneath both the extensor digitorum communis longus and the AIL. It originates from two heads, one from the lateral condyle and the other from the lateral epicondyle of the humerus. The two heads unite over the distal radioulna, and cross the top of the forearm to insert by a broad, thick tendon on the dorsomedial carpus. The ECR is the primary dorsoflexor of the wrist. The non-dimorphic ECU is a large forearm muscle that originates superficially on the lateral epicondyle of the humerus. Its fibers run parallel to the lateral surface of the radioulna and insert on the surface of the lateral carpus (Fig. 1). The ECU is the major lateral wrist extensor.

As is typical in frogs, both male and female bullfrogs hold their wrists with a marked inward rotation during standing and in the landing phase of the hop. The first digit often points straight backwards and the second digit is at close to 90 ° to

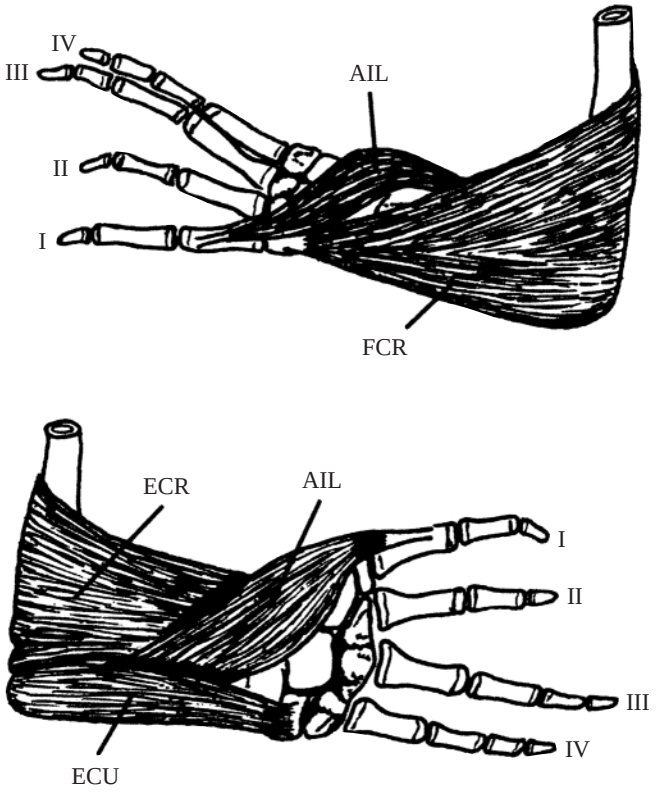

Fig. 1. The right forearm of a male bullfrog (*Rana catesbeiana*) seen from medial (A) and lateral (B) views (in a male bullfrog of 375 g, the distance from the elbow to the tip of the longest finger is approximately 40 mm). In A, the front foot is pronated 90° to show the insertion of the muscles. The muscles examined in this study were the abductor indicus longus (AIL), flexor carpi radialis (FCR), extensor carpi radialis (ECR) and extensor carpi ulnaris (ECU). AIL, FCR and ECR are important in amplexus and are larger in males than in females; the ECU was examined as a non-dimorphic control. The FCR and ECR originate on opposite sides of the distal humerus and insert, respectively, on the ventral and dorsal sides of the medial carpus at the base of the first digit (I). Together, these muscles flex the wrist medially; FCR ventroflexes and ECR dorsoflexes the wrist. These actions keep the wrist in contact with the female's venter and prevent her from moving forwards or backwards relative to the mounted male during amplexus. The AIL originates dorsally on the proximal, lateral side of the radioulna and inserts on the first metacarpal. It abducts the first digit, which is held against the female, and pins her against the male. The ECU is the primary wrist extensor providing lateral dorsoflexion. It originates over the lateral epicondyle of the humerus and inserts on the dorsolateral carpus.

the sagittal plane of the body (see Table 1). The joints are shaped so that flexion and extension of the wrist and elbow occur in a more mediolateral than anterioposterior plane. No detailed kinematic or electromyographic (EMG) studies have been made of amplexus, and descriptions of likely muscle functions are based on musculoskeletal morphology and observed limb positions and movements. During amplexus in bullfrogs, the male clasps the female in the axillary region (Duellman, 1992). The elbow joints are flexed to encircle her girth, and the dorsomedial side of each hand is placed against her belly. During breeding, most male frogs (including bullfrogs) develop enlarged nuptial pads on and near the first digit (Kurabuchi, 1993; Epstein and Blackburn, 1997), and the first digit is abducted against the female's belly by the AIL. The medial side of the radioulna lies against the female as well. The other two dimorphic muscles in this study, the FCR and ECR, lie on the ventromedial and dorsomedial sides of the radioulna, respectively, and either dorsoflex or ventroflex the wrist while moving it medially. Thus, these two muscles are positioned to control a female if she moves either forwards or backwards relative to the male during amplexus. Since both muscles originate on the distal humerus, they may also assist the coracoradialis in flexing the elbow.

Measurement of joint angles and muscle lengths

To compare the different muscles within a functional context, muscle forces were tested over a range of lengths that included the maximum and minimum natural muscle lengths. These lengths were estimated by videotaping (Panasonic VF65, 30 frames s^{-1}) males and females while standing and hopping and during amplexus. The animals were videotaped hopping and standing on a clear Plexiglas runway. A Plexiglas mirror was mounted below the runway at 45° to allow simultaneous recording of both side and bottom views. During hopping, the animals took off from a portion of the runway covered by indoor/outdoor carpet, which allowed good traction, but landed on a clear region so that bottom views of the forelimb during landing could be filmed. The frogs' front feet were relatively dry as a result of prior contact with the carpet, so that in most hop sequences no slipping was observed during landings on the Plexiglas. Only stable landing sequences were used in our joint angle analysis. The hops we filmed were not maximal efforts. Animals typically refused to hop more than 2–3 strides at once, so most measured strides were from standing take-offs in which hop distances ranged from 5 to 7 times body length (6.3±1.4 times body length; mean ± S.E.M., N=22). These hops were somewhat shorter than the mean hopping distance reported for *Rana catesbeiana* of 7.7 times body length (Zug, 1978). These lesser efforts might result in joint angles at maximum forearm flexion during landing that are less flexed in our animals. During maximal hopping, greater joint flexion on landing should result in somewhat longer maximum muscle lengths for the joint extensors (AIL, ECR and ECU) than reported here.

Joint angles during hopping were measured in both males (N=10) and females (N=12). Since there were no differences in the mean and variance of angles used between the sexes, the joint angle data were combined for hopping. The angles of the forelimb during amplexus were measured in males only (N=4), but the corresponding muscle position was tested in both males and females for comparison of isometric properties.

In two pairs of males and females that were not used for contractile measurements, amplexus was stimulated by injection of human chorionic gonadotropin (hCG, 100 mg kg^{-1} body mass) each day for 2 weeks. The animals were monitored several times daily and when they exhibited

reproductive behavior (male calling, mounting attempts), they were placed in an amplexus position while being held by the experimenters. If the male accepted and maintained this position while being held, filming commenced to capture the ventral and side views of the forearm position.

From the ventral and side views of the forelimb during standing, hopping and amplexus, angles were estimated for five joints: the posterior angle between the humerus and the longitudinal body axis (shoulder), the interior angle of the humerus and radioulna (elbow), the anterior angle of the wrist, the angle between digits I and II, and the posterior angle between digit II and the longitudinal body axis. During hopping, these angles were measured with the forelimb in its most extended position at the apex of the leap and with the forelimb maximally flexed at the bottom of the landing. Thus, we measured joint angles during standing, during extended and flexed hopping and in the amplexus position. The joint angles were measured using a stop-frame video cassette recorder (JVC BR9000U) to project the individual frames on a video monitor (Sony PVM1343). The angles were traced on clear acetate and measured with a protractor.

Since the bones forming the joints do not occur in the same horizontal or vertical planes, the real angles between the bones (θ) had to be calculated by combining the lateral (λ) and bottom (β) angles for each joint at each of the natural positions using the following equation:

$$\cos\theta = \frac{1}{\sqrt{(1 + \tan^2\lambda + \tan^2\beta)}} .$$

For those angles where λ and β were greater than 90°, the resultant angle was subtracted from 180° to obtain the real angle, θ (Peters et al., 1996).

Marker ties of black suture silk were attached to the origin and insertion sites of each experimental muscle during surgery, prior to measurement of its contractile properties. The experimental muscles all had fleshy origins, and the origin ties were placed in heavy fascia on the bones directly proximal to the muscle fibers. At the insertions, ties were made close to the junction of the muscle fibers with their tendons. The arms were then clamped with each forelimb angle fixed at the mean angle found to occur during standing. The muscle lengths between the marker ties were measured for each muscle in each experimental specimen using dial calipers with an accuracy of 0.1 mm.

Standing position was chosen as the experimental reference muscle length (L_s) because of the relative ease of clamping the limb in this position and subsequently measuring the length between marker ties. Measurement of the other muscle lengths at extended, flexed and amplexus positions proved difficult with the body and limbs intact. Thus, these muscle lengths were measured on the fresh carcass following the experiments, when the limbs could be removed from the body and clamped in extended, flexed and amplexus joint positions. Preliminary measurements from non-experimental specimens allowed us to estimate the maximum range of muscle length changes between extended and flexed forelimb positions, so that forces gathered for the length/tension curves were tested over a range that included the functional positions.

Measurement of contractile properties

Prior to surgery, each animal was anesthetized with MS222 (tricaine methane sulfonate; 200 mg kg^{-1} body mass; subcutaneous injection), and then pithed. Body mass was measured to allow a comparison of force taking into account body size differences (see below). The forearms were skinned, and the experimental muscles and nerves were dissected. Care was taken to maintain the blood supply to the muscles and nerves, and the forearm was kept moist by frequent irrigation and wrapped with gauze soaked in frog Ringer's solution.

Surgery was conducted in two groups: in one group of seven males and eight females, the FCR and ECR were tested from the left arms, leaving the right arms intact for later measurement of muscle lengths and wet muscle masses. In a second group (six males, six females) only the AIL and ECU were tested; the AIL was used from the left arm and the ECU from the right arm (necessary because ECU overlies AIL and must be removed; also, both are innervated by the same nerve and could not be surgically isolated).

Following isolation and standing length measurements (see above), the insertion tendon of each test muscle was cut and tied, using 2 gauge surgical silk, to a Grass FT10 isometric force transducer mounted on a rack and pinion. This allowed the muscle lengths to be varied from standing to maximum and minimum lengths throughout testing. The animal's arms were immobilized by clamping to a magnetic base, and the body was clamped to a heavy metal frame at the pelvis.

The FCR is innervated by the ulnar nerve, which was exposed superficially on the medial side of the humerus. The ECR is innervated by the radial nerve, which was isolated by careful separation of the long and inner heads of the m. anconeus to expose the nerve as it lies deep, near the humerus. AIL and ECU are both innervated by the radial nerve. Given the complex and deep branching of these nerves, the individual primary branches could not be isolated for any of these muscles. The entire radial and ulnar nerves were severed proximal to the elbow, and the cut nerve ends were stimulated electrically. Non-experimental muscles served by these nerves were either denervated, to prevent contraction, or cut from their insertions, to minimize the effects of any contractions extraneous to the experimental muscles.

Bipolar stainless-steel electrodes (0.1 mm diameter, California Fine Wire, Grover City, CA, USA) were implanted in the belly of each muscle to monitor EMG signals during stimulation. Stimuli were delivered to the radial or ulnar nerves *via* bipolar platinum hook electrodes using a Grass S88 square-wave stimulator. Supramaximal stimuli (2.5× threshold voltage) were delivered, and the force displacement registered by the transducer was displayed on a Tektronix TDS 310 digital oscilloscope and hard-copied to an HP Laserjet III printer.

Stimuli of 0.1 ms duration were delivered to elicit single

twitches from which force, contraction time (T_C) and half-relaxation time ($T_{1/2R}$) were measured. The T_C was measured from the stimulus trigger point to the peak of twitch tension. The $T_{1/2R}$ was measured from the point of peak tension to the point where the twitch force fell to half its peak value. Each muscle was also stimulated to produce maximal tetanic forces using a train of 0.1 ms pulses at a rate of 80 pulses s^{-1} (Hz) for 670 ms. All tests were carried out at room temperature (22±2 °C).

These tests were repeated at 2 mm intervals over a range of lengths that encompassed natural muscle lengths occurring at standing (L_s), at maximum forelimb extension (L_e), at maximum forelimb flexion (L_f) and during amplexus (L_a). Within this range, passive tensions were low or negligible, and the forces reported here are the active twitch and tetanic forces only.

After completion of the length/tension measurements, further tests were performed at the length at which maximum tetanic force occurred (L_0=physiological length). In the force/frequency test, the muscles were stimulated for 670 ms at increasing pulse frequencies from 5 to 80 pulses s^{-1} (at 5-pulse intervals between 5 and 30 pulses s^{-1}; at 10-pulse intervals from 30 to 80 pulses s^{-1}). The muscles were rested for 2 min between each stimulus train to minimize fatigue. In this way, the pulse rate at which tetanic fusion took place could be determined and compared between muscles and sexes. A comparison could also be made of the amount of force generated at each pulse rate, resulting in a force/frequency curve for each muscle.

To test fatiguability, each muscle was stimulated with a 200 ms train of stimuli at a rate of 30 pulses s^{-1} with the trains repeated at 2 s intervals for a total of 4 min. This regime was found to be adequate to reveal muscle fatigue without producing neuromuscular junctional fatigue (as demonstrated by the maintenance of a consistent EMG signal) (Peters, 1994). A fatigue index (FI) (Burke et al., 1971) was calculated as the sum of total force produced during the first 2 min divided by the total force produced over the entire 4 min test multiplied by 100. Thus, an FI of 50 indicates that, during the first half of the test, the muscle produced half the total force; hence, no fatigue. A fatigue index above 50 indicates increasing fatigue (Burke, 1981; Peters, 1994). In addition to the FI, fatigue was compared over the 4 min test by plotting the mean force at each 20 s interval as a percentage of the maximum force. In this way, we could examine the relative amounts of force throughout the fatigue test and compare any decline in force between males and females for each muscle.

At the end of the experiments, the animals were killed, and the muscles from the non-experimental limbs were carefully removed. The tendons were trimmed away close to the muscle tissue, and the muscles were placed on a digital scale (accurate to 0.1 mg) so that mass could be determined. Since muscle force is proportional to the cross-sectional surface area of the muscle fibers, we estimated whole-muscle cross-sectional area by dividing muscle mass (which is proportional to volume) by the muscle length at maximum force (L_0). The values were then used to calculate the tetanic force per cm^2 of muscle cross-sectional area. This method of estimating muscle cross-sectional area is crude in that it does not account for differences in pinnation or for the amount of non-contractile tissue among muscles or between sexes. Future studies that include these measurements would be a valuable addition to our knowledge. For the present, we assume that angles of pinnation are likely to be approximately equal for the same muscle between males and females.

Data analysis

In all comparisons of force values (maximum twitch, maximum tetanus and forces within the length/tension curves), muscle masses and cross-sectional areas, it was necessary to scale the values for differences in body size since female frogs tended to be larger than males (female mean mass 429 g; male mean mass 376 g; see below). In a comparison of body masses between all females and all males, the size difference did not quite reach significance (P=0.06), but in the specimens used for tests of FCR and ECR, the body mass difference was significant (P=0.03). We decided, for consistency, to compare the muscle force, mass and cross-sectional area data in analyses of covariance (ANCOVAs) using a body size factor as a covariate. Both body mass and snout–vent length were examined in logarithmic regressions against maximum tetanic tension and maximum twitch values in grams of tension. In both regressions, there was a significant correlation between tension and these size factors, but the correlation coefficient was significantly higher for body mass (for tetanus, r=0.681, P=0.01) than for snout–vent length (for tetanus, r=0.447, P=0.09). In addition, in ANCOVAs for all four muscles, the body mass factor was significantly correlated (P<0.02) with the dependent variable. Thus, muscle mass, muscle cross-sectional area, twitch force (F_{Tw}) and tetanic force (F_{Te}) comparisons were made between the sexes using an ANCOVA in which body mass was the covariate. In the case of the muscle mass comparisons between male and female ECRs, however, non-homogeneous regression slopes were found in the ANCOVA, so a separate slopes model was used to compare the masses of this muscle.

Contraction times (T_C), half-relaxation times ($T_{1/2R}$), fusion frequencies and fatigue indices (FI) were compared using a one-factor analysis of variance (ANOVA) on the absolute values of each measurement. Two ratios were also calculated and compared: the twitch/tetanus ratio (F_{Tw}/F_{Te}) of maximum twitch force divided by maximum tetanic force, and the maximum tetanic force per cm^2 of whole-muscle cross-sectional surface area. These were compared in a one-factor ANOVA, and Fisher's *post-hoc* test was used for pair-wise comparisons between sexes. All the statistical analyses in this study were subjected to the sequential Bonferroni adjustment for multiple comparisons.

Length/tension curves were constructed for each muscle in each sex by calculating the mean tetanic force for each muscle at standard lengths relative to standing length (from 80 to 120 % of L_s at 10 % intervals). Since the actual lengths at which

tetanic forces were measured (±2 mm increments from L_s) do not correspond to these percentage intervals and differ among individuals as a percentage of L_s, length/tension curves were constructed for each muscle for every individual (best fitted to a second-order polynomial). The forces occurring at these standard length intervals were then extrapolated from the curves, and the mean tetanic force at each interval in each muscle was calculated for males and females (Peters and Nishikawa, 1999). The values reported in Fig. 2 are expressed as tetanic tension in newtons on one scale, but also as force as a percentage of body mass for ease of visual comparison of the differences between the sexes, since females tended to be larger than males. The statistical analysis was performed on the raw data in ANCOVAs using body mass as the covariate to account for the size difference. Force/frequency data were calculated as the mean muscle force at each stimulus frequency (between 5 and 80 pulses s^{-1}) as a percentage of the maximum force. Fatigue over the 4 min fatigue test was calculated at 20 s intervals as mean force as a percentage of maximum force. The amounts of force sustained between tetanic stimuli during the fatigue test were compared at each minute during the 4 min fatigue test and in a subsequent 18 min fatigue test (see Results for details). Values within each of these graphs (see Figs 2–4, 6–7) were compared between males and females using a one-way ANCOVA for the length/tension curves and a one-way ANOVA on the force/frequency and fatigue values at each interval. We then used Fisher's *post-hoc* test to examine the pair-wise differences between the sexes. A repeated-measures ANOVA was not performed because our interest was in determining whether the male and female muscles produced different forces at each comparable length or stimulus, not in how the patterns of repeated measures changed within sexes or in the overall patterns between sexes. For this purpose, it is appropriate to treat each repeated measure as an individual variable to compare between the sexes in one-way ANOVAs.

Results

Data for each of the variables described in this study were collected from 6–8 individuals of each sex that ranged in size from 239 to 462 g (376.3±18.1 g) for males and from 308 to 611 g (428.7±20.5 g) (means ± S.E.M.) for females. Table 1 summarizes the joint angles and their corresponding muscle lengths at the four behavioral positions tested. As noted above, no significant differences were found in the joint angle values between males and females, so the muscle length changes (as a percentage of standing length) that resulted from placing the forelimbs in these mean positions in both males and females were combined. For the wrist/thumb extensors (ECR, ECU and AIL), the longest natural lengths (i.e. excluding the value at L_0) were found at the point of maximum joint flexion, when the frog had just landed. The FCR, however, reached maximum length when the arm was fully extended at the apex of the leap. Note that the muscle lengths changed by approximately 10 % of L_s (range 7.3–13.5 %L_s) between minimum and maximum lengths. In AIL and FCR, amplexus length was 3–4 % shorter than L_s, but in ECR and ECU, amplexus length averaged slightly (<1–2.4 %) longer than L_s.

Table 1. *Joint angles and muscle lengths during hopping, standing and amplexus*

	Joint angle (degrees)			
	Flexion (maximum)	Standing	Amplexus‡	Extension (maximum)
Shoulder	58±2.6	78±3.7	80±2.8	145±4.0
Elbow	63±2.4	92±4.5	66±2.4	171±5.5
Wrist	80±4.0	111±5.6	124±6.4	78±3.4
Digit I/digit II	69±2.8	70±2.6	64±3.2	86±3.0
Digit II/long axis	81±2.6	83±4.1	89±3.2	83±3.2

	Muscle length (%L_s)				
	L_f	L_s	L_a‡	L_e	L_0
AIL (N=12)	104.6±1.6	100	96.1±1.2	97.3±1.6	123.7±12
FCR (N=15)	90.4±0.8	100	96.9±0.7	103.5±1.2	112.8±10.9
ECR (N=15)	108.8±1.3	100	100.8±0.9	97.0±1.6	120.1±11
ECU (N=12)	105.1±1.0	100	102.4±0.7	96.1±1.0	117.1±13.5

Values are means ± S.E.M.

‡Angles were measured in males only, but corresponding muscle lengths were measured in both sexes.

*L_s, muscle length at standing; L_e, muscle length at maximum joint extension; L_f, muscle length at maximum joint flexion; L_a, muscle length during amplexus; L_0, muscle length at maximum tension.

AIL, abductor indicus longus; FCR, flexor carpi radialis; ECR, extensor carpi radialis; ECU, extensor carpi ulnaris.

Force properties

The mean values ± S.E.M. for all of the contractile properties of the four experimental muscles compared between males and females are shown in Table 2. Both maximum twitch and tetanic forces were significantly larger in males for two of the dimorphic muscles (FCR and AIL) (Table 2). Tetanic tension was also significantly larger in males in the other dimorphic muscle (ECR), but the twitch force did not differ between the sexes in the ECR. Among the muscles tested, the greatest maximum tetanic force was produced by the ECR in both males and females. Neither twitch nor tetanic forces differed between the sexes in the ECU.

The twitch/tetanus ratio (F_{Tw}/F_{Te}) was calculated and compared between males and females by dividing the twitch force at physiological length by the corresponding tetanic force. This ratio is thought to relate to the relative activation of force (i.e. the kinetics of Ca^{2+} release), but is also affected by the series elastic component of the muscle. The smaller the F_{Tw}/F_{Te}, the less activation of the muscle occurs with a single twitch stimulus compared with the maximally activated tetanus, and/or the greater is the damping effect of series elasticity during the twitch. The values of F_{Tw}/F_{Te} varied from 0.15 to 0.26. The only significant difference found between the sexes was in the FCR, in which the male ratio (0.15) was less

Table 2. *Muscle sizes and contractile properties*

	AIL			FCR			ECR			ECU	
	Male (*N*=6)		Female (*N*=6)	Male (*N*=7)		Female (*N*=8)	Male (*N*=7)		Female (*N*=8)	Male (*N*=6)	Female (*N*=6)
Twitch (g)	54.3±10.6	*	23.2±3.6	45.8±5.0	*	31.1±6.8	83.5±11.5		109.5±11.1	57.7±9.2	68.2±6.1
(N)	0.53		0.23	0.45		0.30	0.82		1.07	0.56	0.67
Tetanus (g)	239.2±29.5	*	120.2±7.3	324.3±39.4	*	129.2±20.7	554.2±44.3	*	509.8±37.0	382.5±46.0	416.3±8.3
(N)	2.34		1.18	3.18		1.27	5.43		5.0	3.75	4.08
F_{Tw}/F_{Te}	0.26±0.03		0.23±0.01	0.15±0.01	*	0.25±0.02	0.22±0.02		0.23±0.01	0.17±0.02	0.17±0.02
Muscle mass (g)	0.30±0.02	*	0.12±0.004	0.52±0.004	*	0.21±0.01	0.65±0.07	*	0.47±0.03	0.51±0.03	0.52±0.01
Muscle cross-sectional area (cm^2)	0.083±0.006	*	0.033±0.001	0.21±0.03	*	0.10±0.01	0.26±0.03	*	0.18±0.02	0.20±0.01	0.21±0.01
Tetanic tension/muscle cross-sectional area					NS						
($kg\ cm^{-2}$)	2.91±0.39		3.66±0.2	1.59±0.13		1.21±0.11	2.42±0.14		2.91±0.17	1.80±0.17	2.01±0.12
($N\ cm^{-2}$)	28.5±3.8		35.9±2.0	15.6±1.3		11.8±1.1	23.7±1.4		28.5±1.7	17.6±1.7	19.7±1.2
Sexes combined ($N\ cm^{-2}$)	33.4±2.2			13.4±1.0			26.5±1.3			18.6±1.0	
			**			**			**		
					NS						
Fusion frequency ($pulses\ s^{-1}$)	25.8±1.5	*	32.5±1.7	23.0±1.8		25.6±1.8	29.3±1.9		32.5±1.9	29.2±2.4	33.3±3.8
T_C (ms)	99.8±3.1	*	61.7±2.0	82.7±5.3		73.1±2.5	57.8±1.9		58.2±2.9	69.8±3.8	66.7±2.6
$T_{1/2R}$ (ms)	77.9±3.6	*	41.8±1.8	86.8±6.0	*	63.3±2.7	59.1±3.8		39.0±1.6	41.9±1.9	36.5±1.5
Fatigue index	52.7±0.8	*	60.8±0.7	50.4±1.0	*	56.4±1.1	56.0±2.7		58.2±1.5	57.3±0.8	57.0±1.5

Values are means ± S.E.M.

*Denotes differences between males and females within muscles.

**Denotes differences between muscles when male and female data are combined.

To maintain an experiment-wise error rate of $P<0.05$, alpha levels were subjected to a sequential Bonferroni adjustment to determine statistical significance; $k=11$; NS, not significant.

F_{Tw}, twitch force; F_{Te}, tetanic force; T_C, contraction time; $T_{1/2R}$, half-relaxation time.

AIL, abductor indicus longus; FCR, flexor carpi radialis; ECR, extensor carpi radialis; ECU, extensor carpi ulnaris.

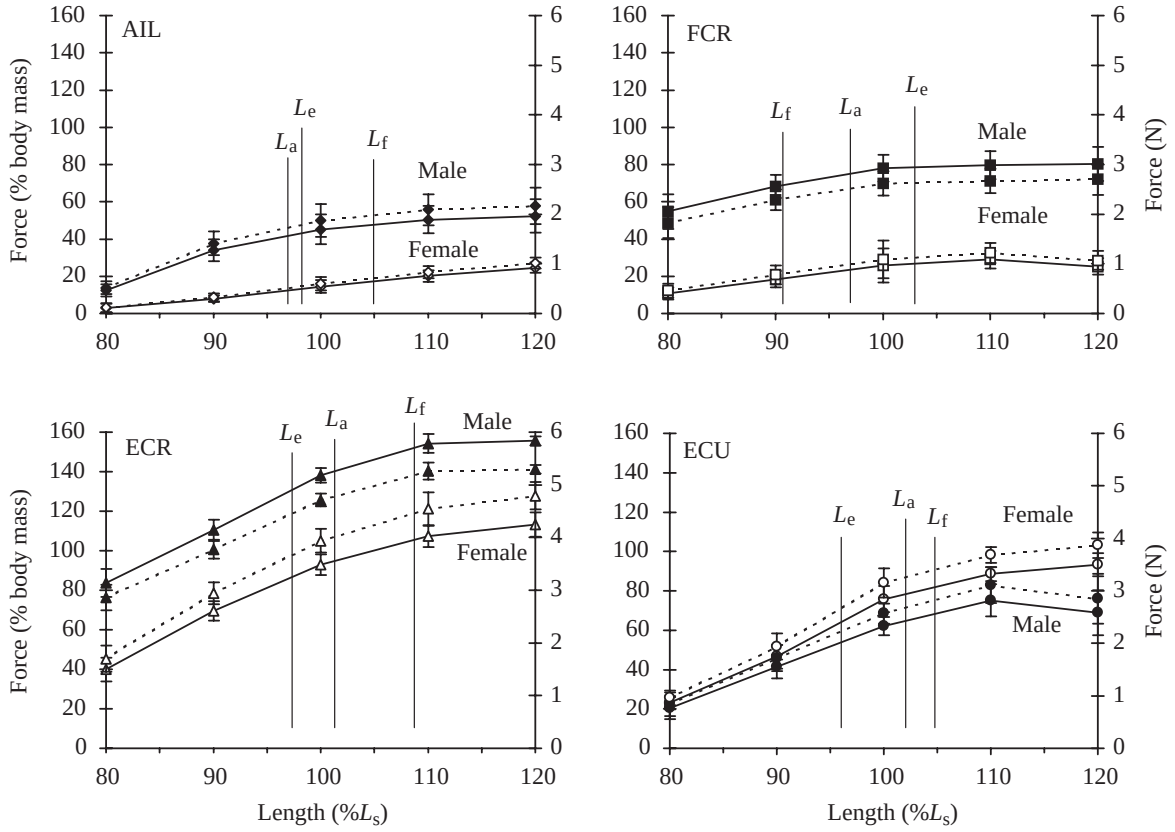

Fig. 2. Length/active tension curves for the four forearm muscles. Forces (in N) are plotted on the right-hand axis (indicated by dashed lines); however, since body size differs between males and females, force is also plotted as a percentage of body mass on the left-hand axis (indicated by solid lines). Forces at each length are shown relative to standing length (L_s=100 %). Statistical tests were used to compare the raw forces in ANCOVAs using body mass as the covariate. In the three dimorphic muscles (AIL, FCR and ECR; for muscle abbreviations, see the legend to Fig. 1), the forces for the males were significantly greater than those for the females at all lengths except at 80 %L_s in the AIL. The values did not differ at any length between the sexes in the ECU. Functionally important lengths are indicated by vertical lines within the length/tension curves: L_e, muscle length at maximum joint extension; L_f, muscle length at maximum joint flexion; L_a, muscle length at amplexus (taken from joint positions measured in males only, but compared with the corresponding lengths in females). Values are means ± S.E.M.; for sample sizes, see Materials and methods.

than in the female (0.25). Thus, for most of the muscles, the ratios scaled in proportion to the differences in force between muscles and sexes.

As noted above, muscle mass has commonly been used in morphological comparisons to estimate relative differences in muscle force. If true cross-sectional areas can be measured, these should be the best estimators of muscle force. Both whole-muscle mass and cross-sectional area data are consistent with the force data in showing that mass and cross-sectional area differed significantly between sexes in the three dimorphic muscles and did not differ in male *versus* female ECU (Table 2). Both muscle mass and cross-sectional area also varied linearly and were highly correlated with maximum tetanic tension (r=0.89 for mass, P<0.0001; r=0.81 for cross-sectional area, P<0.0001). Thus, in these specimens, both mass and cross-sectional area were equally good at predicting the differences in force between sexes. This holds true because the muscle lengths used to calculate cross-sectional areas do not differ between males and females (Table 1), so that variation in muscle mass is a direct reflection of variation in muscle cross-sectional area. When forces were standardized to tetanic tension per cm^2 of muscle cross-sectional area, the differences between sexes disappeared, so that, within muscles, there was no significant difference in the amount of tetanic force generated per cm^2 of muscle cross-sectional area (Table 2). As a result, we combined the male and female data within each muscle and ran an ANOVA to determine whether the force per cm^2 was constant across muscles. The force per cm^2 of muscle area for the FCR and ECU was the same, but significantly smaller than for the AIL and ECR, which did not differ from each other (Table 2). Within a muscle, differences between sexes in tetanic force are, therefore, based strictly on differences in muscle size. The differences in force per cm^2 between muscles may relate either to inaccuracies in estimating true muscle cross-sectional areas or to true morphological and/or physiological differences (see Discussion).

Length/tension and force/frequency

In addition to the maximum force values measured for each muscle, length/tension curves were constructed over the range

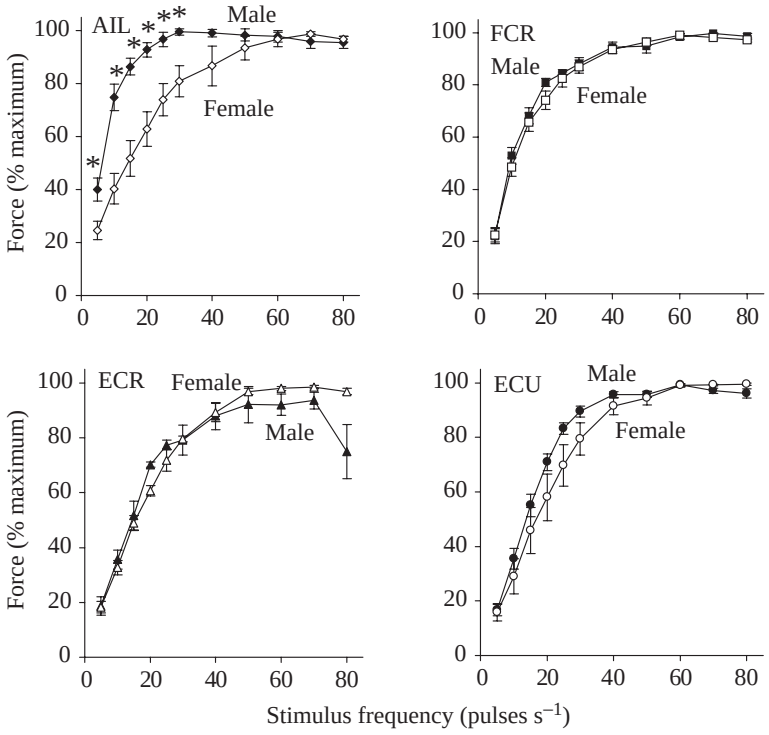

Fig. 3. Force/frequency curves comparing the amount of force (as a percentage of maximal force) generated by the male and female muscles at stimulus frequencies varying between 5 and 80 pulses s^{-1}. The only significant difference (indicated by asterisks) between the sexes was found in AIL, in which the male muscle produced relatively more force than did the female muscle between 5 and 30 pulses s^{-1}. Values are means ± S.E.M.; for sample sizes, see Materials and methods. For muscle abbreviations, see the legend to Fig. 1.

of lengths encompassing the natural behaviors measured (standing, maximum flexion, maximum extension, amplexus). These are shown in Fig. 2, in which the mean tetanic force values are plotted over this range of lengths (as a percentage of standing length). When standardized for differences in body size, dimorphism in magnitude of the curves for the AIL, FCR and ECR over their entire range of lengths is evident. The curves for ECU show the lack of significant difference between the sexes throughout a range of normal lengths. In all four muscles, L_0 is typically 10–20 % longer than L_s (Table 1) and longer than the maximum natural lengths (either L_e or L_f). The extensors develop maximum force on the length/tension curve close to their maximum natural lengths when the limb is fully flexed (L_f); FCR develops maximum force near its length at maximum limb extension (L_e). The muscle lengths during amplexus are shorter than the maximum natural lengths (L_f or L_e) and the forces at amplexus are less than maximum force in both sexes. Note, however, that the length/tension curves are relatively flat, and the forces decrease by only approximately 10–25 % in the region between maximum and minimum natural lengths.

Fig. 3 shows the force/frequency data for each of the four muscles. The mean values ± S.E.M. of the tetanic forces for males and females are plotted as a percentage of maximum force at each stimulus frequency. The curves are similar for males and females for FCR, ECR and ECU. Only in AIL do the males reach greater force at a lower stimulation rate between 5 and 30 pulses s^{-1}.

Contraction and relaxation times

Table 2 shows the mean twitch contraction and half-relaxation times of each muscle in males and females. The T_C values for the FCR, ECR and ECU did not differ significantly between the sexes; only in the AIL were the male T_C values significantly longer than in the females (99.8 ms in males *versus* 61.7 ms in females) (Table 2). In both AIL and FCR, the half-relaxation times were significantly longer in males than in females (Table 2). The non-dimorphic ECU did not differ between sexes in T_C or $T_{1/2R}$, nor did the dimorphic ECR. The fusion frequencies tended to be lower in males than in females in all four muscles; however, only in the dimorphic AIL were they significantly lower (Table 2).

Fatigue and sustained force

The fatigue indices shown in Table 2 indicate that the forelimb muscles in both sexes showed low to moderate fatigue (all FI<61). However, even though the values were not greatly divergent, there were significant differences between the sexes. In both the FCR and AIL, the male FIs were significantly lower (less fatiguable) than in females. Fatiguability in the ECR was not significantly lower in males. The FI values for ECU were also not significantly different between males and females (Table 2).

Fatiguability can be more easily appreciated by following the time course of force decline. Fig. 4 shows force as a percentage of maximum force plotted at 20 s intervals over the entire 4 min fatigue test. Of the three sexually dimorphic muscles, both AIL and FCR showed significantly less decline in force in the males than in the females. Relative amounts of force were significantly greater in males over the last 1–2 min of repetitive stimulation. In the ECR, as expected from its FI, the decline in force did not differ significantly between the

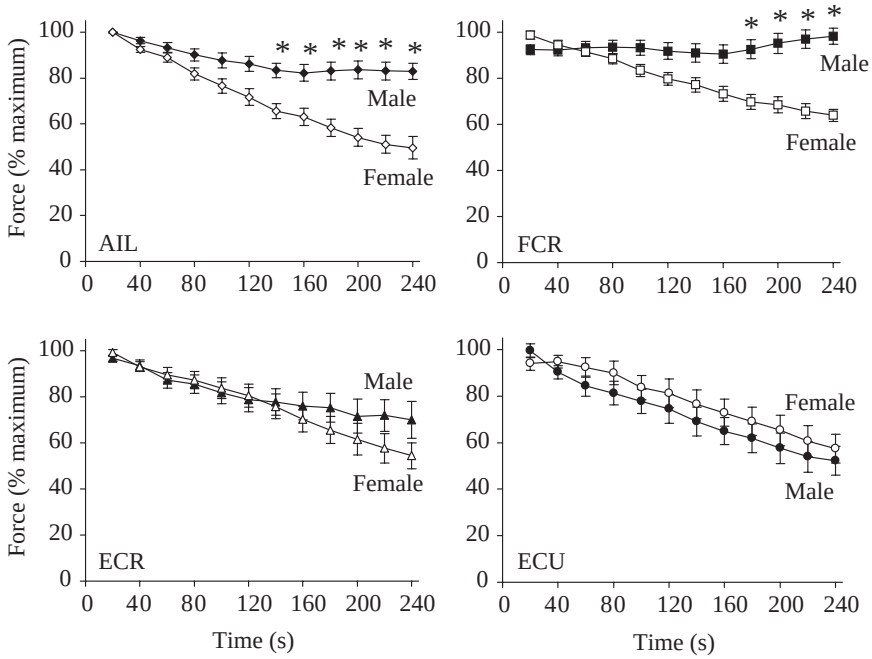

Fig. 4. Fatigue over time for each muscle as the mean force at 20 s intervals as a percentage of maximal force. In both AIL and FCR, force declined less over the final 1–2 min in males than in females. Significant differences between sexes are marked with an asterisk. There was no significant difference between sexes in the force decline of the ECR and ECU. Values are means ± S.E.M.; for sample sizes, see Materials and methods. For muscle abbreviations, see the legend to Fig. 1.

sexes. The decline in force in the ECU was also identical between males and females (Fig. 4).

During the fatigue tests on our experimental muscles, we observed the phenomenon illustrated in Fig. 5. In all the sexually dimorphic muscles, we found that by 2–3 min into the 4 min test, two major changes had occurred in the force profiles. First, the shape of each force waveform broadened at the base. In addition, the baseline to which the trace returned between stimuli became elevated. At the conclusion of the fatigue test, when the stimulator was turned off, the baseline always returned to its resting position within 5–10 s, so we were confident that the elevation was part of the muscle response during stimulation. This response is the result of unrelaxed or sustained force maintained by the muscle between trains of stimuli (see Discussion). The force values used to calculate FIs and total force at each time point (Fig. 4) were measured from the resting baseline to the peak of active force. In many cases, the majority (60–80 %) of this force was made up of the sustained force of the muscles. Fig. 6 shows the amounts of sustained force as a percentage of the total force at 1, 2, 3 and 4 min during the fatigue test. Although most

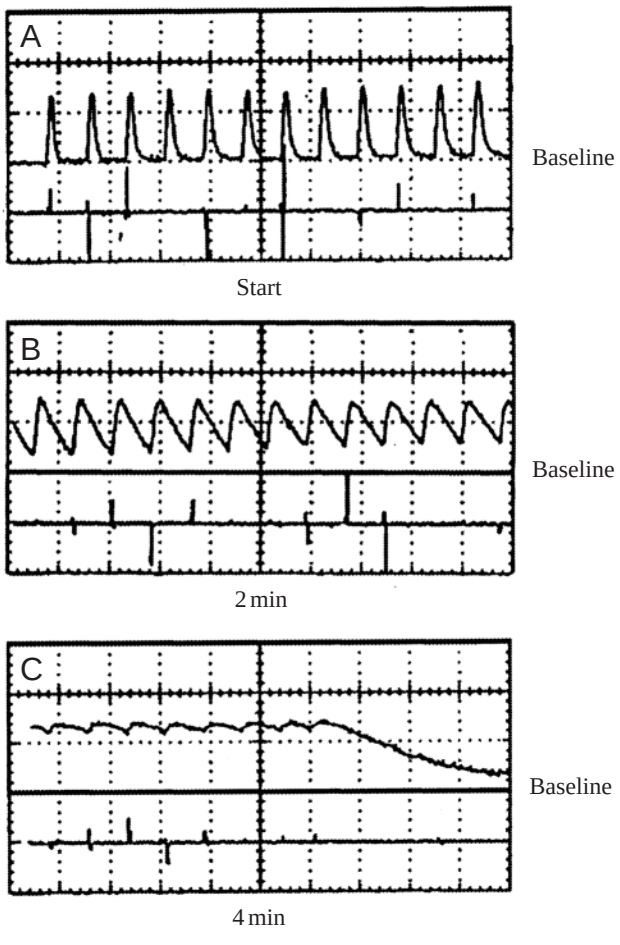

Fig. 5. Oscilloscope traces showing the common pattern of fatigue in the flexor carpi radialis (FCR) of a male bullfrog. Force is shown in the top trace, and the lower trace within each panel is the electromyogram. The muscle was stimulated once every 2 s with a tetanic train that lasted for 200 ms at a pulse rate of 30 pulses s^{-1} for 4 min. Note that, within the starting panel (A), the force waveform begins to expand at the trailing edge and there is a slight elevation of the baseline. By 2 min into the test (B), the waveform has broadened dramatically, indicating marked slowing of relaxation. In addition, the level to which the trace falls between stimulus trains has become significantly elevated above the starting baseline. By 4 min (C), the electrically stimulated force is reduced to a scalloped ridge, while the force sustained between stimuli (sustained force) makes up more than 80 % of the total force. Note that the total force level remains nearly constant. The background grid represents time intervals of 2.5 s per division.

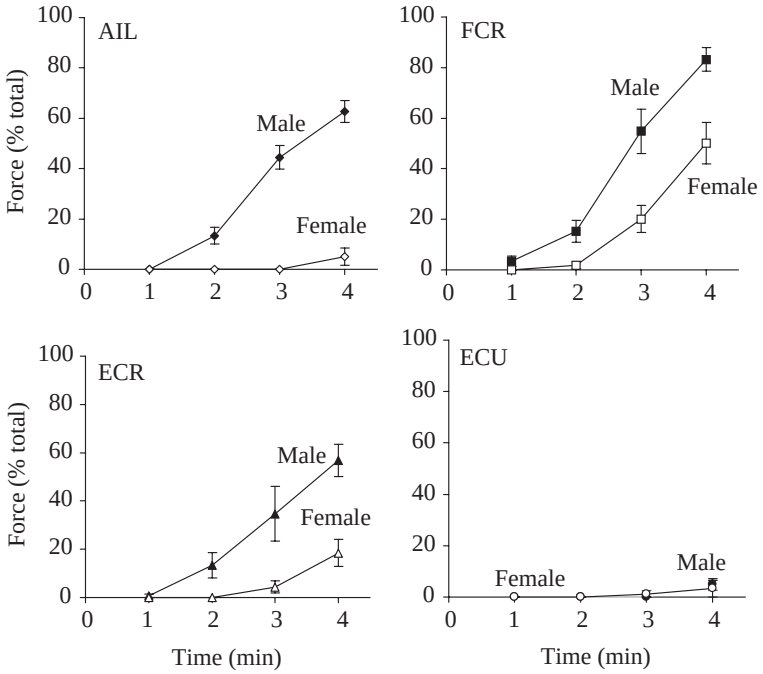

Fig. 6. Sustained force (as a percentage total force) plotted at each minute within the 4 min fatigue test and compared in all four muscles between males and females. In each of the sexually dimorphic muscles (AIL, FCR and ECR), both males and females produced sustained force, but the force in males was significantly larger than that in females. A small amount of sustained force was produced in male and female ECU (roughly equivalent to the female AIL), but the force did not differ between the sexes. Values are means ± S.E.M.; for sample sizes, see Materials and methods. For muscle abbreviations, see the legend to Fig. 1.

pronounced in the male AIL, FCR and ECR, this was also true to a lesser degree for these muscles of the females. In females, the sustained force was typically less than half of that reached in the males. The tiny amounts (less than 5 % of total force) of sustained force in the ECU did not differ between males and females (Fig. 6).

To study sustained force over a period longer than 4 min, we performed a separate series of experiments on the FCR in males (N=4) and females (N=4). First, we ran the standard 4 min fatigue test (one train of stimuli per 2 s at 30 pulses s^{-1}; 200 ms duration). At the end of 4 min, we decreased the pulse rate to 10 pulses s^{-1} and continued these short (200 ms) intermittent trains (one train per 2 s) for 10 min. We then raised the pulse rate back to 30 pulses s^{-1} and continued this stimulation for a further 4 min. The *in vivo* stimulus intensities and durations are not known, and it is unlikely that normal motor unit recruitment is as high as in our *in vitro* tests; however, this 18 min test provides a time-course of stimulation that we chose to mimic as closely as possible the pattern that might happen during amplexus. We assume that when a male clasps a female the natural stimulation rate would be relatively high during their initial contact. Once amplexus has been established, the male should be able to reduce the number of motor units activated and, perhaps, decrease the stimulation rate to the active motor units. We assume that he cannot totally relax these muscles during amplexus, so that low-rate input is likely over prolonged periods. At times during amplexus, the pair may move to the oviposition site within the male's territory or they may be disturbed by predators (Howard, 1978). In these circumstances, the male would probably have to increase the stimulus input to remain in amplexus.

The results of a patterned 18 min fatigue test of the FCR are shown in Fig. 7. During the first 4 min of the fatigue test, the total force declines by 25 % in males and by 35 % in females. In this same period, the sustained force rises to 57 % of total force in the male, but to only 23 % of total force in the female. Following a 10 min period of very low stimulus input (at 10 pulses s^{-1}), the total force in both males and females recovers to near-initial levels upon renewed stimulation at higher frequency (30 pulses s^{-1}). In addition, the sustained force rises to a higher proportion of total force than in the initial 4 min period. In females, the maximum sustained force initially was 23 % of total force; at the end of 18 min, it was 28 % (P<0.01). In males, the initial sustained force reached 57 % of total force; by the end of 18 min, it rose to 69 % of total force (P<0.001). In fact, compared with the initial 4 min period, during renewed stimulation at a higher rate, the sustained force as a percentage of total force was significantly higher at all times after 1 min (Fig. 7). Thus, the proportion of the total force made up of sustained force is re-established quickly and rises to higher percentages than in the first 4 min of the test. This is due primarily to the fact that we see somewhat more fatigue in the electrically evoked component of the total force over the last 4 min, whereas the actual forces (in grams) sustained at 4 min and 18 min are nearly identical (mean male, 12.2 g *versus* 10.3 g; mean female, 2.4 g *versus* 2.1 g).

Discussion

The goal of this study was to compare the isometric contractile properties of dimorphic forelimb muscles in male and female bullfrogs to identify differences that may relate to their dimorphic functions. In summary, our data confirm that, in bullfrogs, three of the four forelimb muscles examined were dimorphic in mass, being larger in males than females. In addition, we found that three of the four male muscles had

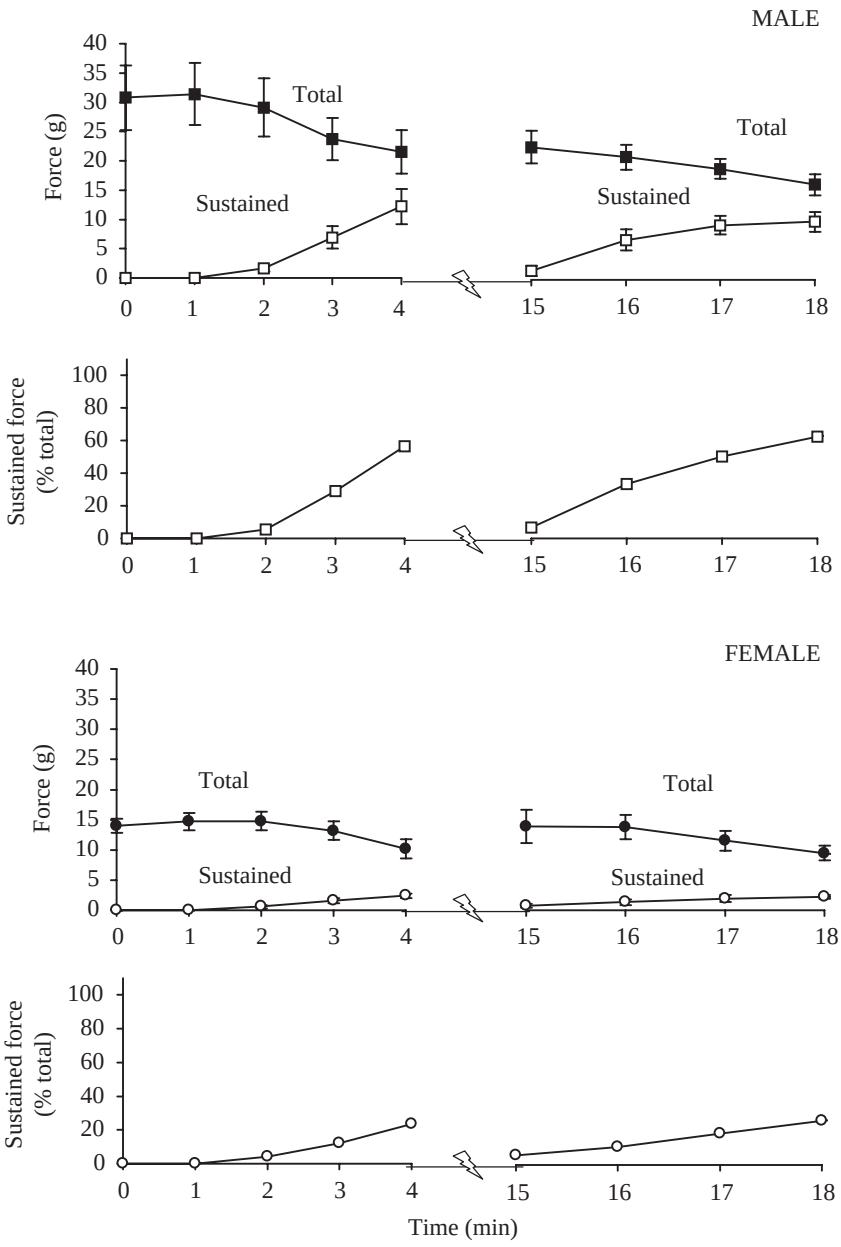

Fig. 7. Force profiles produced by male and female flexor carpi radialis (FCR) over the course of an 18 min fatigue test (see text for details). Actual force is shown in the upper graphs for each sex, including total force (filled symbols) and the amount of this total made up of sustained force (open symbols). The lower graphs, plotted for each sex, show sustained force as a percentage of total force. In both sexes, sustained force becomes an increasing percentage of total force over the first 4 min of the test (stimulated at 30 pulses s^{-1}). Following a 10 min period of reduced stimulation (at 10 pulses s^{-1}), a higher stimulation rate (30 pulses s^{-1}) was reapplied for the final 4 min. Note that in both sexes sustained force rises more rapidly during minutes 15–18 than during the initial 4 min test. The amount of sustained force (as a percentage of total force) is also higher at each minute in these final 4 min than in each of the first 4 min. The pattern of development of the sustained force is the same in the both males and females; however, the amount of sustained force is significantly higher in males. Values are means ± S.E.M., N=4, of each sex.

larger muscle cross-sectional areas and produced greater isometric tetanic forces than those of females. The tetanic forces scaled directly with both muscle mass and cross-sectional area, so that the larger force from male muscles was attributable to relatively larger size. Since muscle lengths (L_0) did not differ between the sexes, the larger masses of the three dimorphic muscles was a direct reflection of their greater cross-sectional area in males. Thus, force differences were due to larger muscle size in males rather than to intrinsic physiological differences. Other isometric properties that showed dimorphism were twitch contraction and relaxation times, with some male muscles showing slower contraction kinetics than females. One muscle, AIL, had a lower fusion frequency in males and produced relatively more force at low-to-moderate stimulus frequencies than in females. Finally, two of the dimorphic male muscles (AIL and FCR) were markedly less fatiguable than their female counterparts. A major component of the force produced during the fatigue test comes from force that is sustained between tetanic stimulus trains. We devised an 18 min test which confirmed that, over a prolonged period of low-level stimulation, male muscles can maintain a large percentage of initial force as a result of the sustained force. This sustained force is present in the dimorphic muscles of both males and females, but is much greater in magnitude in males.

Forearm function

The forelimbs do not appear to contribute significantly to propulsion during either hopping or swimming in most anurans. In terrestrial frogs, the main behavior requiring forelimb force is support for the body during landing from a hop (Peters et al., 1996). Not surprisingly, in bullfrogs of both

sexes, the wrist extensors (ECR and ECU) and the wrist flexor (FCR) produced large forces, since their combined actions would stabilize the wrist during landing.

The sexual size dimorphism in selected forelimb muscles is probably related to their use by the males during amplexus and male–male aggression. Bullfrogs are territorial breeders. The largest males are able to establish territories that are used to attract females and serve as oviposition sites (Emlen, 1976; Howard, 1978). Territorial males often fight off intruders by grappling with them, usually using the forelimbs to grasp the other male and hold him under the water. This clasp is quite similar to that used in amplexus and probably involves the same muscles. Since young males typically cannot effectively compete with older males for territories, they often use subterfuge, either by mimicking a territorial male when a territory has been vacated or by lurking near an occupied territory and trying to intercept females as they enter it (Howard, 1978, 1984). Older females will typically try to dislodge young males, but the yearling males occasionally succeed in establishing amplexus and in mating. These alternative mating strategies may well place a premium on the early development of forelimb muscle size, especially for being able to control an unwilling female and, later, for repelling competing males. In these activities, both size and endurance could be very important.

Contractile properties

The results of our study confirm that the muscles involved in amplexus in males have greater wet masses and greater whole-muscle cross-sectional areas than in females. These muscles also generate higher forces compared with females (Table 2), and differences in muscle mass and cross-sectional area scale directly with the differences in force. In spite of the significantly greater total forces produced by the dimorphic muscles in males, when standardized to whole-muscle cross-sectional area, we found no differences in tetanic tension per cm^2 of muscle cross-sectional area between the sexes. In most of the muscles (except FCR), in fact, the female force per cm^2 tended to be slightly larger than in males. This may reflect differences in muscle fiber populations between the sexes (e.g. males with greater numbers of smaller, oxidative fibers). The longer T_C and $T_{1/2R}$ values and the significantly lower FIs in some of the male muscles suggest that such differences occur. Further examination of the histochemical and functional properties of individual fibers and motor units are necessary to examine these differences.

Muscle mass and/or cross-sectional area are commonly used to estimate muscle forces (e.g. Strickland, 1978; Spector et al., 1980; Sacks and Roy, 1982; Oka et al., 1984; Yekta and Blackburn, 1992). Since the force per cm^2 of muscle cross-sectional area differed between muscles, however, this should not be relied on for more than rough estimates of actual muscle forces. We assume that differences in these values between muscles arise from the lack of data on true muscle fiber cross-sectional areas, so that differences in proportions of connective tissue, fat and other non-contractile elements, and also differences in muscle fiber types between the muscles, affect the predictive value of our force per cm^2 data.

The length/tension plots (Fig. 2) show that the dimorphic muscles of the male bullfrog produce greater force than those of the female at almost all lengths. However, the muscles changed length very little over the normal range of hopping movement (Table 1), and the forces produced between maximum and minimum muscle lengths varied little. The force changed between maximum muscle length and amplexus length by less than 20 % in males and by 15–35 % in females across all four muscles. The muscles were used at lengths that were somewhat shorter than physiological length, but at the maximum hopping and amplexus lengths, the force was 70 % or more of maximum isometric tension in all cases. Isometric length/tension data can only supply an estimate of the potential forces available at different lengths. Although isometric values may be an appropriate comparison for amplexus, if a muscle is actively shortening or lengthening, the forces would differ from isometric values. Forces measured at these static whole-muscle lengths also do not take into consideration the effects of the elastic tissues of the arm, either in stabilizing the joints or in possible recoil from rapid stretching on landing from a hop. However, elastic effects would probably not play a role in amplexus. More detailed studies of forelimb actions during these activities and of *in vivo* length and force changes are needed to put our data into a more meaningful context.

Previous studies (Peters, 1994; Peters and Nishikawa, 1999) found that frog muscles with longer T_C and $T_{1/2R}$ values tended to have lower fusion frequencies and to produce a greater proportion of their maximum force at lower stimulus frequencies. In the present study, only the male AIL had both a longer T_C and a longer $T_{1/2R}$ than the female. The AIL also had a significantly lower fusion frequency (Table 2) and generated higher force at low stimulus input in the male (Fig. 3). Thus, a correlation between slow T_C and $T_{1/2R}$ and the relatively greater force at low-to-moderate stimulus frequency is supported by our data.

Mammalian studies (e.g. Goslow et al., 1977; Burke, 1981; Peters et al., 1984) and some studies of amphibians (e.g. Luff and Proske, 1976, 1979; Ridge and Thomason, 1980) have found a correlation between T_C, force and fatiguability, in which the largest motor units contract fastest but are the most fatiguable. Smaller, slower motor units tend to be most resistant to fatigue. In general agreement with this, our study found that the slower male muscles (AIL and FCR) were less fatiguable than the faster female muscles (Table 2; Fig. 4). Peters (1994) also noted, in a study of two frog hindlimb muscles, that the slower motor units were less fatiguable, but they were the motor units that produced the largest forces. Among tongue muscles in three frog species (Peters and Nishikawa, 1999), no correlation among force, contraction speeds and fatiguability was found. Thus, the physiological mechanisms that determine contraction times are not necessarily linked to those affecting fatiguability. These and the force properties may vary according to the muscle under study.

It is clear from our study that the contractile differences between males and females are not based only on size. The tendency for slower contraction and relaxation times and lower fatiguability in males indicates that their muscles are not simply scaled-up female muscles, but are physiologically different. Are the differences between males and females based on differences in the muscle fiber types present? Several studies (Sassoon et al., 1987; Regnier and Herrera, 1993b; Dorlochter et al., 1994) have compared muscle fiber properties between dimorphic muscles of castrated males and those of castrated, testosterone-treated males. They found that testosterone has a marked effect on increasing muscle fiber size and oxidative capacity. Studies that compare muscle fiber types between normal males and females in these dimorphic muscles (Thibert and Nicolet, 1975; Melichna et al., 1972; Rubinstein et al., 1983; Oka et al., 1984) have looked at a single muscle only (usually the FCR). The results have been inconsistent across the variety of species examined. For instance, in their study of *Rana temporaria*, Melichna et al. (1972) found that, in the FCR of females, the muscle fibers are all of the fast-twitch type; in addition to these, the male muscles also had tonic fibers. In *Bufo japonicus*, both male and female muscles had equal proportions of twitch and tonic muscle fibers (Oka et al., 1984). If we assume that bullfrogs have mixed populations of muscle fibers in these muscles, during amplexus one would expect that only tonic or slow fibers would be active. Presumably, during initial contact and when disturbed, males would recruit the fast-twitch fibers. Our experiments could not identify or differentially recruit these fibers, so that much more attention needs to be paid to muscle fiber studies in these dimorphic muscles and to species-specific differences to gain a better understanding of the relationship between muscle fiber diversity and functional diversity.

Fatiguability and sustained force

Generally, in the species studied, dimorphic male muscles have greater fatigue resistance (inferred from the higher staining intensity for oxidative enzymes, e.g. succinate dehydrogenase) than the female muscles (Sassoon et al., 1987; Regnier and Herrera, 1993b). Our fatigue data show that, on direct measurement, two of the dimorphic forelimb muscles in males (AIL and FCR) were significantly less fatiguable than in females (Fig. 4). Since several studies have shown that resistance to fatigue and/or oxidative capacity increase with increasing testosterone levels (Thibert and Nicolet, 1975; Norris et al., 1980; Regnier and Herrera, 1993b), it is possible that this difference is enhanced during the breeding season and that the other dimorphic muscle (ECR) might also be less fatiguable in males during the breeding season. Comparisons between males and females need to be made when both are in breeding condition to assess fully the effects of hormonal changes on muscle properties and functions.

The most intriguing result from our work is the observation that, during repetitive stimulation, high levels of unrelaxed or sustained force are maintained between stimulus trains (Figs 5–7). The typical response of muscle to prolonged stimulation is fatigue, which is defined by declining force production, a slowing of shortening velocity and an elongation of relaxation time (e.g. Lannergren and Westerblad, 1989; Westerblad and Lannergren, 1991). In the dimorphic muscles we tested, females responded to repetitive stimulation with these expected changes. However, in the male muscles, under isometric conditions similar to those expected during amplexus, the force did not decline dramatically over the period of stimulation (Fig. 4). This was due largely to the extreme elongation of relaxation time, which minimized the decline in force between stimulus trains (Fig. 5).

Elongation of relaxation time with prolonged stimulation has been well described in both amphibians (Cannell, 1986; Curtin, 1986; Allen et al., 1989; Westerblad and Lannergren, 1990) and mammals (Edwards et al., 1975; Gillis, 1985; Westerblad and Lannergren, 1991). Most studies have concentrated on elucidating the biochemical and cellular mechanisms that explain the increased relaxation times. Since increased relaxation time decreases the rate at which a muscle can be reactivated for repetitive movements, it is normally seen as a negative effect of fatigue. However, we are unaware of any previous reports showing that increased relaxation times resulted in the prolonged and high levels of sustained force such as we found during repetitive stimulation in the dimorphic muscles of bullfrogs. Our results show that, in spite of a clear reduction in the stimulus-activated component of total force over a 4 min fatigue test, the increase in sustained force allows high levels of total force to be maintained (Figs 5, 7). In addition, our results show that, while this response is present in the dimorphic muscles of both sexes, it is much greater in the males (Figs 6, 7).

One could argue that the intermittent stimulus regime we used over only 4 min is inadequate to evaluate these effects in a prolonged behavior such as amplexus. For this reason, we tested the FCR in several males and females using an 18 min regime of stimulation. As explained above (see Results), an initial 4 min of intermittent stimulation at 30 pulses s^{-1} was followed by 10 min of low-level stimulation (at 10 pulses s^{-1}) and then by another 4 min of increased stimulation frequency (at 30 pulses s^{-1}). Although natural amplexus may last much longer, this pattern of high–low–high stimulation frequency for 18 min was considered adequate as a first attempt to examine the dynamics of the sustained force. Our isometric procedure in which the whole muscle is maximally activated is unnatural in two ways: (i) it constricts all the capillaries at once, depriving all muscle fibers of blood flow for brief periods, and (ii) all the motor units are activated at once. If, in normal behavior, alternative pools of motor units turn on and off to sustain adequate force (Burke, 1981), then blood can continue to flow into most regions of the muscle, and some motor units can be rested while others are active. Thus, an 18 min period of continuing intermittent contraction could be magnified into many hours if the motor units take turns at being active. The results of this test (Fig. 7) clearly show that low-level, intermittent stimulation maintains the cellular environment that causes sustained force and allows the muscle to respond quickly to increased demand.

The pattern of sustained force we found is the same in the dimorphic muscles of both males and females, but males produce much more sustained force [a maximum of 1.6 (FCR) to 10 (AIL) times more]. Since the relaxation times of forelimb muscles increase with increasing testosterone levels (Regnier and Herrera, 1993a; Melichna et al., 1972), this response is very probably affected by hormone levels and may be enhanced during breeding. Increased relaxation times, and perhaps the sustained force response, are probably caused either by a decrease in cross-bridge cycling time (Edwards et al., 1975; Westerblad and Lannergren, 1991) or by the increased levels of free Ca^{2+} that would occur if there were a decrease in the rate of Ca^{2+} sequestration in the sarcoplasmic reticulum (Allen et al., 1989; Westerblad and Lannergren, 1991). The high cytosolic [Ca^{2+}] would then result in prolonged cross-bridge formation. The force sustained in the muscle between stimulus trains may therefore occur when cross-bridges form continuously (either by not disengaging or by reforming as fast as they disengage), leading to prolonged periods of force production without nerve input (2–10 s was typical in our data).

Whatever the physiological basis for this response, the functional result in the male bullfrog is to keep total force levels high during long-term stimulation (Fig. 7). If the cross-bridges are locked rather than cycling, this mechanism could save energy. If there is slowed cross-bridge release, energy would also be saved during prolonged isometric contractions, both at the muscle fiber level and by the nervous system because only brief, intermittent stimulation would be required. Thus, a male can grab the female and control her with muscle contractions stimulated at normal levels, but then maintain a contracture-like condition with decreased stimulation.

Much work remains to be done to examine this phenomenon fully. Is it due to the same molecular mechanisms that underlie increased relaxation time during normal fatigue or are unique mechanisms involved in the maintenance of such high levels of unrelaxed force? Do all or most muscle fiber types in males respond in this way, or is this a property found only in tonic fibers? In an effort to test whether tonic muscle fibers might account for the response in bullfrogs, we tested two males and one female by perfusing the FCR with a solution containing a high concentration of K^+ (100 mmol l^{-1}) known to stimulate contracture in tonic fibers (Carrier, 1989). In neither sex was any measurable force produced, suggesting that the levels of sustained force (up to 90 % of total force in some FCRs tested) cannot be accounted for by the action of tonic fibers alone.

In summary, our results support and extend previous findings that the dimorphic forelimb muscles of bullfrogs are much larger, slower-contracting, slower-relaxing and slower to fatigue in males than in females. We describe the phenomenon of sustained force for the first time, and hypothesize that sustained force may save energy at both the muscle and neural levels in muscles that are called upon to maintain a postural position for prolonged periods. Whether this response results from greater numbers of tonic muscle fibers in males (or another specialized type yet to be described) or whether different muscle fiber types show the effect is unknown. It is clear that comprehensive studies of these muscles that include kinematics and electromyography, muscle fiber histochemistry and biochemistry and contractile properties are necessary before we fully understand the basis for the sexual dimorphism in forearm muscles of frogs and the role played by that dimorphism in amplexus.

Our thanks go to Jennifer Warren for her valuable work on the prolonged tests of sustained force. We appreciate the technical help of Sandra Zane and Steven Clark in producing the final figures. Many thanks are due to Sharon Emerson, Kiisa Nishikawa, David Bashor and Elizabeth Meischer-Clemens for their thoughtful comments on an early draft of the manuscript. This work was supported in part by a Grant-in-Aid of Research from the Society of Sigma Xi and by funds provided by the University of North Carolina at Charlotte.

References

Allen, D. G., Lee, J. A. and Westerblad, H. (1989). Intracellular calcium and tension during fatigue in isolated single muscle fibres from *Xenopus laevis*. *J. Physiol., Lond.* **415**, 433–458.

Burke, R. E. (1981). Motor units: Anatomy, physiology and functional organization. In *Handbook of Physiology*, section I, vol. 2, *The Nervous System: Motor Control* (ed. V. B. Brooks), pp. 345–422. Bethesda, MD: American Physiological Society.

Burke, R. E., Levine, D. N., Zajac, R. E., Tsairis, P. and Engel, W. K. (1971). Mammalian motor units: Physiological–histochemical correlation in three types in cat gastrocnemius. *Science* **174**, 709–712.

Cannell, M. B. (1986). Effects of tetanus duration on the free calcium during relaxation for frog skeletal muscle fibres. *J. Physiol., Lond.* **376**, 203–218.

Carrier, D. R. (1989). Ventilatory action of the hypaxial muscles of the lizard *Iguana iguana*: a function of slow muscle. *J. Exp. Biol.* **143**, 435–457.

Curtin, N. A. (1986). Effects of carbon dioxide and tetanus duration on relaxation of frog skeletal muscle. *J. Muscle Res. Cell Motil.* **7**, 269–275.

Dorlochter, M., Astrow, S. H. and Herrera, A. A. (1994). Effects of testosterone on a sexually dimorphic frog muscle: Repeated *in vivo* observations and androgen receptor distribution. *J. Neurobiol.* **25**, 897–916.

Duellman, W. E. (1992). Reproductive strategies of frogs. *Scient. Am.* **267**, 80–87.

Edwards, R. H. T., Hill, D. K. and Jones, D. A. (1975). Metabolic changes associated with the slowing of relaxation in fatigued mouse muscle. *J. Physiol., Lond.* **251**, 287–301.

Emlen, S. T. (1976). Lek organization and mating strategies in the bullfrog. *Behav. Ecol. Sociobiol.* **1**, 283–313.

Epstein, M. S. and Blackburn, D. G. (1997). Histology and histochemistry of androgen-stimulated nuptial pads in the leopard frog, *Rana pipiens*, with notes on nuptial pad evolution. *Can. J. Zool.* **75**, 472–477.

Gaupp, E. (1896). *Anatomie des Frosches* (ed. A. Ecker and R. Wiedersheim). Braunschweig: Friedrich Vieweg und Sohn.

Gillis, J. M. (1985). Relaxation of vertebrate skeletal muscle. A

synthesis of the biochemical and physiological approaches. *Biochem. Biophys. Acta* **811**, 97–145.

Goslow, G. E., Jr, Cameron, W. E. and Stuart, D. G. (1977). Ankle flexor muscles in the cat: Length–active tension and muscle unit properties as related to locomotion. *J. Morph.* **153**, 23–38.

Herrera, A. A. and Regnier, M. (1991). Hormonal regulation of motor systems: how androgens control amplexus (clasping) in male frogs. In *Visual Structures and Integrated Functions, Research Notes in Neural Computing* (ed. M. A. Arbib and J.-P. Ewert), pp. 369–379. New York: Springer-Verlag.

Howard, R. D. (1978). The evolution of mating strategies in bullfrogs, *Rana catesbeiana*. *Evolution* **32**, 850–871.

Howard, R. D. (1984). Alternative mating behaviors of young male bullfrogs. *Am. Zool.* **24**, 397–406.

Howell, A. (1935). Sexual difference in the muscles of Salientia. *Copeia* **4**, 188–189.

Kirby, A. C. (1983). Physiology of the sternoradialis muscle: sexual dimorphism and role in amplexus in the leopard frog (*Rana pipiens*). *Comp. Biochem. Physiol.* **74**A, 705–709.

Kurabuchi, S. (1993). Fine structure of nuptial pad surface of male ranid frogs. *Tissue& Cell* **25**, 589–598.

Lannergren, J. and Westerblad, H. (1989). Maximum tension and force–velocity properties of fatigued, single *Xenopus* muscle fibres studied by caffeine and high K^+. *J. Physiol., Lond.* **409**, 473–490.

Luff, A. R. and Proske, U. (1976). Properties of motor units of the frog sartorius muscle. *J. Physiol., Lond.* **258**, 673–685.

Luff, A. R. and Proske, U. (1979). Properties of motor units of the frog iliofibularis muscle. *Am. J. Physiol.* **236**, C35–C40.

Melichna, J., Gutmann, E., Herbrychova, A. and Stichova, J. (1972). Sexual dimorphism in contraction properties and fibre pattern of the flexor carpi radialis muscle of the frog (*Rana temporaria* L.). *Experientia* **28**, 89–91.

Muller, E. R. A., Galavazi, G. and Szirmai, J. A. (1969). Effect of castration and testosterone treatment on fiber width of the flexor carpi radialis muscle in the male frog (*Rana temporaria* L.). *Gen. Comp. Endocr.* **13**, 275–284.

Nagaya, N. and Herrera, A. A. (1992). Facilitation compensates for lowered synaptic efficacy in a sexually dimorphic muscle. *Soc. Neurosci. Abstr.* **18**, 235.

Norris, D. O., Jackson, R. L. and Silins, M. H. (1980). Measurement of muscle performance *in vitro* following *in vivo* steroid hormone treatment of immature female frogs. *Comp. Biochem. Physiol.* **67**A, 687–689.

Oka, Y., Ohtani, R., Satou, M. and Ueda, K. (1984). Sexually dimorphic muscles in the forelimb of the Japanese toad, *Bufo japonicus*. *J. Morph.* **180**, 297–308.

Peters, S. E. (1994). Properties of twitch motor units of the ankle extensor muscles in the bullfrog, *Rana catesbeiana*. *J. Morph.* **221**, 121–131.

Peters, S. E., Kamel, L. T. and Bashor, D. P. (1996). Hopping and swimming in the leopard frog, *Rana pipiens*. I. Step cycles and kinematics. *J. Morph.* **230**, 1–16.

Peters, S. E., Mulkey, R., Rasmussen, S. A. and Goslow, G. E., Jr, (1984). Motor units of the primary ankle extensor muscles of the opossum (*Didelphis virginiana*): Functional properties and fiber types. *J. Morph.* **181**, 305–317.

Peters, S. E. and Nishikawa, K. C. (1999). Comparison of isometric contractile properties of the tongue muscles in three species of frogs, *Litoria caerulea*, *Dyscophus guinetti* and *Bufo marinus*. *J. Morph.* **242**, 107–124.

Pette, D. and Vrbova, G. (1985). Neural control of phenotypic expression in mammalian muscle fibers. *Muscle Nerve* **8**, 676–689.

Regnier, M. and Herrera, A. A. (1993a). Changes in contractile properties by androgen hormones in sexually dimorphic muscles of male frogs (*Xenopus laevis*). *J. Physiol., Lond.* **461**, 565–581.

Regnier, M. and Herrera, A. A. (1993b). Differential sensitivity to androgens within a sexually dimorphic muscle of male frogs (*Xenopus laevis*). *J. Neurobiol.* **24**, 1215–1228.

Ridge, R. M. A. P. and Thomason, A. M. (1980). Properties of motor units in a small foot muscle of *Xenopus laevis*. *J. Physiol., Lond.* **306**, 17–27.

Rubinstein, N., Eruldar, S. and Schneider, G. (1983). Sexual dimorphism in the fibers of a clasp muscle of *Xenopus laevis*. *Exp. Neurol.* **82**, 424–431.

Sacks, R. D. and Roy, R. R. (1982). Architecture of the hind limb muscles of cats: Functional significance. *J. Morph.* **173**, 185–195.

Sassoon, D. A., Gray, G. E. and Kelley, D. B. (1987). Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. *J. Neurosci.* **7**, 3198–3206.

Smith, C. (1938). The clasping reflex in frogs and toads and the seasonal variation in the development of the brachial musculature. *J. Exp. Biol.* **15**, 1–9.

Spector, S. A., Gardiner, P. E., Zernicke, R. F., Roy, R. R. and Edgerton, V. R. (1980). Muscle architecture and force–velocity characteristics of cat soleus and medial gastrocnemius: Implications for motor control. *J. Neurophysiol*. **44**, 951–960.

Strickland, N. C. (1978). Muscle weights and succinic dehydrogenase distribution in the hind limb musculature of two rodents (*Thryonomys gregorianus* and *Pedetes capensis*) with different locomotory habits. *Acta Anat.* **102**, 203–208.

Thibert, P. and Nicolet, M. (1975). Tonic properties of the flexor carpi radialis muscle of the male frog (*Rana temporaria*). *Pflügers Arch.* **356**, 253–265.

Wells, K. D. (1977). The social behaviour of anuran amphibians. *Anim. Behav.* **25**, 666–693.

Westerblad, H. and Lannergren, J. (1990). Decreased Ca^{2+} buffering contributes to slowing of relaxation in fatigued *Xenopus* muscle fibres. *Acta Physiol. Scand.* **139**, 243–244.

Westerblad, H. and Lannergren, J. (1991). Slowing of relaxation during fatigue in single mouse muscle fibres. *J. Physiol., Lond.* **434**, 323–336.

Yekta, N. and Blackburn, D. (1992). Sexual dimorphism in mass and protein content of the forelimb muscles of the northern leopard frog *Rana pipiens*. *Can. J. Zool.* **70**, 670–674.

Zug, G. R. (1978). Anuran locomotion – Structure and function. II. Jumping performance of semiaquatic, terrestrial and arboreal frogs. *Smithson. Contr. Zool.* **276**, 1–31.