Season and testosterone affect contractile properties of fast calling muscles in the gray tree frog *Hyla chrysoscelis*

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Running Head: Testosterone effects on high-frequency muscle

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Abstract
In anurans, circulating levels of androgens influence certain secondary sexual characteristics that are expressed only during the breeding season. We studied the contractile properties of external oblique muscles (used to power sound production) in a species of North American gray tree frog, *Hyla chrysoscelis*, during the breeding season and also in testosterone-treated captive males and females after the breeding season. As compared to the muscles of breeding-season males, the trunk muscles of post-breeding-season males have 50% less mass, 60% longer twitches, and 40% slower shortening velocities. Testosterone levels similar to those found in breeding-season male hylid frogs restore the contractile speed and mass of male trunk muscles and also convert the small slow trunk muscles of females into larger fast-contracting muscles. We conclude that androgens are likely play a key role in altering the contractile properties of these muscles in males during the annual cycle, allowing them to operate in the breeding season at the frequencies required to produce the characteristic rapidly pulsed calls of this species. Females as well as non-breeding-season males do not produce advertising calls and therefore the slower muscles found in these animals may allow more economic operation of these muscles. The effects of testosterone on female trunk muscles indicate the potential of this hormone in contributing to the sexual dimorphism in size and contractile properties of these muscles, but this dimorphism is likely due to the interaction of more than one hormone.

Introduction
The effects of androgens on skeletal muscle have been of great interest in part because of the controversial question of whether human muscle size and strength are enhanced by exogenous testosterone (3). In the broader context of sexual dimorphism in vertebrates the effects of testosterone on human muscle are perhaps best viewed as resulting from the evolution of sexual dimorphism in primates (35). In other vertebrates, a number of sexually dimorphic neuromuscular structures that underlie reproductive behaviors are known to be androgen-sensitive either in a developmental context or acutely (6-8,11, 20, 26, 33, 48, 53). The high sensitivity of these structures to androgens is related to the expression of a high numbers of androgen receptors (7, 13, 25), which presumably trigger specific genes regulating muscle size and contractile properties.
Muscles used for male-specific reproductive behaviors have been found to be sexually dimorphic in a number of amphibian species. Laryngeal muscles used to produce calls in *Xenopus* are sexually dimorphic and have been extensively studied in the past decade (20-25, 43, 61). Flexor carpi radialis, a primary forelimb muscle used by male amphibians for clasping during mating, also has been shown to be sexually dimorphic in size, fiber type, and contractile properties (32, 39, 40, 42). In North American toads *Bufo fowleri*, a sexual difference in the thickness of the trunk wall is seen throughout the year, but becomes more marked during the breeding season (4). Marsh and Taigen (29) documented marked sexual dimorphism in size and enzymatic capacities of the trunk muscles in North American gray tree frog, *Hyla versicolor*. Seasonal variation in the degree of sexual dimorphism may be caused by seasonal changes in levels of androgens (26). Androgen levels in males of seasonally breeding amphibians increase during the breeding season and seem to correlate with expression of secondary sexual characters such as clasping behavior and production of advertisement calls (26, 32).

Communication using loud calls is an important aspect of the annual reproductive behavior of many anurans. Males produce advertising calls to attract gravid females. Vocalization is also used to mediate aggressive signals to conspecific males (15, 24, 55). Breeding activity in anuran species found in temperate climates is limited to spring and summer, thus, the vocal chorusing also shows a similar seasonal pattern (55). Vocalization during advertising is one of the most energetically demanding activities, requiring up to 20 times the energetic cost experienced at rest (36, 37, 49). Sound production in most anurans is powered by cyclical contraction of trunk muscles (external and internal oblique muscles) (17, 30, 31). In addition, laryngeal muscles have been also shown in several species to be involved in modulating the call structure (44, 45). In pipid frogs including *Xenopus* the laryngeal muscles provide the major power source for vocalization (20). The muscles, which are used in sound production, are quite different from typical amphibian muscles. They consist of 100% fast oxidative glycolytic fibers having high citrate synthase activity, high mitochondrial and capillary densities and high ATPase activity (29, 41).

Two recent studies focused on understanding contractile properties of trunk muscle (external obliques) in two species of North American gray tree frogs, *Hyla versicolor* and *Hyla chrysoscelis* (18, 27). *Hyla versicolor* is a tetraploid species that has most likely evolved from the diploid *Hyla chrysoscelis* (38). These two species are morphologically identical, but differ in
their call structures (16, 17). *In vivo* operating frequencies of these muscles are relatively high (operating frequencies at 25°C are 25 Hz and 50 Hz for *Hyla versicolor* and *Hyla chrysoscelis*, respectively) and are matched with the pulse frequency within a call (18). *In vitro* studies have shown that at similar temperatures the external oblique muscles have short twitch durations and high intrinsic shortening velocities to match the *in vivo* operating frequencies (18, 27). Power output measured from these muscles is also high, thus making them suitable for providing energy to produce loud calls (18). Most of these measurements of contractile properties, enzymatic activities and ultra-structures of the trunk muscles of hylid frogs have been done during the breeding season, and thus, there is little information available regarding the seasonal control of these properties. However, in his study Marsh (27) reported a significant decrease in shortening velocities recorded from an animal two weeks after the calling season, suggesting a possibility of seasonal changes in contractile properties of these muscles.

We hypothesized that testosterone might have the potential to acutely modify the contractile properties of the calling muscles in gray tree frogs, thus making it a likely candidate for a natural signal seasonally controlling the properties these muscles in breeding frogs. A previous study has shown that trunk muscles in frog undergo hypertrophy in response to androgens (13). However, influences of steroids on the contractile properties of trunk muscle used in calling have not been investigated to date. Testosterone control of the contractile properties of the trunk muscles is particularly interesting because these rapidly contracting muscles function quite differently than the flexor carpi radialis muscle, in which androgens cause a slowing of contraction (12, 39, 40). In our present study, we used *Hyla chrysoscelis* to systematically compare the contractile properties of trunk muscles in animals during breeding season with those seen in animals during the post-breeding time of the year, and to examine the effects of exogenous testosterone on these muscles in post-breeding season males and females.

**Methods**

**Animals**

*H. chrysoscelis* Cope were collected in Wilson County, Tennessee by a commercial supplier. One group of males was collected in mid-May for breeding-season studies. Another group of males and females were collected in early July (while males were still chorusing in the field) and maintained in the laboratory for studies during the post-breeding season (during Fall).
Collection of animals during the breeding season followed by laboratory housing was required because of the difficulty of collecting animals from the field in the post-breeding season. Males collected in May and July were similarly sized (6.89 ± 0.3 g and 5.96 ± 0.23 g respectively). Females had a mean mass of 10 ± 1.26 g. Animals were housed in glass aquaria with beds of moist sphagnum moss and a water source. Frogs were fed crickets at least twice a week. The crickets were coated with powdered calcium carbonate and multi-vitamins. Frogs studied during the breeding season were maintained at 25°C in a L:D cycle of 15:9 hours and contractile studies were completed within two weeks after the animals arrived in the laboratory. The frogs held for post-breeding season studies were maintained at 25°C in a light dark cycle of 12:12 hours. All procedures were undertaken under a protocol approved by the Northeastern University Animal Care and Use Committee.

Muscle preparation

The animals were killed by destroying the brain and this procedure was followed by a spinal pith. The external and internal obliques muscles are closely apposed sheet-like muscles surrounding the anuran trunk (31). They originate on the vertebral spines and insert near the mid-line on the ventral surface. Approximately 3-mm-wide muscle strips were dissected from origin to insertion parallel to the orientation of the fibers of the external oblique. The strip consisted of intact external oblique fibers and adherent small fragments of fibers from the internal oblique. However, these fragments are short and non-contractile. Following the contractile measurements, the muscle length was measured at the length resulting in maximum isometric force (L₀). The fragments of internal oblique fibers and small amounts of connective tissue were dissected away from the intact external oblique fibers under a dissecting microscope. After blotting, the mass of the external oblique strip was determined using a Mettler analytical balance. Cross-sectional area of the active fibers muscle was estimated from these measurements assuming a density of 1 gm cm⁻³.

Measurement of contractile properties during breeding season

Similar contractile measurements were performed with both breeding season and post-breeding season animals. During the measurements the muscles were placed vertically in a Plexiglas chamber and bathed with circulating oxygenated Ringer’s solution (115 mM NaCl, 2.5
mM KCl, 1.0 mM MgSO4, 20 mM imidazole, 1.8 mM CaCl2, 11 mM pyruvic acid, pH 7.9). This solution was oxygenated for at least an hour before the experiment and maintained at 25°C. The dorsal end of the muscle was secured with a silk thread to a stainless steel hook at the bottom of the chamber. A lightweight silver chain was tied to the ventral end of the muscle with silk thread. The chain was used to attach the muscle to the lever of a Cambridge Technology ergometer (model 300B) lever. Force and length outputs were digitized by a MacAdios II, 12-bit A-D converter running in a Macintosh computer. Sampling frequency was 2000 Hz. The muscle was supra-maximally stimulated using two parallel platinum plate electrodes. Square-wave stimuli of 0.5 ms were produced by an audio power amplifier connected to a Grass S48 stimulator, which generated the stimuli under computer control.

The muscle was allowed to recover from the dissection for approximately thirty to forty-five minutes before any contractile measurements were done. After the recovery period, optimal length (L₀) of the muscle was determined using a series of twitches and tetani. The optimum length of the muscle was defined as the length that yielded maximal tetanic force (P₀). At L₀, time to peak force in a twitch (tₚₜw) and time to half relaxation (t₅₀%R) were determined. Maximal force produced was measured in isometric tetani. A rest period of one and three minutes was allowed between twitches and tetani, respectively.

Subsequently the force-velocity characteristics of the muscles were determined by subjecting them to 10-12 after-loaded isotonic contractions starting at L₀. The forces in these isotonic contractions ranged from 0.9 P₀ to as low as 0.01 P₀. The data were described by fitting a three parameter hyperbolic - linear equation (28):

\[ V = \frac{B (1-P/P₀)}{(A+ P/P₀)} + C(1-P/P₀) \]

where, V is the shortening velocity in muscle lengths per second (L₀ s⁻¹) and P is the force in N cm⁻², B and C are constants with dimensions of L₀ s⁻¹ and A is a constant with no dimensions. These curves were fitted using a non-linear curve fitting routine in the application Igor (WaveMetrics). Two statistical methods were used to describe these data. First, a composite curve was fitted to the data collected from all the animals. These curves provide a good representation of the average properties of the muscle. Additionally, data from each animal was fitted individually allowing us to generate mean values to use in statistical comparisons of the groups. Maximum shortening velocity (V_max) was estimated by extrapolating the curve to zero force. Maximum isotonic power output was calculated from the force-velocity relationships. The power ratio (R_p),
which is a measure of curvature of the force velocity relation, was calculated by dividing the maximum isotonic power by the product of \( V_{\text{max}} \) and \( P_0 \) after conversion to appropriate units.

*Post-breeding season studies of contractile properties and effects of exogenous testosterone*

During August, males were arbitrarily assigned to either a testosterone treated group or a control group. Testosterone treatment was done as previously described (51, 39 and 40). Testosterone propionate (a testosterone ester which is reconverted *in vivo* to free testosterone) was packed in silastic tubing (i.d. 0.3 mm and o.d. 0.6 mm, Dow Corning) and made into small pellets with ~3 mm of testosterone-filled length by sealing the ends with silicone cement. Animals to receive a pellet were anesthetized by immersing them in 0.5% aqueous 3-aminobenzoic acid ethyl ester (Sigma, USA). Eight animals in the testosterone group received the testosterone pellet in their intra-peritoneal cavity through a small ventral abdominal incision of about 4 mm. The incisions were sutured with silk thread. Of the animals in the control group, four animals received empty pellets made of the silastic tubing and the other four were left un-operated and received no implant. Animals with either an empty pellet or no implant showed similar properties and were combined to form the “untreated” post-breeding season group. Six female frogs were also included in the study. Half of them received testosterone propionate pellet, one received an empty pellet and other two were left un-operated. All animals recovered within 1 or 2 hours after the surgery. After the operation they were treated with tetracycline (0.5 mg / 30 g of body weight) using a stomach tube once daily for seven days after the operation. Following the operation, frogs were kept in individual containers for 12 weeks before any contractile measurements were done. We selected this time period based on a previous study (26).

*Measurement of testosterone levels, muscle mass and contractile properties:*

When the animals were sacrificed for contractile studies approximately 0.25 ml of blood was drawn from each with a heperanized micro hematocrit tube. The tubes were centrifuged and the plasma was stored at -20°C until analyzed. Plasma testosterone levels were measured by RIA on ether extracted, non-chromatographed, plasma samples using a commercial kit, the BiotrakTM testosterone/dihydrotestosterone [3H] assay system from Amersham. Relative
muscle size was determined by measuring the combined mass of the two sets of trunk muscles (internal and external obliques) and expressing the data as a percent of total body mass.

Statistics

All data are presented in this study as means ± standard errors. For comparison of values obtained for contractile properties measured in different groups, mean values were compared using one-way ANOVA, with the different treatment group as the factor. Pair-wise comparisons were done with the Bonferroni-Dunn post-hoc procedure.

Results

Plasma testosterone levels

Plasma testosterone levels for the treated male frogs during post-breeding season were much higher (49 ± 3.79 ng ml⁻¹) than the levels in untreated animals (both un-operated and operated), which were non-detectable (<3 ng ml⁻¹). The three testosterone-treated females had similar testosterone levels as found in the treated males (49.5 ± 5.09 ng ml⁻¹). Control females also had a non-detectable amount of testosterone.

Size of the trunk muscle

The trunk muscles of male *H. chrysoscelis* experience a significant atrophy (p<0.0001) after the breeding season is over, decreasing to about one-half the mass of the muscles in breeding-season males (Fig. 1). Even at this reduced size, the trunk muscles in post-breeding season males weighed more than twice as much as these muscles in untreated females (Fig. 1). Testosterone evoked a dramatic increase in relative size of the trunk muscles in post-breeding season males as well as in females. Testosterone treatment increased muscle mass by 2.2 fold in treated post-breeding season males (p<0.0001) and by 2.8 fold in treated females (p=0.0008) (Fig. 1).
**Isometric properties**

External oblique muscles in breeding-season males have significantly shorter twitch duration than that found in the same muscle in post-breeding season males (Fig. 2A, Fig. 3). Both the time to peak tension ($t_{ptw}$) and the 50% relaxation time ($t_{50\%R}$) were shorter in breeding-season males, and overall the twitch duration (sum of $t_{ptw}$ and $t_{50\%R}$) was 24% shorter. Testosterone treatment of post-breeding season males restored twitch times to values that are not significantly different from breeding-season males. Twitch duration of the external oblique muscles of these treated males was 30% shorter than the value for untreated post-breeding season males, a highly significant difference. Twitch kinetics in testosterone treated females were very different than those seen in the control female group (Fig. 2B, Fig. 3). The muscles of untreated females had twitches even longer than those of post-breeding season males. Testosterone treatment reduced the twitch times found in female muscles by half resulting in values similar to those in breeding season or testosterone treated males.

Peak isometric forces ($P_0$) measured per cross-sectional area of muscle recorded in the testosterone treated males and untreated males in the post-breeding season were similar to each other (7.56 ± 0.3 N cm$^{-2}$ and 7.86 ± 1.05 N cm$^{-2}$ respectively, $p=0.79$). These values were significantly lower than those measured during the breeding season (10.4 ± 2.6 N cm$^{-2}$, $p=0.0267$). In females, $P_0$ values in control and testosterone treated animals were not significantly different (9.72 ± 0.31 N cm$^{-2}$ and 7.68 ± 0.76 N cm$^{-2}$, respectively, $p=0.0899$).

**Isotonic properties**

At 25°C there was a significant ($p<0.0001$) decline in the maximum shortening velocities ($V_{max}$) measured in post-breeding season animals (8.60 ± 0.20 L$_0$ sec$^{-1}$) compared to $V_{max}$’s measured during the breeding season (13.35 ± 0.58 L$_0$ sec$^{-1}$) (Fig. 4A and D; Table 1). The mean $V_{max}$ increased in response to testosterone treatment (12.46 ± 0.29 L$_0$ sec$^{-1}$) (Fig. 4B). This value was significantly greater than that measured in untreated post-breeding season animals ($p<0.0001$) and was similar to the mean $V_{max}$ of breeding-season males ($p=0.61$) (Fig. 4D). The $V_{max}$’s measured in testosterone treated females (11.61 ± 0.83 L$_0$ sec$^{-1}$) were significantly ($p<0.0001$) higher than those obtained for the control females (6.34 ± 0.33 L$_0$ sec$^{-1}$) (Figs 4C and D).
Maximum isotonic power outputs were estimated based on the force-velocity curve. A significant decline (p<0.0001) occurred in maximum isotonic power output in post-breeding season males compared to the breeding-season males (96.55 ± 6.2 W kg⁻¹ and 223.4 ± 9.5 W kg⁻¹ respectively) (Table 1). In testosterone treated males, maximum powers (164.5 ± 14 W kg⁻¹) increased significantly over those measured in post-breeding season untreated males (p<0.0042). Maximum isotonic power measured for testosterone treated females was 147.8 ± 15 W kg⁻¹ and that for control females was 69.19 ± 2.23 W kg⁻¹ (p=0.0025).

Discussion

The present study clearly shows marked seasonal variation in the size and contractile properties of trunk muscles in male tree frogs, demonstrates sexual dimorphism of these properties, and provides evidence for the control of these properties by testosterone. These data do not demonstrate that testosterone is the signal that determines sexual or seasonal differences in these properties in natural populations, but they clearly show that this hormone has the potential to do so at physiological levels. Seasonal variation in testosterone has not been documented in *Hyla*, but males in other genera of anurans show substantial seasonal changes in this hormone, e.g., 15 fold in *Rana esculenta* (34). To our knowledge, testosterone levels during breeding season have not been measured in *H. chrysoscelis*. However, testosterone implanted animals in this study had plasma testosterone levels, 49 ng ml⁻¹, that were very similar to those reported for chorusing breeding-season males in other species of *Hyla*, 32-36 ng ml⁻¹ (9, 46).

Comparative data from other anurans

In anurans, most previous studies have focused on two groups of sexually dimorphic muscles, the laryngeal muscles in *Xenopus* (5, 20, 21, 43, 51, 54) and the clasper muscles in *Xenopus* and also in other anurans (32, 39, 40, 48, 50). The larynx of *Xenopus* and other pipid frogs is quite unusual in structure, and sound production is apparently powered directly by the laryngeal muscles rather than the trunk muscles as in many anurans including tree frogs (14, 20). The clasper muscles are used by male frogs and toads to grip and hold females during amplexus. Both of these muscle groups are androgen-sensitive; however, the influence of androgens on the laryngeal muscles differs from that on the clasper muscles. Androgens seem to bring about permanent organizational changes in the structure and function of the laryngeal muscles during a
critical period in development (54). These muscles in adult males show no seasonal changes in structure and function and also do not show altered mass or fiber type in response to androgen deprivation (43, 47). In other vertebrates, androgen is known to have similar developmental influences on sexually dimorphic muscles (6, 53). In contrast to the solely developmental role of androgens in the laryngeal muscles of *Xenopus*, these hormones play an acute role in altering the properties of the clasper muscle. Structural and functional properties of these muscles vary with the levels of circulating androgens have been concluded to mediate seasonal changes in mature animals (21, 39, 40, 48, 54). Our data suggest that androgens also acutely alter the size and contractile properties of the trunk muscles of male hylids during the breeding season. Whether androgen also plays an organizational role during development of these muscles or not is presently unknown, although we have shown that the trunk muscles of untreated post-breeding-season males are considerably larger than those of females.

*Twitch kinetics*

The twitch properties of the trunk muscles of males measured during the breeding season were very different from those seen during the post-breeding season. Also, the twitch properties show sexual dimorphism, with females having twitch durations almost twice as long as those seen in seasonal males. Overall twitch duration of external oblique muscles during the breeding season in *H. chrysoscelis* is ~23 ms at 25°C, which is well matched with the operating frequency 40-50 Hz at 25°C) of these muscles (18, 27). The twitches measured in post-breeding animals are ~35% longer when compared to those measured in breeding season animals. Because muscles must activate and deactivate during the time available for shortening (18), it seems likely that the muscles of post-breeding-season males would not be capable of operating at 40 to 50 Hz. However, during the post-breeding period of the year the trunk muscles are not used in high frequency contraction and therefore, lengthening the twitch time may function to reduce energy expenditure during contraction.

Testosterone significantly decreased the twitch time in both post-breeding-season males and in females (Figs. 2 and 3A). In testosterone treated males the overall twitch time decreased to ~21 ms, which is very close to the mean values measured during the breeding season. The twitch durations were dramatically shorter in testosterone treated females compared to the untreated females (~20 ms and 40 ms respectively) (Fig. 2B).
Testosterone treatment influences the twitch kinetics of sexual dimorphic muscles in ways that seems to adapt these muscles for their specific roles in successful mating. Our data show that testosterone shortens contraction time in hylid trunk muscle, a change necessary for high frequency operation during calling. Sassoon and Kelly (43) have reported faster twitches in laryngeal muscles (involved in sound production) in *Xenopus* in response to increasing levels of plasma testosterone during post-metamorphic development. In contrast, the twitch durations become longer in the fibers of flexor carpi radialis (one of the clasper muscles) in response to testosterone treatment (42). Slowing of this muscle presumably adapts it to maintain grip with minimal fatigue for prolonged periods of time (42). Twitch duration in this muscle in *Rana temporaria* is shortest during summer (the post-breeding season for this species) when the endogenous levels of testosterone are low and it lengthens with rising levels of androgen during the breeding season (31).

**Shortening velocity**

Our study is the first to supplement knowledge of androgen effects on twitch kinetics with information on the intrinsic velocity of shortening as measured in isotonic contractions. Maximum isotonic shortening velocity reflects the kinetics of the interaction between myosin and actin and influences the potential for power output by the muscles. Assessing isotonic properties is thus important in comparing the performance of different muscles. Muscles used at high frequencies for power output need to have shortening velocities fast enough to allow substantial work output in each contractile cycle (18). Conversely a reduced V\text{max}, along with lengthened twitch times, should result in lower energy use during muscle use in the post-breeding season.

High shortening velocities were measured in the external oblique muscles during the breeding season (Fig. 4A). High intrinsic velocities and flat force-velocity curves allow this muscle to produce the high power output required for sound production at high operating frequencies (18). Maximum isotonic power measured in females and in the post-breeding-season males were much lower than those recorded in males during the breeding season. Testosterone treatment caused significant increases in V\text{max} and isotonic power output in both post-breeding season males and in females (Figs 4B, C and D).
The changes in shortening velocity must result from altered myosin function under the influence of testosterone. Myofibrilar ATPase activities in flexor carpi radialis of *Rana temporaria* have been reported to be altered along with contractile properties in response to androgen (32). However, several later studies have reported contradictory results with no change in ATPase activity seen in flexor carpi radialis either in *Rana* or in *Xenopus* (8, 40, 55). An androgen-induced myosin heavy chain isoform has been identified in a sexually dimorphic muscle in guinea pigs (26). Also a laryngeal specific, androgen induced, myosin heavy chain has been reported in *Xenopus laevis* (10), however, ontogenetic and hormonal regulation are different in the laryngeal muscles of *Xenopus* compared to the clasper muscles and the oblique muscles of tree frogs. Whether the changes in maximum shortening velocities reported in the present studies are correlated with expression of different myosin heavy or light chain isoforms, or are due to other changes that influence myosin function requires further study.

**Sexual dimorphism**

We have demonstrated in the present study that treatment of adult females with exogenous testosterone transforms the external oblique muscles substantially, resulting in muscles with contractile properties similar to those of males, although the muscles remain smaller than those in breeding season or testosterone-treated males. We have no data on contractile properties of breeding-season females, but the trunk muscles of wild-caught *Hyla* females in the breeding season are small and similar in appearance to the control females in our study (29). However, the determination of the sexually dimorphic properties of these muscles in natural populations is likely to be more complex than simple determination by testosterone level. Other species of female frogs are known to have high levels of androgen during the breeding season (12, 34), however, estrogen levels are also high during this time of the year, which in turn may inhibit the effects of androgen (34, 48). The remarkable changes in the external oblique muscles of females in response to exogenous testosterone documented here may have occurred because the present study was done after the breeding season and the endogenous levels of estrogen were therefore low. These results contrast with observation on the laryngeal system of testosterone-treated adult female *Xenopus laevis* which show only partial masculinization of the laryngeal muscles (19, 43, 52). Further work on trunk muscle system is required to sort out the hormonal control mechanisms in females, but our results clearly demonstrate the responsiveness
of these muscle to testosterone, when administered to captive animals in the post-breeding season.

Conclusions

The results from our study demonstrate atrophy and slowing of contraction in the trunk muscles of male gray tree frogs in the post-breeding season. Administering exogenous testosterone, which restores plasma testosterone levels to values similar to those found in breeding males of other species, was sufficient to restore the properties that allow these muscles to produce high frequency calls during the breeding season. We conclude that differences in circulating levels of testosterone, which have been seen seasonally in other frog species, likely plays a role in seasonal changes in size and contractile properties in the external oblique muscles of *H. chrysoscelis*. During the breeding seasons, these muscles, which are responsible for production of mating calls, are four times larger in males than in females. During the breeding season they have contractile kinetics that enable them to contract at 40-50 Hz and produce high power output at these frequencies. Call parameters such as loudness, pulse repetition rates, and call durations are very important in determining the reproductive success of males of a large number of anuran species (1, 16). Measurements of contractile properties in males during the post-breeding season show much slower twitch kinetics and lower maximum velocity of shortening compared to the properties measured in the breeding season. Maximum isotonic power output also declines during the post-breeding season. The enhanced muscle properties during the breeding season appear adaptive because these muscles operate at high frequencies only during the mating season. Reducing the mass and contractile speed of these muscles in the non-breeding times of the year likely saves energy. Our results also show that the trunk muscles of females are responsive to testosterone, but determining the role of this hormone in females will require further work on interactions of testosterone with estrogens in these animals.

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Table 1. Effect of season and testosterone on the isotonic contractile properties of the external oblique muscle in *Hyla chrysoscelis*.

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>n</th>
<th>$V_{\text{max}}$, L(_0) s(^{-1})</th>
<th>$R_p$</th>
<th>$W_{\text{max}}$, W kg(^{-1})</th>
<th>$P_0$, N cm(^{-2})</th>
<th>A</th>
<th>B, L(_0) s(^{-1})</th>
<th>C, L(_0) s(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Males</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breeding season(^1)</td>
<td>5</td>
<td>13.35 ± 0.58</td>
<td>0.17 ± 0.01</td>
<td>223.4 ± 9.5</td>
<td>10.4 ± 2.6</td>
<td>0.91 ± 0.021</td>
<td>0.51 ± 0.03</td>
<td>5.9 ± 0.46</td>
</tr>
<tr>
<td>Non-breeding season</td>
<td>8</td>
<td>8.60 ± 0.20</td>
<td>0.16 ± 0.004</td>
<td>96.55 ± 6.2</td>
<td>7.86 ± 1.05</td>
<td>0.63 ± 0.036</td>
<td>4.24 ± 0.051</td>
<td>5.53 ± 1.07</td>
</tr>
<tr>
<td>Testosterone treated</td>
<td>7</td>
<td>12.46 ± 0.29</td>
<td>0.14 ± 0.01</td>
<td>164.5 ± 14</td>
<td>7.56 ± 0.3</td>
<td>0.28 ± 0.06</td>
<td>1.46 ± 0.27</td>
<td>2.11 ± 0.28</td>
</tr>
<tr>
<td><strong>Females</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>3</td>
<td>6.34 ± 0.33</td>
<td>0.17 ± 0.01</td>
<td>69.19 ± 2.23</td>
<td>9.72 ± 0.31</td>
<td>0.32 ± 0.03</td>
<td>2.01 ± 0.17</td>
<td>7.143 ± 0.41</td>
</tr>
<tr>
<td>Testosterone treated</td>
<td>3</td>
<td>11.61 ± 0.83</td>
<td>0.11 ± 0.002</td>
<td>147.8 ± 15</td>
<td>7.68 ± 0.76</td>
<td>0.21 ± 0.08</td>
<td>1.39 ± 0.29</td>
<td>1.04 ± 0.36</td>
</tr>
</tbody>
</table>

n= sample size; $V_{\text{max}}$ = maximum shortening velocity at zero force; $R_p$ = power ratio; $W_{\text{max}}$ = maximum isotonic power; A, B, C = constants from hyperbolic linear equation (see methods) fitted to the composite data for each group.

\(^1\)Data for breeding-season males are from Girgenrath and Marsh (18).
Fig. 1. Muscle mass (external and internal obliques) expressed as percent of body mass for male *H. chrysoscelis* during breeding season, testosterone treated (+T) and untreated (-T) males from non-breeding season and testosterone treated (+T) and untreated (-T) females. Hatched bar represents breeding-season males. Open bars represent testosterone treated non-breeding-season males and females. Gray bars are for untreated males and females. Muscle mass decreased significantly in post-breeding-season males compared to that seen in breeding season males (p<0.0001). Testosterone treatment induced muscle hypertrophy in both males and females when compared to their respective control groups (male, p<0.0001; female, p<0.0008).
Fig. 2. Representative twitch contractions of external oblique muscles of *H. chrysoscelis* at 25 °C. (A) Twitch traces from the muscles of three representative males (breeding season, untreated from the non-breeding season, and testosterone treated from non-breeding season) and (B) Twitch traces from two females, one treated with testosterone and one untreated. The latent periods between stimulation and force rise for the males were similar; however the traces have been offset for clarity.
Fig. 3. Mean twitch kinetics of external oblique muscles of *Hyla chrysoscelis* (A) Time to peak force during a twitch and (B) time from peak force to 50% relaxation in a twitch. Mean values for breeding-season males are shown as hatched bars. Values for non-breeding-season males and females are shown as gray bars for untreated animals (-T) and open bars for testosterone treated animals (+T).
Fig. 4. Composite force velocity data for the different experimental groups: (A) breeding-season males; (B) non-breeding-season males; (C) females. For non-breeding-season males and females open symbols and dashed lines indicate untreated animals and closed symbols and solid lines indicate testosterone treated animals. (D) Composite force-velocity curves from the various groups. The constants for the hyperbolic-linear curves are given in Table 1. Data for breeding-season males are from Girgenrath and Marsh (1999). Decrease in Vmax was significant for post-breeding season animals compared with those measured in breeding season animals (p<0.0001). In response to testosterone, there was a significant increase in Vmax males (p<0.0001) as well as females (p< 0.0001) compared to their respective controls.