

Sexual dimorphism and testosterone responsiveness in hypaxial muscles of the northern leopard frog, *Rana pipiens*

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Abstract. Abdominal hypaxial muscles of male anurans function in vocalization by causing forcible expulsion of pulmonary air through the larynx. The hypaxial muscles were studied in the leopard frog, *Rana pipiens*, to test whether functions of these muscles in males are reflected in sexual dimorphism and in androgen responsiveness. The combined abdominal oblique muscles and the rectus abdominis showed significant sexual dimorphism in wet mass and dry mass. However, the abdominal oblique muscles were less concentrated in protein content in males than in females. In overwintering males, exogenous testosterone cypionate stimulated a significant increase in mass and protein content of the abdominal oblique and rectus abdominis muscles. Castration had no effect on muscle mass or on the response to androgen treatment.

Introduction

Among anurans, vocalization plays an essential role in reproduction. By calling, males attract females and establish and protect their breeding territories from other males (Savage, 1934; Wells, 1977, 1978). Although calling is not confined to males in all species, and has been lost evolutionarily in some frogs (Emerson, 1996), the typical anuran pattern is one in which vocalization is predominantly a male attribute (Wells, 1977; Duellman and Trueb, 1986). Vocalization commonly is accomplished through forcible expulsion of air from the lungs through the larynx, powered by contraction of the abdominal musculature (Martin, 1972; Martin and Gans, 1972; Schneider, 1988). The specialized function of the male hypaxial muscles in some hylid frogs is reflected in sexual dimorphism in muscle mass and biochemistry (Taigen et al., 1985; Marsh et al., 1987; Given and McKay, 1990; Wells and Taigen, 1992; also see Bevier, 1995; Ressel, 1996). Consequently, one could postulate that circulating androgens stimulate development of specialized characteristics of the hypaxial muscles in male anurans.

In the North American leopard frog, *Rana pipiens*, differential functions of the fore-limb muscles in males and females are paralleled by a marked sexual dimorphism in

muscle mass, protein content, and fiber composition (Yekta and Blackburn, 1992; Blackburn, 1994). Males use their forelimbs to clasp females during a prolonged period of amplexus, and have correspondingly enlarged forearm adductors and flexors of the elbow and carpus. Experimental treatment with exogenous testosterone stimulates hypertrophy of these same muscles in overwintering frogs, and the degree of sexual dimorphism of the muscles correlates with their sensitivity to testosterone (Blackburn et al., 1995; Sidor and Blackburn, 1998). The present study extends to the abdominal musculature questions about intersexual differences and their endocrinological basis in *R. pipiens*.

Our investigation was designed to test two hypotheses: (1) that differential functions of the hypaxial muscles of male and female *Rana pipiens* are reflected in sexual dimorphism in mass and composition of these muscles outside of the breeding season; and (2) that testosterone affects muscle mass and protein content of the hypaxial muscles in overwintering males. Our rationale stems from the existence of testosterone responsiveness in other sexually-dimorphic features of male anurans. These features include the forelimb muscles (Thibert, 1972; Regnier and Herrera, 1993; Dorlöchter et al., 1994; Blackburn et al., 1995), as well as the nuptial pads (e.g., Obert, 1975; Kanamadi and Saidapur, 1982; Lynch and Blackburn, 1995), skin glands (Thomas and Licht, 1993), and larynx (Tobias et al., 1993; Watson et al., 1993). If the hypaxial muscles of male ranid frogs are sexually dimorphic as well as testosterone-responsive, they would have to be included as a secondary sex characteristic whose attributes are affected by the endocrine system. Given the importance of the abdominal muscles to reproduction, studies of their morphology and physiology have important implications for an understanding of the physiological ecology, behavior, and evolution of anurans (Taigen et al., 1985; Marsh and Taigen, 1987; Wells and Taigen, 1992; Bevier, 1995; Ressel, 1996).

Materials and methods

For the study of androgen responsiveness, adult male *Rana pipiens* (sensu Hillis, 1988) that had been collected from northern Wisconsin, U.S.A. were obtained from a commercial supplier in January. The captive frogs had been housed at cold temperatures, and nuptial pad histology (Lynch and Blackburn, 1995) indicated that they were not in an active reproductive state. The frogs were housed in a customized frog facility containing tilted aluminum troughs with running water (18 C) at an increasing photoperiod of 12:12 LD to 14:10 LD, and were fed earthworms and crickets weekly.

Fifty frogs were randomly divided into four groups 12-13 frogs each: Group I: Sham-operated + placebo; Group II: Sham-operated + testosterone; Group III: Castrated + placebo; Group IV: Castrated + testosterone. A fifth group of untouched frogs was maintained to indicate effects of the surgery. Castration was conducted under urethane-anesthesia, via a lateral abdominal incision; in sham-operated animals, the testes were surgically manipulated only. Experimental animals were then treated with two successive three-week, time-release pellets that contained 5 mg of either testosterone cypionate or

cholesterol placebo (Innovative Research, Toledo, Ohio, U.S.A.), administered subcutaneously into the dorsal lymph sac. Previous work has shown that this androgen dosage induces hypertrophy of sexually-dimorphic forelimb muscles (Blackburn et al., 1995), as well as development of nuptial pads that are histologically and histochemically like those of breeding frogs (Lynch and Blackburn, 1995; Epstein and Blackburn, 1997). After six weeks of hormone treatment, frogs were sacrificed by an overdose of urethane, wrapped in plastic and aluminum foil, and frozen at -70°C . The study of sexual dimorphism was based on adult male and female *R. pipiens* that had been collected by a commercial supplier from Lake Champlain, Canada in October; these were sacrificed and stored as described above.

Specimens were thawed in warm water, and the hypaxial muscles were dissected from each. The specimens from the testosterone experiment had been thawed twice prior to this study, to harvest other muscles (Blackburn et al., 1995; Sidor and Blackburn, 1998). Because the external and internal oblique muscles could not be separated, they were dissected free as a single sheet, and are treated as a single muscle unit herein. The abdominal obliques were trimmed along the lateral border of the rectus abdominis; dorsal to it, they blend with the aponeurosis that sheaths the rectus. The broad sheet of the rectus abdominis that lies caudal to the sternum was analyzed; segments that lie dorsal to the sternum, where the muscle is associated with the sternohyoid complex, were excluded. Dissected muscles were cleaned of adherent fascia, patted dry with paper towels, weighed on an analytical balance, and stored temporarily at -18°C . Muscle samples were lyophilized for 6 hr at -60°C in a Lyphlock freeze dry system (Labconco, Kansas City, Missouri, U.S.A.), and weighed to the nearest 0.01 mg. Protein analyses were done on the abdominal muscles of five males and females in the sexual dimorphism study, as well five frogs from each of the five groups in the testosterone experiment. Following previously-used procedures (Yekta and Blackburn, 1992; Sidor and Blackburn, 1998), muscle samples were homogenized for 30 minutes in 0.1 N NaOH at a concentration of 1 mg/ml to extract the protein. Samples were analyzed spectrophotometrically using the BCA assay (Pierce, Rockford, Illinois), with albumin standards. Data from these studies were analyzed by using Statistica software (Tulsa, Oklahoma) and Statview software (Berkeley, California).

Results

The hypaxial muscles in *Rana pipiens* largely conform to descriptions by Gaupp (1896) for *R. esculenta*. However, we found the external oblique muscle and internal oblique muscle (the “transversus abdominis” of Gaupp) to be inseparably fused in both sexes; here they are collectively termed the “abdominal oblique muscle layer”. The presence of both muscles was confirmed by fiber orientation. The fibers of the external oblique muscle run posteroventrally. Those of the internal oblique are oriented relatively vertically (dorsoventrally); the most cranial fibers are oriented somewhat anteroventrally, and the

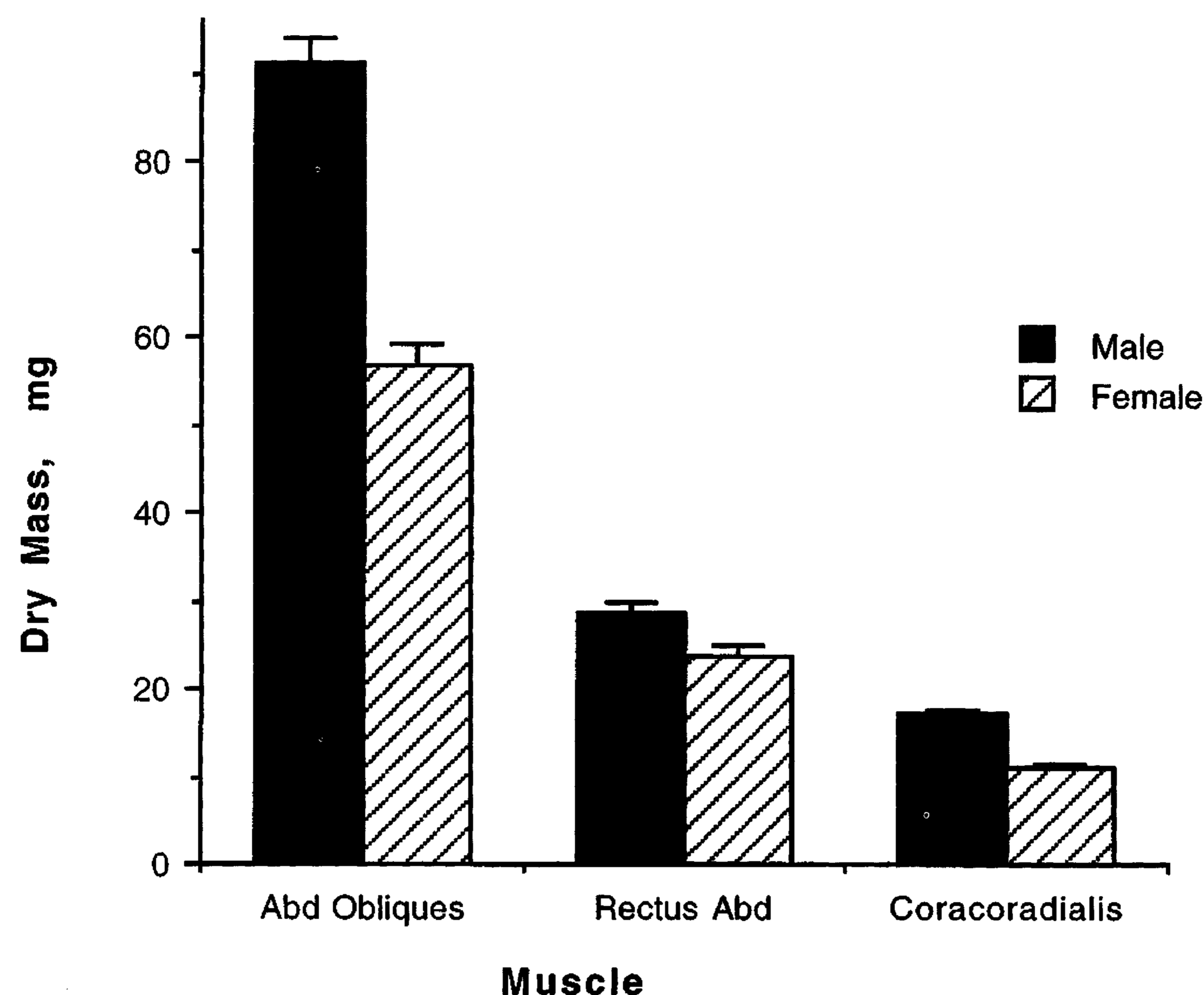


Figure 1. Sexual dimorphism of the hypaxial muscles in male and female *Rana pipiens*. Bars = mean dry mass + $s_{\bar{x}}$. "Abd Obliques" = the combined external oblique and internal abdominal oblique muscles; "Rectus Abd" = the rectus abdominis muscle. The coracoradialis, a prominent forearm adductor that is involved in amplexus, is included for comparison (Yekta and Blackburn, 1992). All three muscles are sexually dimorphic in wet and dry mass ($P < 0.001$ in each case).

caudal fibers, in a posteroventral direction. In some male specimens, fibers of the internal and external oblique muscles were separable in the anterior region of the trunk where the muscles were thicker, but not posteriorly.

In terms of mean dry mass, sexual dimorphism was exhibited by both the abdominal oblique muscle layer (two-tailed t -test: $F = 90.49$, $P < 0.001$) and the rectus abdominis muscle ($F = 10.91$, $P < 0.001$). Mean dry mass of the male hypaxial muscles averaged 160% (oblique muscles) and 121% (rectus abdominis) times the mass of corresponding muscles in females (fig. 1). These values may underestimate the intersexual differences, because males in our study were significantly smaller (two tailed t -test; $P < 0.0001$) than females in both snout-vent length (males, $76 \text{ mm} \pm 0.6 s_{\bar{x}}$; females, $81.9 \text{ mm} \pm 0.8$) and body mass (males, $37.3 \text{ g} \pm 0.82 s_{\bar{x}}$; females, $48.0 \text{ g} \pm 1.82$). When controlled for body mass via ANCOVA, mean dry mass of the abdominal oblique and rectus muscles in males were respectively 192% and 149% those of females. Data on wet mass paralleled the dry mass data in magnitude and statistical significance. Expressed as a percentage of body mass, muscle wet mass in males was larger than in females ($P < 0.0001$) for both the abdominal obliques (males, $0.75\% \pm 0.03 s_{\bar{x}}$; females, $0.38\% \pm 0.01$) and the rectus abdominis (males, $0.25\% \pm 0.01 s_{\bar{x}}$; females, $0.14\% \pm 0.01$).

With regard to the dry mass/wet mass ratio, the abdominal oblique muscles did not differ between the sexes ($F = 1.96$, $P < 0.18$), whereas the rectus abdominis showed a

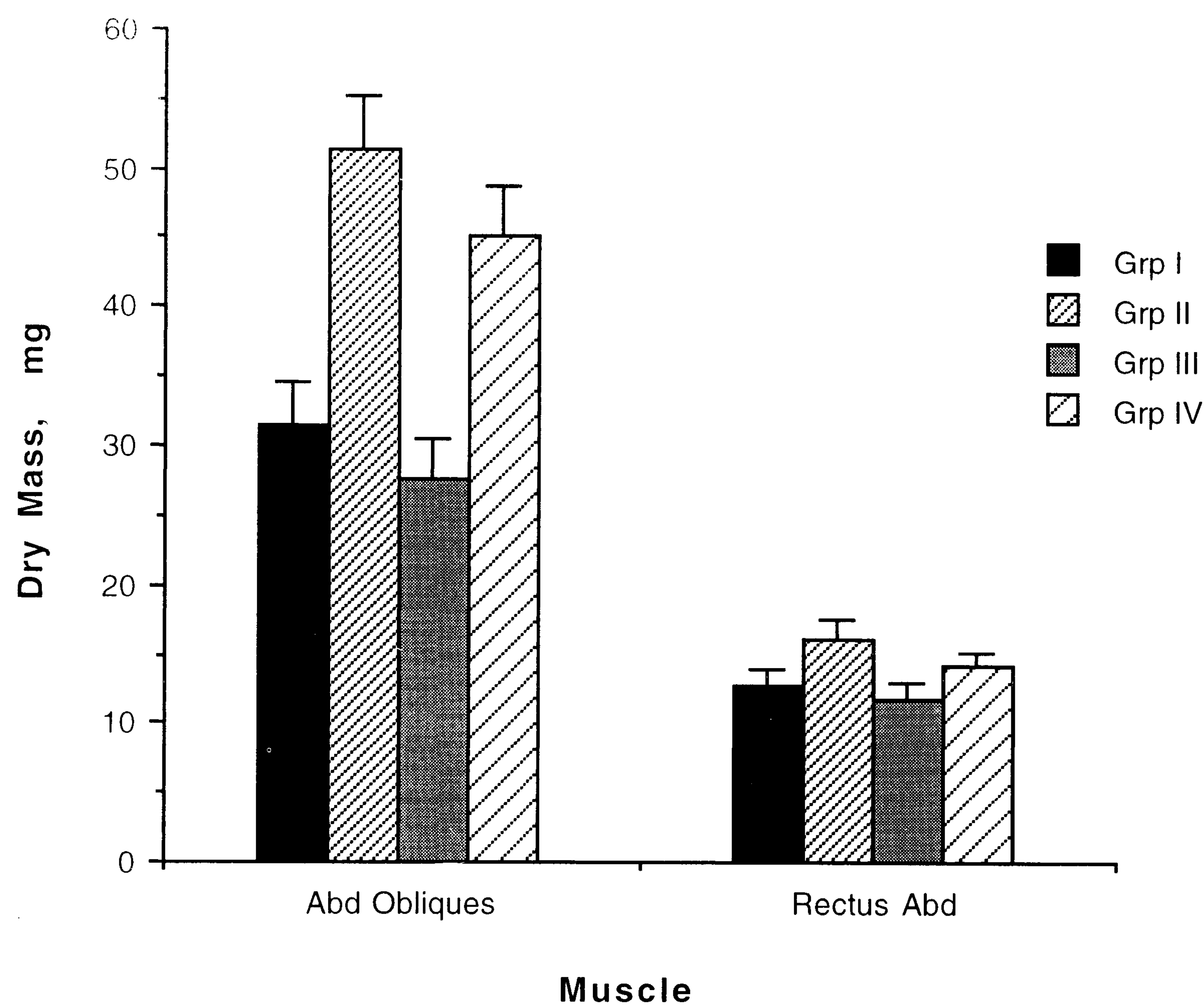


Figure 2. Androgen responsiveness of the hypaxial muscles in male *Rana pipiens*. Bars = mean dry mass + $s_{\bar{x}}$. Group I = sham-operated control frogs; Group II = testosterone-treated, sham-operated frogs; Group III = placebo-treated castrates; Group IV = testosterone-treated castrates. Muscle abbreviations are as in fig. 1. Both muscles in the testosterone treated frogs differed significantly from those of the placebo-treated controls ($P < 0.001$ in each case).

very small intersexual difference ($F = 14.04$, $P = 0.002$) (males = $30.6\% \pm 0.77 s_{\bar{x}}$; females = $34.3\% \pm 0.62$). The abdominal oblique muscle did show a significant intersexual difference ($F = 6.66$, $P = 0.03$) in measurable protein content (males = $553.8 \mu\text{g}$ protein/mg dry mass $\pm 32.9 s_{\bar{x}}$; females = $658.7 \mu\text{g} \pm 24.0$). However, protein content of the rectus abdominis did not differ statistically ($F = 0.99$, $P = 0.35$) between males ($617.1 \mu\text{g}$ protein/mg dry mass ± 17.8) and females ($668.6 \mu\text{g} \pm 48.4$).

ANCOVA of the experimental groups (Groups I-IV) with body mass as the covariate revealed that treatment with testosterone increased the abdominal oblique muscles in terms of both wet mass ($F = 32.44$, $P < 0.0001$) and dry mass ($F = 37.65$, $P < 0.0001$) (see fig. 2). Treatment also affected the rectus abdominis in wet mass ($F = 5.00$, $P < 0.01$) and dry mass ($F = 4.38$, $P < 0.01$). Scheffe post hoc tests revealed significant differences ($P < 0.05$) between the testosterone treated groups and their respective controls (Group II vs. I; Group IV vs. III) in wet and dry mass of each of the muscles. In testosterone treated, sham operated frogs, mean dry mass of the abdominal oblique muscles and rectus abdominis muscles were respectively 163% and 126% that of the controls (Group II vs. I). Analysis of the adjusted means after controlling for body

mass via ANCOVA yielded almost identical values (164% for the oblique muscles and 125% for the rectus abdominis).

Experimental treatment affected the ratio of abdominal oblique wet mass to body mass (ANOVA, $F = 29.41$, $P < 0.001$); in testosterone-treated groups, the mean ratio of muscle mass to body mass was 146% times that of the controls (e.g., mean ratio of Group II = $0.76\% \pm 0.02 s_{\bar{x}}$; Group I = $0.52\% \pm 0.02$). ANOVA suggested that treatment also affected the ratio of muscle mass to body mass ($F = 4.79$, $P < 0.01$) for the rectus abdominis (mean ratio of Group II = $0.26\% \pm 0.02 s_{\bar{x}}$; Group I = $0.22\% \pm 0.02$); however, the Scheffe post hoc tests were negative ($P > 0.05$).

Castration did not affect muscle mass (Group I vs. III) or the response to exogenous testosterone (Group II vs. IV) in either muscle ($P < 0.05$) (fig. 2). In terms of % water content, experimental treatment affected neither the oblique muscles (ANOVA, $F = 2.17$, $P > 0.1$) nor the rectus abdominis ($F = 0.37$, $P = 0.77$). Treatment also did not affect protein concentration (μg protein/mg dry mass) of the oblique muscles (ANOVA, $F = 0.80$, $P > 0.5$) or rectus abdominis ($F = 0.18$, $P > 0.90$).

Discussion

Sexual dimorphism in the forelimb muscles of anurans has been known for over a century (Gaupp, 1896), and intersexual differences have been demonstrated in forelimb muscle mass, protein content, fiber composition, and contractile properties (Melichna et al., 1972; Rubinstein et al., 1983; Oka et al., 1984; Yekta and Blackburn, 1992). Sexual dimorphism also has been documented in the laryngeal musculature of certain frogs (Kelley, 1986; Sassoon and Kelley, 1986; Marsh and Taigen, 1987). In contrast, the possibility of intersexual differences in the hypaxial musculature of anurans has only recently been considered, and relevant studies are confined to a few species. Thus, in *Hyla crucifer* and *H. versicolor*, for example, sexual dimorphism is manifested in mass, lipid content, and oxidative capacity (Marsh and Taigen, 1987; Wells and Taigen, 1992). Males of certain other hylid and leptodactylid species also exhibit biochemical and ultrastructural specializations (Bevier, 1995; Ressel, 1997) that presumably are lacking in females. The hormonal basis for the intersexual differences has not been determined.

This study has demonstrated sexual dimorphism in mass and protein content of the abdominal musculature of non-breeding *Rana pipiens*, one of the most commonly used amphibians in experimental studies. The rectus abdominis and abdominal oblique muscles were significantly larger in males in terms of mean dry mass both with and without statistical control for body mass. Expressed as a percentage of body mass, the combined wet mass of these muscles in males was double that of females. Because the frogs were sacrificed in autumn, the dimorphic muscles may not have been at maximum size in males, particularly if the muscles cycle seasonally (see below). On the other hand, because the females contained ovarian eggs, ratios based on measurements of body mass underestimate the contribution of the hypaxial muscles to somatic body mass in females.

However, the values do allow rough comparison to data from the literature on other species, which also are based on females with eggs.

Intersexual differences in mass of the oblique muscles clearly are less pronounced in *Rana pipiens* than in certain *Hyla*, in which the proportion of body mass represented by the abdominal obliques in breeding males is 4.5-5 \times that of females, and in which the oblique muscles in males can represent 6-15% of body mass, depending on the species (Taigen et al., 1985; Marsh and Taigen, 1987). The differences in *R. pipiens* are more comparable to values for *R. virgatus* during the breeding season, in which wet mass of the oblique muscles in males are about 2.4 \times the mass of the female muscles (Given and McKay, 1990). The differences between the hylids and ranids probably reflect differences in male calling effort. In a recent summary of data on several anurans, the North American *R. virgatipes* (Given, 1988) ranked lowest in calling effort, and along with *R. sylvatica*, among the lowest in mass of the oblique muscles in males, relative to body mass (Wells and Taigen, 1992; also see Bevier, 1995; Ressel, 1997).

In terms of magnitude of the intersexual differences, the hypaxial muscles of *R. pipiens* lie in the middle of the range of sexually dimorphic muscles of the forelimb (see Yekta and Blackburn, 1992). With regard to the male/female dry mass ratio, sexual dimorphism of the abdominal oblique muscles is equivalent to that of the coracoradialis, a major adductor of the forelimb and flexor of the elbow that plays an important role in amplexus (Kirby, 1983). This muscle has been studied by several authors, and is included for comparison in fig. 1. Intersexual differences in mass of the rectus abdominis are similar to that of the flexor antibrachii medialis, a minor elbow flexor (Yekta and Blackburn, 1992).

The rectus abdominis and abdominal obliques of male *R. pipiens* responded strongly to androgen treatment. In testosterone-treated frogs, the mean dry mass increase of these muscles (Group II vs. I) was equivalent to that of the aforementioned coracoradialis and flexor antibrachii medialis muscles (see Sidor and Blackburn, 1998). Experimental work has shown a strong correlation between the extent of sexual dimorphism and androgen sensitivity in muscles of the forelimb, as measured in frogs outside of the breeding season (Blackburn et al., 1995; Sidor and Blackburn, 1998). The present study suggests that the hypaxial muscles also show a relationship between degree of sexual dimorphism and responsiveness to exogenous testosterone. Extension of this relationship to the hypaxial muscles further supports the postulate that androgens are responsible for stimulating the increased size of the muscles in males.

That castration had no effect on hypaxial muscle mass might seem surprising; castration can lead to forelimb muscle atrophy in other anurans, a response prevented by testosterone treatment (Thibert, 1972; Regnier and Herrera, 1993; Dorlöchter et al., 1994). However, our findings are consistent with data on the sexually dimorphic forelimb muscles in overwintering *R. pipiens* (Blackburn et al., 1995; Sidor and Blackburn, 1998). The lack of effect of castration may reflect the possibility that the muscles were in seasonal regression at the time of the experiment, due to lowered androgen levels. This inference is supported by the observation that the nuptial pads in the untouched

controls were inactive (Lynch and Blackburn, 1995). Although seasonal cycles have not been searched for in the hypaxial musculature of *R. pipiens*, temperate ranids do exhibit cyclicity in forelimb muscles (Muller et al., 1969; Melichna et al., 1972). A less likely alternative to the above explanation is that the hypaxial muscles do not respond to androgen withdrawal, like the laryngeal muscles of *Xenopus laevis*. The laryngeal muscles of *X. laevis* do not show seasonal cyclicity, and once androgens have organized the phenotypic pattern seen in males, the muscles are relatively insusceptible to altered androgen levels (Sassoon et al., 1987; Segil et al., 1987; Marin et al., 1990). However, unlike *R. pipiens*, *X. laevis* is not a seasonal breeder.

The distinction between organizational vs. activational roles of hormones (Moore, 1981) may be key to understanding our findings. The presence of intersexual differences in the hypaxial muscles in *R. pipiens* outside of the breeding season may reflect organizational effects of androgens on the muscles during development. In contrast, responses by these muscles to exogenous testosterone (and a lack of response to castration) may reflect an activational role for circulating androgens during seasonal cycles. Organizational and activational effects have been invoked to explain a similar pattern of sexual dimorphism and androgen sensitivity in the forelimb muscles (Sidor and Blackburn, 1998).

The fact that androgen treatment and castration had no effect on muscle composition is consistent with work on the forelimb muscles (Sidor and Blackburn, 1998). Interestingly, however, the abdominal oblique muscles contained a significantly smaller concentration of measurable protein in males than in females. The value for the female oblique muscle lies in the range exhibited by the rectus abdominis of both sexes, as well as the forelimb muscles (Yekta and Blackburn, 1992; Sidor and Blackburn, 1998). Thus, the abdominal oblique muscles of males seem to be unusual in protein concentration. Although the functional significance of the intersexual difference is not known, it might reflect a greater proportion of lipid in male hypaxial muscles. Lipids reportedly account for 45% of trunk muscle mass in male *Hyla crucifer* at the beginning of the breeding season, and provide energy for calling in early spring in this species (Wells and Taigen, 1992). In contrast, lipids constitute 14% of the muscle mass in male *H. versicolor* (Marsh and Taigen, 1987). The possibility that intersexual differences in muscle composition in *R. pipiens* may reflect different functional roles or capabilities deserves examination.

A relatively minor point is that muscles in both parts of this investigation showed a diminished water content (66-77% water by mass) compared to forelimb muscles (82% water) in the study by Yekta and Blackburn (1992). These differences probably reflect the greater surface area/volume ratio of the broad, thin abdominal muscles; more water tends to be extracted from these muscles as they are patted dry before being weighed. The issue illustrates the importance of using lyophilized samples rather than wet mass to measure muscle size.

In conclusion, this investigation has demonstrated that the abdominal hypaxial muscles of *Rana pipiens* exhibit typical characteristics of a male secondary sex characteristic, notably sexual dimorphism and androgen sensitivity. That androgens have organizational

effects on the hypaxial muscles is suggested by the existence of sexual dimorphism in muscle mass outside of the breeding season. An activational role for testosterone is suggested by the fact that this hormone stimulates muscle hypertrophy in over-wintering frogs.

Like the forelimb musculature, the hypaxial muscles appear to offer a model system for studies on the effects of androgens on muscle tissue and the mechanism by which they exert those effects. Given the importance of male vocalization in the competition over territory and females, individual and interspecific variation in muscle attributes have significant implications for amphibian reproductive biology, ethology, ecology, and evolution. Current work in our laboratory is examining intersexual differences at cytological levels, to provide a basis for studies on the effects of androgens and the mechanism of hormone action.

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