

**Androgen Regulation of the Calling Muscles in the gray tree frog**

*Hyla versicolor*

BIOS 568

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1999

## ABSTRACT

As in most anuran amphibians male gray tree frogs (*Hyla versicolor*) use advertisement calls to attract mates during the mating season. Since females appear incapable of producing these vocalizations it is possible that differing effects of plasma androgens on calling muscles play a role in their regulation. We examined the effect of testosterone treatment on the dilator, posterior constrictor, external obliques, and semimembraneous to determine the its role in maintaining muscle fiber size and ratio. The muscles used in calling, the dilator, posterior constrictor, and external obliques showed high ratios of fast-twitch oxidative/glycolytic fibers while the semimembraneous consisted mostly of fast-twitch glycolytic fibers. Although it is known that skeletal muscles involved in mating behaviors are sensitive to androgens, no significant differences were recognized in muscle fiber size or distribution between testosterone-treated and control groups.

## INTRODUCTION

Male anuran amphibians use advertisement calls to attract females during mating seasons (Wells 1977). Previous studies have shown that *H. versicolor* males are capable of sustaining these loud mating calls for many hours (Marsh and Tiagen, 1987). The ability to sustain such calls for an extended period without fatigue requires a great deal of energy output to the laryngeal muscles. Work by Bucher et al (1982) and Tiagen and Wells (1985) has shown that *H. versicolor* males consume oxygen at a high rate with little lactic acid build-up during calling. Marsh and Tiagen (1987) demonstrated that the laryngeal muscles of the species consist entirely of fast oxidative glycolytic (FOG) fibers, as characterized by histochemical analysis. This type of muscle fiber is capable of quickly oxidizing energy sources for use and does not fatigue easily.

Because only males are capable of exhibiting calls during mating (Capranica 1968), it is conceivable that regulation and development of these muscles is under hormonal control. Many skeletal muscles in amphibians involved in reproductive behaviors are highly sensitive to androgens. Steroid hormones have been shown to influence both the size and fiber type in certain adult skeletal muscles as well as their development in juveniles. For example, in *Xenopus leavis* testosterone was found to affect the number of tonic muscle fibers in the arm, an area used extensively in mating behaviors for amplexic clasping of the mate (Rubenstein et al, 1983). Furthermore, the laryngeal muscles of *Xenopus* (Seigal et al 1987) and *Rana catesbeiana* (Boyd et al, 1999) males have shown to have a higher number of androgen receptors than their female counterparts. Sassoon et al (1987) demonstrated that *Xenopus* juveniles subjected to testosterone treatment showed an increased rate of maturation. These results suggest that

dimorphisms in reproductive behaviors are at least partially due to sex-differences in the endocrine control of certain skeletal muscles.

In the present study we characterized the effects of testosterone treatment on four muscles in adult male *H. versicolor*. The androgen effects on SDHase staining patterns was examined in the external obliques, dilator laryngis, and the posterior constrictor, which all contribute to male advertisement vocalization, as well as the semimembraneous. In addition, the effects of the androgen treatment on whole muscle and muscle fiber size were observed.

## **MATERIALS AND METHODS**

*Animals.* Adult male *Hyla versicolor* were captured in Gogebic county, Michigan between May 27 and July 6, 1999. Males were housed in 10 gallon aquaria, which were kept at a temperature comparable to outside (approx 18°C). The animals were fed crickets and had access to fresh water.

*Hormone Treatment.* Captured males were randomly divided into two groups. Testosterone-treated males (n=9) were given intra-peritoneal injections of 1µg of testosterone in 0.05 ml of vegetable oil for 14 continuous days. Similarly, oil-treated males (n=8) were given 0.05 ml of vegetable oil during the same period of time.

*Sacrifice.* Within 24 hours of the final injection, animals were transported to the University of Notre Dame campus. The males were deeply anesthetized (0.2% benzocaine) and sacrificed within 48 hours of arrival. The forelimbs, hind limbs,

laryngeal complex, and oblique muscles were extracted. All tissues were fresh frozen at -80°C.

*Laryngeal Anatomy.* The laryngeal complex was thawed from -80°C. Measurements of the width and length of the dilator and posterior constrictor muscles were taken before muscle extraction. In some cases, measurements of the larynx length and width were made after the dilator and posterior constrictor had been removed from one side. Larynx length was determined by measuring from the base of the hyoid cartilage at the anterior arytenoid cartilage to the posterior end of the arytenoid cartilage. Larynx width was measured from the attachment of the dilator muscle to the same point on the other side. Dilator length was taken from its connection at the arytenoid cartilage to its attachment at the cricoid cartilage. Dilator width was taken at its widest point.

*SDHase Procedure.* Chunks of the dilator, posterior constrictor, semimembraneous, and external oblique muscles were embedded in Histoprep (Fisher) and frozen at -80°C. All tissues were cryo-sectioned (50 um) at -18°C. The dilator, posterior constrictor, and semimembraneous muscles were sectioned across the fiber length while the external oblique muscles were cut along the fibers. Sections were thaw mounted on alternating subbed slides so that matched pairs of sections were produced. Both sets of slides were stored at -20° C.

Within 24 hours of sectioning, one set of slides was treated for SDH. These slides were incubated in Nitro BT solution (40 ml Tris buffer, pH 7.4, 2 ml dH<sub>2</sub>O, 0.01 g

Nitro BT) at 37°C for 45 min. Slides were then rinsed for 1 min in dH<sub>2</sub>O and dehydrated in an ethanol gradient followed by submersion in Hemo-De solution for 5 min. The slides were mounted with Permount.

*Muscle Fiber Size and Ratio.* Visual inspection under a light microscope at 40X and 100X was used to make estimates of fiber size and ratio in each of the four muscle tissues. Analysis of photographs taken using a Nikon 4X digital camera were also used in fiber size and ratio determination.

## **RESULTS**

There was no significant difference in animal size and weight before treatment between the testosterone and oil-treated groups (Table 1). Similar body sizes between groups allows for the assumption that differences in muscle and muscle fiber size is not due to differences in animal size. Measurements of the dilator length, dilator width, larynx length, and larynx width taken after treatment showed no significant differences between the treated and control groups in an unpaired t test (Table 1).

SDHase staining of the dilator, posterior constrictor, semimembraneous, and the external obliques showed two distinct fiber types in the four skeletal muscles. Both lightly staining large fibers and smaller, darker fibers were present to some degree in each of the four muscles. In the semimembraneous light fibers were 1.4-2 times larger than the dark fibers.

There was clear differences in the ratio of light to dark fibers in the muscle types. The semimembraneous was comprised of mostly light fibers, demonstrating

**Table 1**

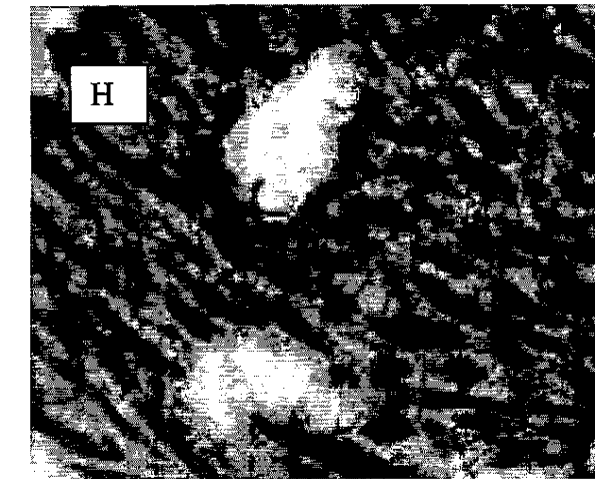
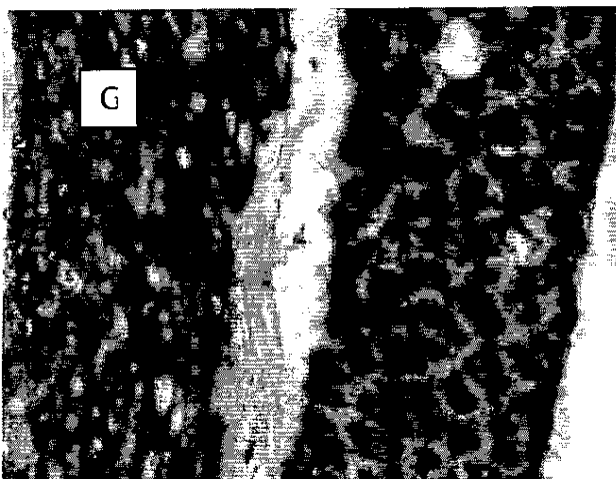
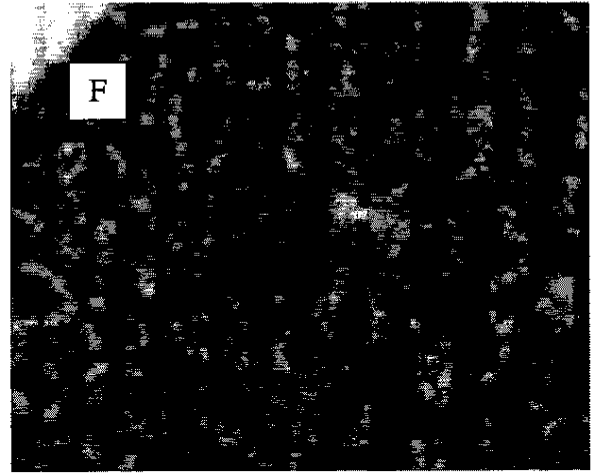
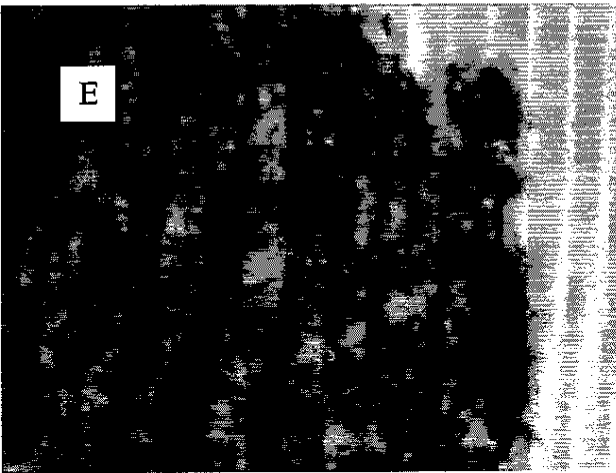
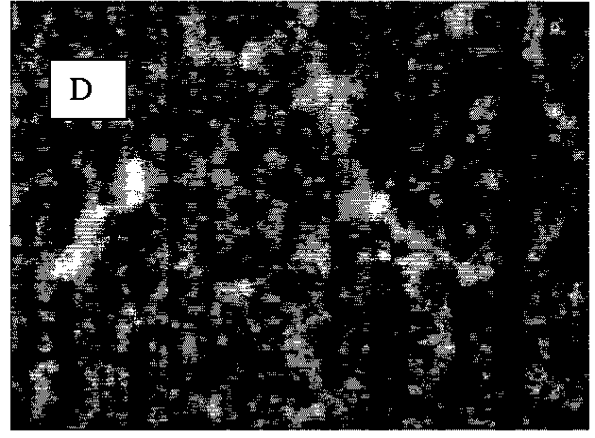
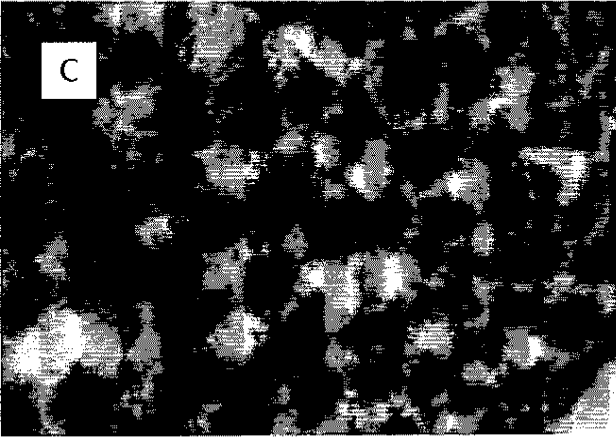
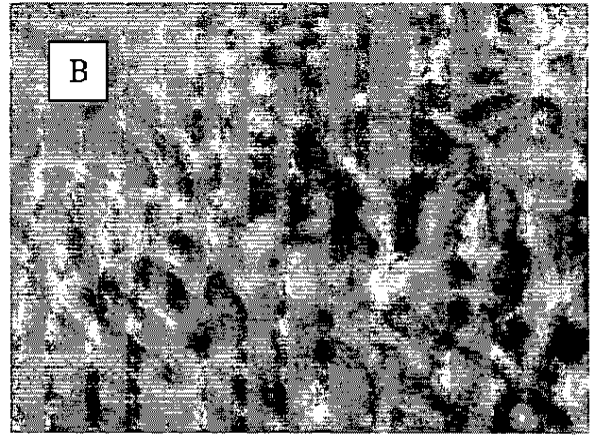
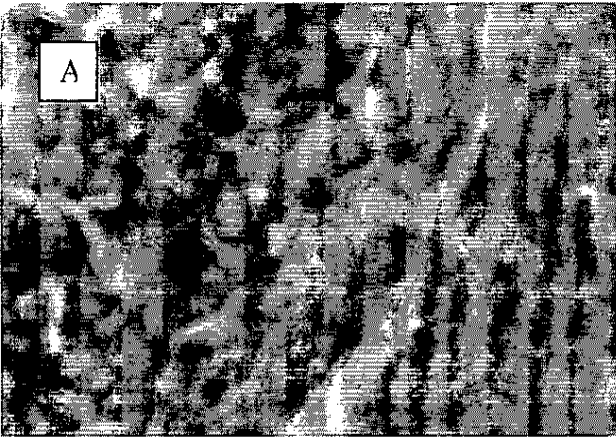
Morphological Parameters of the Larynx in Testosterone-Treated (n=9) and Control Group (n=8)

Parameter	Testosterone-treated	Control
Snout/vent length (cm)	4.6 ± 0.05	4.4 ± 0.1
Weight (g)	7.7 ± 0.3	7.4 ± 0.5
Larynx length (mm)	8.6 ± 0.1	8.7 ± 0.07
Larynx width (mm)	8.1 ± 0.1	8.2 ± 0.06
Dilator length (mm)	4.7 ± 0.1	4.5 ± 0.04
Dilator width (mm)	3.4 ± 0.1	3.4 ± 0.1

(Means ± SEM); Unpaired t test between treated and control groups showed no significant differences ( $P \geq 0.05$ ) in all parameters.

approximately two of the light fibers for each of the dark (Figure 1 A and B). The posterior constrictor, dilator, and external oblique muscles showed an overwhelming proportion of dark fibers, comprising as much as 85% of the total muscle fibers (Figure 1 C-H). Furthermore, relative sizes of similar fiber types varied in the four tissues. A comparison of dark fibers from the dilator and posterior constrictor showed that they were approximately half the size of the dark fibers in the semimembraneous.

**Figure 1.** SDHase staining of *Hyla Versicolor* semimembraneous in (A)treated group (B) control group, dilator in (C) treated group (D) control group, posterior constrictor in (E) treated group (F) control group, and external obliques in (G) treated group (H) control group. The semimembraneous (A and B) showed a predominance of light fibers while the dilator (C and D), posterior constrictor (E and F), and external obliques (G and H) exhibited mostly dark fibers.





Although fiber ratio clearly varied between different muscle tissues there did not appear to be any significant differences in fiber size or ratio between the control and testosterone treated groups in any of the four muscle tissues. Furthermore, muscle fiber size, for both dark and light fibers appeared to remain unchanged in testosterone treated animals as compared to the control groups in each of the four muscle types (Figure 1).

## DISCUSSION

The energy requirements for prolonged calling in anuran amphibians is very high. Marsh and Tiagen (1987) showed that the citrate synthase activity, an enzyme used in the production of energy during the Krebs's Cycle, in the laryngeal muscles of *H. versicolor* is among the highest recorded for ectothermic vertebrates. These results demonstrate the energy-producing capabilities of the tissue.

The large, light staining fibers as demonstrated by SDHase staining are likely fast twitch glycolytic fibers corresponding to amphibian type 1 or mammalian type IIb fibers (Gans and De Gueldre, 1992). The predominance of this type of muscle fiber in the semimembraneous in the present study is consistent with the results of Marsh and Tiagen (1987) who reported that skeletal muscles other than laryngeal muscles of anuran amphibians consist of 80-90% fast-twitch glycolytic fibers. This muscle type is a relatively slow consumer of energy as compared to fast twitch oxidative/glycolytic fibers and is susceptible to fatigue.

The fibers of muscles used in calling, the posterior constrictor, dilator, and external obliques demonstrated a high ratio of darkly staining fibers indicating a high level of energy producing enzymes. These small, dark fibers are likely fast

oxidative/glycolytic (FOG) fibers corresponding to amphibian type 2 or mammalian type IIa (Gans and De Gueldre, 1992). These fibers are capable of prolonged activity with little lactic acid build-up. The predominance of this type of fiber in muscles used for calling in the present study is consistent with the findings of Marsh and Tiagen (1987) who reported that the dilator of *H. versicolor* consisted entirely of FOG fibers.

The size of laryngeal muscles in both *Rana catesbeiana* and *Hyla versicolor* have demonstrated sexual dimorphisms in both muscle size and fiber distribution (Boyd et al, 1999; Marsh and Tiagen, 1987). Sex differences in calling in anuran amphibians may therefore be due to differing effects of plasma androgens on the muscles used for calling. The presence of 13% more androgen receptors in the dilator of male bullfrogs as compared to females suggests that steroid hormones play a role in regulating the muscle. Furthermore, dilators of adult male *Xenopus* demonstrated an increased ratio of FOG fibers when treated with testosterone (Sassoon et al, 1987). It is thus puzzling that the effects of testosterone treatment in the present study appeared to show no significant effect on muscle fiber size or ratio in *H. versicolor* calling muscles.

It has been demonstrated that skeletal muscles of amphibians used in mating behaviors are sensitive to androgens (Rubenstein et al, 1983; Seigal et al, 1987; Boyd et al, 1999). The apparent absence of androgen effects on calling muscles in the present study may be due to the qualitative method of analysis used to determine differences in fiber size and ratio between testosterone-treated and control groups. Furthermore, plasma levels of testosterone in the control group may have been sufficient enough so that no significant difference could be detected between control and treated groups. Clearly for

any definite conclusions to be drawn about the regulation of muscle fiber size and ratio by androgens to be made a more qualitative approach to the analysis must be carried out.

#### **ACKNOWLEDGEMENTS**

The author wishes to thank especially Michelle Tito for help in performing and interpreting these experiments and Dr. Michelle Whaley for use of equipment. Alecia Mingo and Amy Evans assisted with animal collection. This study was funded by the Bernard J. Hank Family Endowment.

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