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Androgen Receptors and Sexual Dimorphisms in the Larynx of the Bullfrog

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As in most anuran amphibians, both male and female bullfrogs (*Rana catesbeiana*) vocalize. Sex differences in vocalizations in the bullfrog may be due to sex differences in the larynx. We examined the laryngeal muscle to determine whether it possessed androgen receptors and whether there were morphological sexual dimorphisms in the larynx. Using a polyclonal antibody and immunocytochemistry, we found androgen receptors in the laryngeal dilator muscle of both sexes. Males possessed approximately 13% more receptor-positive muscle nuclei than females. We also stained the dilator muscle for the presence of succinate dehydrogenase. Density of staining for the enzyme was significantly greater in male muscle than in female muscle, indicating greater oxidative capacity of muscle in males. This procedure also showed both a significantly greater cross-sectional area for the dilator muscle in males and a greater area for individual fibers. Male muscle consisted almost entirely of fast-twitch oxidative/glycolytic fibers. Female muscle contained a mixture of fast-twitch glycolytic fibers and two subclasses of fast-twitch oxidative/glycolytic fibers. Finally, both the length and width of the entire laryngeal complex and the length and width of the dilator were significantly greater in males than in females. In summary, laryngeal muscle of bullfrogs possessed androgen receptors and is thus likely to be androgen sensitive. Androgens, during development or at adulthood, may be responsible for the anatomic and enzymatic sexual dimorphisms in the larynx.

Most male and female anuran amphibians vocalize, although there is usually a sex difference in the type of vocalization. In the majority of species, an ''advertisement call'' is given exclusively by males, but both sexes often give a ''release call.'' This sexual dimorphism in calling behavior may be a function of sexual differences at several levels. First, these dimorphisms may be due to differences in the primary vocal organ which is the larynx with its associated cartilages and muscles. Differences may also occur in secondary contributors to the vocalization, including the vocal sacs, buccal cavity, and thoracic musculature. Finally, behavioral dimorphisms may be due to sex differences in nervous or endocrine control of vocal effectors. The extent to which sexual differences in vocalizations are due to peripheral anatomical differences between males and females, or to neural or hormonal differences, is not well understood.

In bullfrogs, as in most other anurans, only males ever give an advertisement call (Capranica, 1968). Female bullfrogs possess a functional larynx and can give release calls so the lack of advertisement calling in females is clearly not due to a complete lack of the appropriate peripheral tissues. Morphological differences in the larynx may nonetheless constrain calling in female bullfrogs. There are significant structural differences between male and female larynges in many anuran amphibian species. In *Rana pipiens*, for example, overall laryngeal size is about two times greater in males than in females (McClelland and Wilczynski,

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1989). The larynx of male frogs has been uniformly described as larger, stronger, and more complex in representatives from most families (Ridewood, 1897, 1900; Trewavas, 1933; Schmidt, 1972b; Eichelberg and Schneider, 1974; Kusunoki *et al*., 1986; Marsh and Taigen, 1987; McClelland and Wilczynski, 1989; Ryan and Drewes, 1990; Kelley, 1996; McClelland *et al*., 1997). Sex differences in the types of calls given by male and female bullfrogs may be due to sexual differences in larynx morphology.

Larger larynges in males have led to the common assumption that this dimorphism is androgen dependent. Yet evidence for androgen mediation comes from studies on only one species, the South African clawed frog *Xenopus laevis* (reviewed in Kelley, 1996). The larynx of *Xenopus* is strikingly sexually dimorphic in overall size, muscle and cartilage mass, and muscle fiber number and type. Gonadectomy, steroid-replacement therapy, and antisteroid treatment experiments have convincingly shown that these dimorphisms are dependent on the presence of androgens during development. Androgen receptors have been demonstrated in the *Xenopus* larynx using both radioligand binding methods and molecular methods (Segil *et al*., 1987; Kelley *et al*., 1989; Fischer and Kelley, 1991; Fischer *et al*., 1993, 1995). The presence of androgen receptors in the larynx or androgen dependence of larynx structure has not been shown in any other amphibian.

Whether androgens might control larynx morphology in other anurans is unclear because the vocal apparatus of *Xenopus* diverges significantly from that of other frogs and toads (Ridewood, 1897, 1900; Witschi *et al*., 1953; Rabb, 1960; Gans, 1973; Kelley, 1996). Pipids lack vocal cords altogether but the dilator muscle and both the cricoid and the thyrohyal cartilages are greatly enlarged. Arytenoid cartilages are modified into rods and sound is produced by "clicking" these cartilages apart. In the majority of other anurans, including bullfrogs, sound is produced when the dilator muscle opens the glottis and allows air flow which causes vibration of the vocal cords and associated structures (McAlister, 1959; Martin, 1972; Schmidt, 1972a; Gans, 1973). We therefore examined the larynx of the bullfrog because the mechanism of sound production and function of the dilator muscle in this amphibian is more typical of anurans (Schmidt, 1965, 1973; deJongh and Gans, 1969). Specifically, we sought to determine whether the bullfrog larynx possessed androgen receptors and whether any sex differences existed in larynx morphology.

MATERIALS AND METHODS

Androgen Receptor Distribution

The entire laryngeal complex was removed from adult bullfrogs (*Rana catesbeiana*) and stored at −80°C. Bullfrogs were obtained from C. D. Sullivan (Nashville, TN) and killed with an overdose of the anesthetic benzocaine (0.2%) before dissection. There were no significant differences in body weight between males and females (mean ± SEM: males 334 ± 25 g and females 361 ± 44 g). Laryngeal dilator muscle tissue was dissected free, embedded in OCT compound (Miles, Inc), and sectioned (20 μm) in a cryostat at −17°C. Every other section was placed on a separate slide to generate matched pairs of sections. One member of each pair was used for androgen receptor immunocytochemistry and the other member, for hematoxylin and eosin staining. Sections were thaw mounted on subbed slides and immediately frozen at −20°C.

Representative slides (each containing two sections) from every animal were stained together, using the same reagent preparations, to reduce technical variability. All steps took place at room temperature unless otherwise noted. Slides were dried for 10 min and then fixed for 10 min in 4% paraformaldehyde (with 10% saturated picric acid in 0.1 M phosphate buffer). They were next rinsed in phosphate-buffered saline (PBS; 0.05 M with 0.9% NaCl) twice for 5 min each time and incubated in 0.5% H_2O_2 in PBS for 30 min. They were rinsed in PBS/gel (PBS with 0.1% gelatin) twice for 10 min each time and blocked with 4% normal goat serum (NGS) plus 20% avidin D (avidin/biotin blocking kit No. SP-2001 from Vector Laboratories, Burlingame, CA) for 30 min. After blocking serum was poured off, slides were incubated overnight at 4°C with the primary antibody solution. Primary antibody was the affinity-purified rabbit polyclonal antibody PG-21 (Prins *et al*., 1991; 2 μg/ml antiserum in PBS/gel with 0.3% Triton-X 100, 2% NGS, and 20% biotin from Vector avidin/biotin blocking kit). The next day, slides were rinsed twice (5 min each) in PBS/gel then

incubated with the biotinylated secondary antibody for 30 min (Vector ABC kit No. PK-6101). After three rinses (5 min each) in PBS/gel, slides were incubated in Vector ABC reagent (per kit instructions) for 5 min and rinsed again three times (5 min each) in Tris–HCl. Peroxidase was visualized by incubating sections for 30 min in 0.05% DAB with 0.1% NiCl and 0.3% H_2O_2 in Tris–HCl. Finally, sections were rinsed in Tris–HCl (5 min) and distilled water (5 min) and then dried (37°C) and mounted with Permount. Control sections from the same animals were treated identically, except for the following changes: (1) omission of the primary antibody; (2) primary antibody preadsorbed with the peptide used to generate the PG-21 antibody (25-fold excess; amino acids 1–21 of the rat androgen receptor); (3) primary antibody preadsorbed with a distant, unrelated peptide of the same size (25-fold excess; amino acids 462–478 of the receptor); or (4) nonimmune rabbit IgG (Sigma No. R-5001; 56 µg/ml) substituted for the primary antibody.

Slides with alternate sections were stained with hematoxylin and eosin (Delafield progressive method; Humason, 1972). The number of cellular nuclei was counted on both immunocytochemically stained sections (to yield number of androgen receptor-positive nuclei) and hematoxylin and eosin-stained sections (to yield total number of nuclei). Nuclei in three different fields (each 0.6 mm in diameter) were counted on two sections per slide for each animal. These values were averaged to yield a single value for each parameter for each animal so that *n* in Table 1 represents the number of animals ($n = 9$ males and $n = 7$ females).

TABLE 1

Androgen Receptor-Positive Nuclei in the Laryngeal Dilator Muscle of Male and Female Bullfrogs, *Rana catesbeiana*

Parameter	Males ($n = 9$)	Females ($n = 7$)
Total number of muscle nuclei	111 ± 7.3	100 ± 7.4
Androgen receptor-positive nuclei	42.8 ± 3.6	27.5 ± 3.6[a]
Percentage of total nuclei which were positive for androgen receptor immunoreactivity	41.2 ± 4.8	27.4 ± 3.2[b]

Note. Means ± SEM; in a fixed field of 0.6 mm diameter.

[a] Unpaired *t* test comparison of males and females (two-tailed), $P \leq 0.009$.

[b] Unpaired *t* test comparison of males and females (two-tailed), $P \leq 0.032$.

Laryngeal Anatomy

Adult, sexually mature bullfrogs were sacrificed by overanesthetization in benzocaine (0.1%). Animals were deliberately chosen to be similar in body size and there were no significant differences in size between males and females (mean ± SEM body weight: males 320 ± 17 g and females 325 ± 13 g). The entire laryngeal complex was dissected free of the frog before measurements but muscles were measured while still attached. Larynx length was measured 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 outer attachment of the dilator muscle (m. dilator laryngis) at the lateral process of the cricoid cartilage to the outer attachment on the opposite side. Dilator length was measured from the connection of the dilator muscle at the arytenoid cartilage to its attachment at the lateral process of the cricoid cartilage. Dilator width was taken at its widest point. (See Schneider, 1988, for anatomical detail).

Succinate Dehydrogenase (SDHase) Distribution

Histochemical staining for SDHase was used to determine overall level of oxidative capacity of the dilator muscle and fiber type composition (Sperry, 1981; Putnam and Bennett, 1983; Marsh and Taigen, 1987; Rowlerson and Spurway, 1988). After anatomical measurements (consistently within 3 min), dilator muscles were dissected free of the laryngeal complex and frozen at −80°C embedded in OCT. Dilators were sectioned (50 µm; through entire muscle) in a cryostat at −16°C, thaw mounted on subbed slides, and used immediately for demonstration of SDHase using the nitroblue tetrazolium method of Humason (1972). Slides were incubated in Nitro BT solution (Nitro BT from Sigma Chemical Co.; dissolved in 1 ml distilled water rather than dimethyl formamide) at 37°C for 45 min. They were then rinsed for 1 min in distilled water, dehydrated, cleared, and mounted.

The Drexel University PC-based image analysis system was used to determine dilator muscle cross-sectional area and intensity of SDHase staining. To determine maximum cross-sectional area, the outline

of the two largest sections from each animal was drawn and the area was computed (in mm^2) by the software, based on prior calibration with a stage micrometer. Both area measurements were averaged for each animal. Relative overall intensity of SDHase staining was measured by determining the gray level within a 1.4-mm^2 box, centered over each section (two sections per animal measured). A box this size would include between 20 and 100 fibers. Gray-level values ranged from 0 (darkest) to 230 (lightest). Lighter intensity SDHase staining thus has a higher gray level value than darker intensity SDHase staining.

The NTS5 tracing software system (Eutectics, Inc) was used to determine fiber diameter on tissue stained for SDHase. The outline of at least 20 fibers per animal was drawn (40× power) and each fiber was coded as having dark, medium, or light staining for SDHase (Sassoon *et al*., 1987). The software computed fiber area based on prior calibration. Both male and female muscle possessed an eccentric area with smaller and darker fibers. Data presented are only from the more central, homogeneous area which comprised the bulk of the muscle.

RESULTS

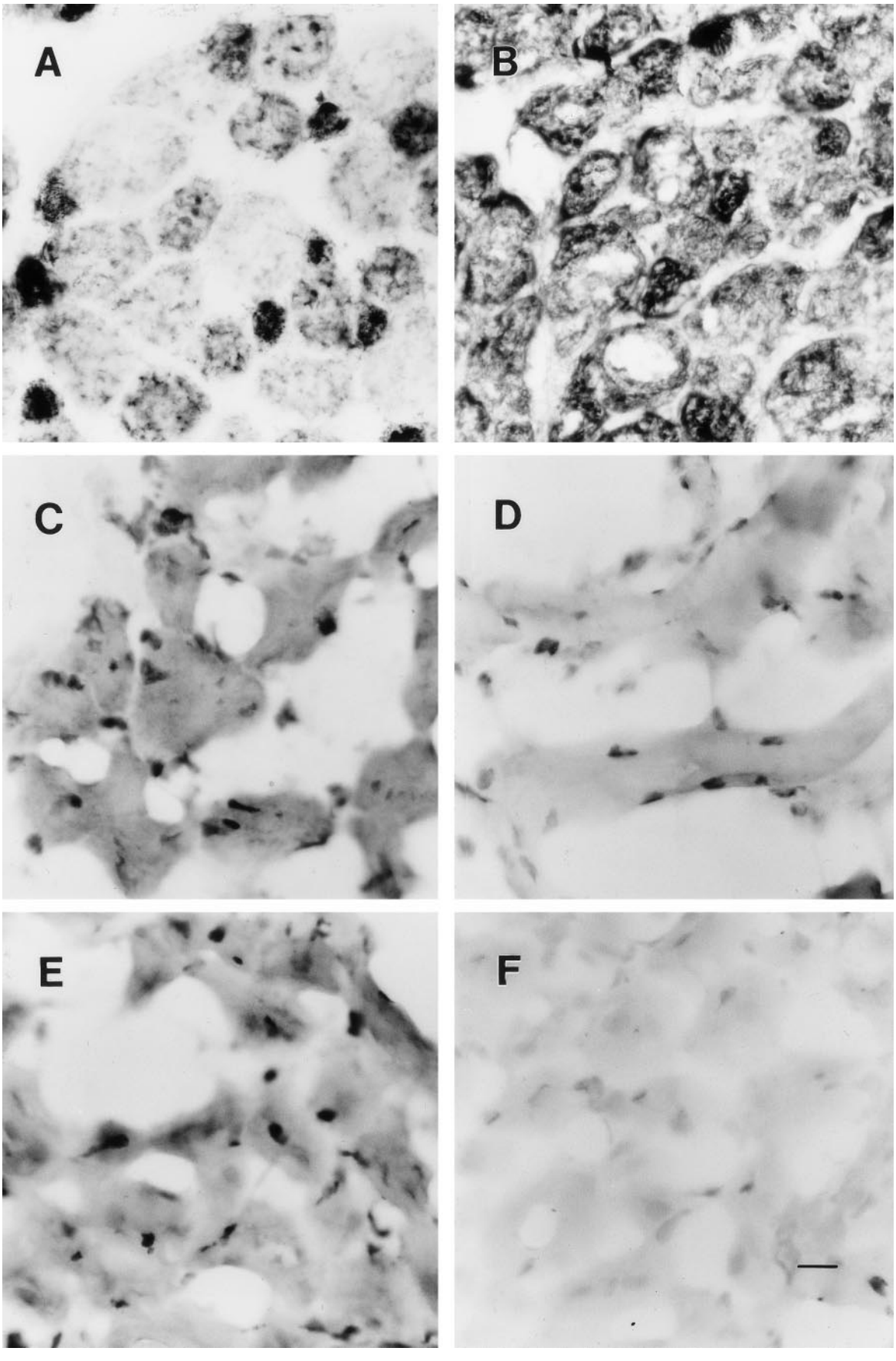
The laryngeal muscle of bullfrogs contained significant levels of androgen receptor-like immunoreactive material (Figs. 1C and 1D). This immunoreactivity was located primarily over muscle fiber nuclei and was found in both sexes. Light immunoreactivity was present over fiber cytoplasm. Immunoreactivity was likely specific because staining was abolished when the antibody was preadsorbed with the peptide used to produce the antibody (Fig. 1F), when nonimmune rabbit serum IgG replaced the primary antibody, and when primary or secondary antibodies were omitted (data not shown). Staining was not affected when primary antibody was preadsorbed with another peptide which was also part of the rat androgen receptor but not used for antibody production (Fig. 1E). Further, immunoreactivity was also observed in another bullfrog laryngeal muscle, the anterior constrictor, but not in the bullfrog esophageal smooth muscle (data not shown).

The distribution of androgen receptor-like immunoreactivity was sexually dimorphic in bullfrog laryngeal dilator muscle (Table 1; Figs. 1C and 1D). Males had significantly more immunoreactive nuclei than females. The overall density of muscle nuclei in males and females (determined on a fixed-size field of hematoxylin and eosin-stained sections) was not significantly different. When the number of immunoreactive nuclei was expressed as a percentage of total nuclei in that field, the significant difference between males and females remained. Males had approximately 13% more androgen receptor-positive nuclei than females.

There were significant sex differences also in larynx morphology (Table 2). The length and width of the entire larynx and also of the laryngeal dilator muscle was significantly greater (by about 30%) in male bullfrogs, compared to females. The cross-sectional area of the dilator muscle was 42% larger in males than in females. Enzymatic activity in the laryngeal dilator muscle, as determined by overall intensity of staining for SDHase, was significantly greater in males (Table 2; Figs. 1A and 1B).

Finally, there was a sex difference in the size and type of muscle fibers which made up the bullfrog dilator. Overall, the cross-sectional area of individual fibers on SDHase-stained sections was significantly greater in males than in females (Table 2; Figs. 1A and 1B). In addition, SDHase staining in male muscle appeared more homogeneous (Figs. 1B and 2A), with 98% of cells having a medium-intensity staining and very light-staining fibers being virtually absent. In males, fibers with different intensities of staining did not have significantly different areas (Fig. 2A). In

FIG. 1. Histochemical staining of the bullfrog laryngeal dilator muscle. (A) Succinate dehydrogenase (SDHase) staining in a female. (B) SDHase staining in a male. (C) Androgen receptor-like immunocytochemical staining in a male. (D) Androgen receptor-like immunocytochemical staining in a female. (E) Staining remains when primary antibody is preadsorbed with a peptide from elsewhere in the androgen receptor (see Materials and Methods). (F) Specific staining disappears when the antibody is preadsorbed with the peptide used to create the antibody. Gross histology differs between A, B, and C–F because tissue stained for SDHase was cut in cross-section while that stained for androgen receptor was cut longitudinally. Bar, 50 μm.



females, three classes of fibers with different staining intensity could be recognized (Figs. 1A and 2B). Dark-stained fibers represented about 4.3% of the total fiber sample, medium-stained fibers represented 39.4% (comparable to most male fibers), and light-stained fibers represented 56.3% of the fibers. In female bullfrog muscle, there were significant differences in fiber area for fibers with different staining intensities (Figs. 1A and 2B; one-way ANOVA *df* 2,157; $F = 17.822$; $P < 0.001$). Mean fiber area (±SEM) for dark fibers in females was 697 ± 127 μm^2, mean area for medium-stained fibers was 1354 ± 89 μm^2, and mean fiber area for light fibers was 1878 ± 71 μm^2. Areas of each of the three fiber types in females (dark, medium, and light) differed significantly from all other types (post hoc Fisher's LSD test).

DISCUSSION

Sex differences in laryngeal morphology of bullfrogs may be due to effects of plasma androgens on the larynx. The larynx muscle tissue of both male and female bullfrogs contained specific androgen receptor-like immunoreactivity, but males exhibited a significantly higher density of androgen receptors. This is the first time that androgen receptors have been located in the anuran larynx with immunocytochemistry. Previously, ligand-binding and molecular methods were used to identify androgen receptors in the larynx of *Xenopus* (Fischer and Kelley, 1991; Kelley, 1996). Our findings in bullfrogs support the hypothesis that androgen sensitivity of the larynx is a common feature of anurans. Spectral and temporal properties of frog vocalizations are dependent on larynx muscle fiber type and mass of laryngeal structures (McAlister, 1961;

TABLE 2

Morphological Parameters of the Larynx in Male ($n = 5$) and Female ($n = 8$) Bullfrogs (Means ± SEM)

Parameter[a]	Males	Females
Larynx length (cm)	2.26 ± 0.05	1.49 ± 0.02
Larynx width (cm)	2.52 ± 0.10	1.87 ± 0.05
Dilator muscle length (cm)	1.48 ± 0.08	1.08 ± 0.04
Dilator muscle width (cm)	0.66 ± 0.05	0.43 ± 0.02
Dilator cross-sectional area (mm^2)	174.9 ± 14	100.7 ± 9
Density of SDHase staining (gray level)	113.8 ± 4	160.4 ± 4
Muscle fiber cross-sectional area (all fiber types; μm^2)	2080 ± 121	1620 ± 59

[a] Unpaired *t* test comparison between males and females found significant differences ($P \leq 0.001$) in all parameters.

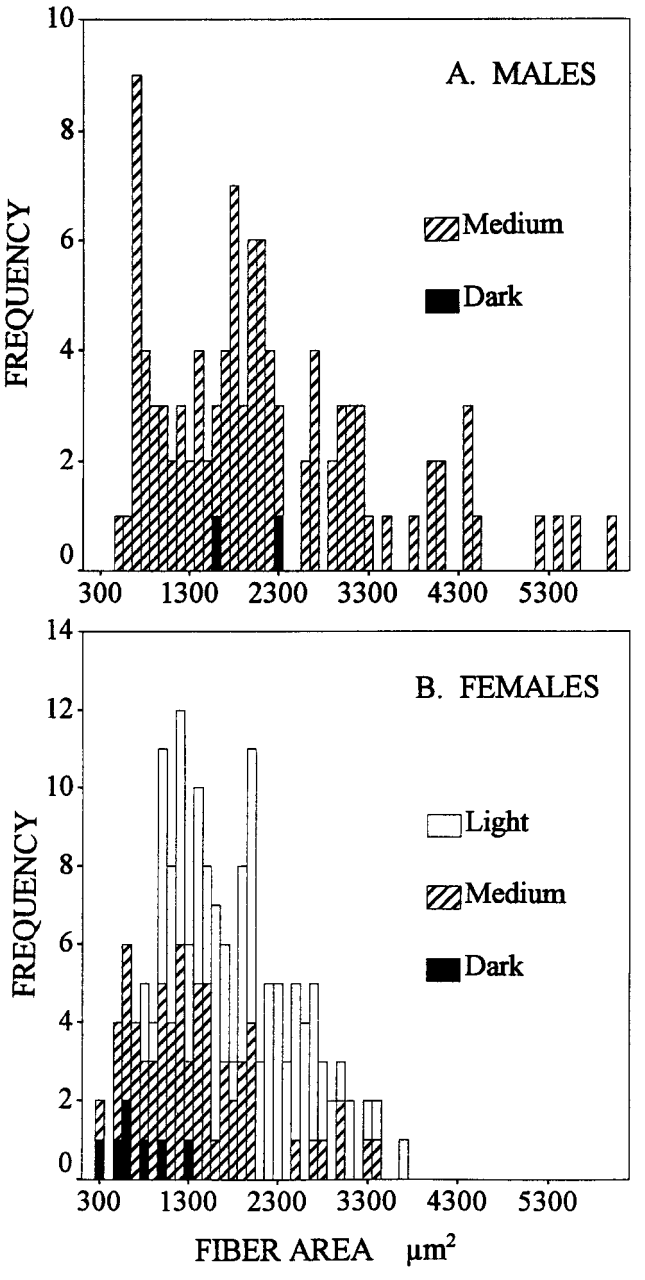


FIG. 2. Frequency histograms of individual muscle fiber cross-sectional area in the laryngeal dilator muscle of male (A) and female (B) bullfrogs. Legend indicates the darkness of staining of individual fibers for SDHase. Bin width was 100 μm^2.

Drewry *et al*., 1982; Ryan, 1986; Tobias and Kelley, 1988; McClelland *et al*., 1996). Sexual differences in larynx morphology may thus contribute to sexual differences in calling.

The antibody we used likely recognized an authentic androgen receptor protein in bullfrogs because the structure of the androgen receptor is relatively conserved across vertebrates. The amphibian androgen receptor has been cloned from *Xenopus* (GenBank Accession No. U67129). Within the DNA-binding region, rat and frog receptors are identical except for one amino acid substitution (He *et al*., 1990; Fischer *et al*., 1993). Our antibody was raised against the first 21 amino acids of the rat androgen receptor (Prins *et al*., 1991). There is 71% homology between the first 21 amino acids of the rat and frog receptors. On the other hand, there is minimal overlap in this same region between the *Xenopus* androgen receptor and other *Xenopus* steroid hormone receptors (e.g., Weller *et al*., 1987). It is thus unlikely that this antibody recognized another steroid receptor in bullfrogs. This antibody has been previously used to identify androgen receptors in other tissues in amphibians (Dorlochter *et al*., 1994; Matsumoto *et al*., 1996). Similarity of androgen receptor structure between amphibians and rats is also supported by cross-reactivity of other antibodies (Davis and Moore, 1996), by ligand-binding studies (Lupo *et al*., 1993), and by cDNA probe hybridization experiments (Varriale and Serino, 1994).

Sex differences in androgen responsiveness may underlie morphological differences in the bullfrog larynx. Male bullfrog laryngeal dilator muscle contained 13% more androgen receptor-positive cell nuclei than female muscle. This difference alone may account for sexual differences in larynx morphology and the call which is ultimately produced. In *Xenopus*, androgens stimulate myogenesis, increases in fiber area and sex-typical changes in fiber type composition (Kelley, 1996). Androgens also increase fiber diameter and change fiber type in forelimb muscles involved in the sexual amplectic clasp (Muller *et al*., 1969; Rubinstein *et al*., 1983; Dorlochter *et al*., 1994). Bullfrogs were sacrificed during the breeding season when plasma androgens are high (Licht *et al*., 1983; Boyd, 1992). At this time of year, plasma androgens are usually equivalent in both sexes so sex differences in circulating androgens are unlikely to account for all differences in calling behavior.

Muscle fiber type in the bullfrog larynx was also sexually dimorphic. Based on the area and SDHase staining of fibers in the bullfrog larynx, the male larynx muscle contained primarily fast-twitch oxidative/glycolytic fibers (FOG fibers; corresponding to amphibian type 2 or mammalian type IIa fibers; nomenclature of Gans and De Gueldre, 1992). In size and staining, male bullfrog laryngeal fibers were most similar to those in the frog hindlimb muscle although ratio of fiber types differs (Lannergren and Smith, 1966; Smith and Ovalle, 1973; Putnam and Bennett, 1983; Rowlerson and Spurway, 1988). Male bullfrog larynx may also contain fast-twitch glycolytic fibers because the fibers with the largest diameter were also lightest staining but statistical analysis did not prove the existence of a distinctly different set of fibers. Laryngeal muscles of both *Xenopus* and *Hyla versicolor* have been described as consisting entirely of FOG fibers (Marsh and Taigen, 1987; Sassoon *et al*., 1987). Our finding of this predominant fiber type in bullfrog larynx muscle is thus similar to two other anuran species.

Muscle fiber types in laryngeal dilator muscles differed significantly between male and female bullfrogs. Female bullfrog dilator muscle contained three distinct classes of fibers (based on Lannergren and Smith, 1966; Smith and Ovalle, 1973; Putnam and Bennett, 1983; Lannergren and Hoh, 1984; Rowlerson and Spurway, 1988; Rowlerson, 1994). Large-diameter and light-staining fibers were likely fast-twitch glycolytic fibers (corresponding to amphibian type 1 and mammalian type IIB; Gans and De Gueldre, 1992). Medium-size and medium-staining fibers were likely FOG fibers and similar to those in male bullfrogs. Small, dark fibers were probably also FOG fibers but from a subgroup with a slower twitch which corresponds to amphibian type 3 (and mammalian type I; Rowlerson and Spurway, 1988; Gans and De Gueldre, 1992; Rowlerson, 1994). In *Xenopus* and *Hyla arborea*, females also possess a mixture of fiber types in laryngeal muscle (Eichelberg and Schneider, 1974; Sassoon *et al*., 1987).

Species differences in call rate, call amplitude, and mechanisms of sound production are likely to be correlated with variation in the composition of laryngeal muscles. Bullfrog laryngeal dilator muscle fibers

were significantly larger than fibers in the same muscle in *Xenopus* (2080 μm^2 area in bullfrogs compared with 5–25 μm^2 in *Xenopus*; Sassoon *et al*., 1987). It is unlikely that this difference is due to differences in body size between the two species because muscle fiber size is generally unrelated to body size (Putnam and Bennett, 1983; Marsh and Taigen, 1987). The intensity of staining for SDHase also differed significantly between male *Xenopus* (''very dark'' corresponding to high oxidative capacity; Sassoon *et al*., 1987) and male bullfrogs (''medium''). Fiber type composition and metabolic enzyme levels of muscle are related to activity levels in frogs. This is true across species, within species, and within individuals (Engel, 1965; Bennett, 1974; Cummings, 1979; Sperry, 1981; Given and McKay, 1990). Very small fiber diameter and very dark staining for SDHase in *Xenopus* larynx may represent specializations for high call rates. The advertisement call of male *Xenopus* requires contraction of the dilator muscle at a rate of about 71 times per second (Kelley and Gorlick, 1990). In contrast, the bullfrog advertisement call is much slower, with the dilator muscle contracting only about twice a second (Capranica, 1965). The energetic costs of calling in bullfrogs are modest, compared to some other anurans (e.g., Taigen *et al*., 1985), and this may be associated with lower levels of SDHase in laryngeal muscle.

The laryngeal dilator muscle plays a critical role in call production by opening the glottis and allowing air flow from the lungs to the buccal cavity (deJongh and Gans, 1969). Androgen receptor localization in this muscle supports the hypothesis that this tissue is androgen sensitive. Plasma androgens in bullfrogs may thus be responsible for morphological sexual differences in the larynx and sex differences in calling behavior. In male bullfrogs, the dilator muscle is larger overall, individual fibers have larger diameters and oxidative capacity is greater, compared to the female bullfrog dilator muscle. Male laryngeal muscle therefore has the potential to generate more force which could, in turn, result in greater call duration and longer calling bouts. The advertisement call of males does show these differences from the female release call (Capranica, 1965). It is unlikely however that these muscle differences represent absolute constraints on advertisement calling by female bullfrogs. Females give no advertisement call whatsoever—not even one that is shorter and simpler. Dimorphisms in the central nervous system thus likely account for the lack of an advertisement call in female bullfrogs.

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