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The Sexually Dimorphic Larynx of *Xenopus laevis*: Development and Androgen Regulation

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ABSTRACT The aims of this study were to characterize sexual dimorphism in the larynx of adult *Xenopus laevis* and to determine how sex differences arise during postmetamorphic development. The larger male larynx is a result of greater cell numbers in both cartilage and muscle. The dilator laryngis muscle of the male larynx has 6–7 times more muscle fibers than that of the female. At metamorphosis, the larynx is sexually monomorphic and feminine in phenotype. The DNA content of the male larynx doubles during the first 6 months following metamorphosis; there is no net DNA increase in the female larynx during this time. Both sexes experience a marked increase in laryngeal DNA content and mass between 6 months and adulthood. The number of muscle fibers in the male larynx increases at an average rate of 150 fibers a day during the first 10 months of postmetamorphic development. There is no net change in fiber numbers in the female larynx from metamorphosis to adulthood. Administration of the antiandrogen Flutamide to metamorphic frogs prevents the net addition of laryngeal muscle fibers in males. Thus, we propose that addition of postmetamorphic laryngeal muscle fibers in males is dependent upon the presence of circulating androgens. Exogenous testosterone administration results in an increase in laryngeal mass, DNA content, and cellular proliferation in juvenile frogs. Using [^{3}H]thymidine injections to probe ongoing, as well as testosterone-induced, cell proliferation, we conclude that cellular proliferation is regulated differently in males and females during development. Thus androgen-induced proliferation is one cellular mechanism responsible for the sexual dimorphism observed in adults.

In several species of the anuran genus *Xenopus* sounds are produced by movement of paired cartilagenous disks within a highly specialized larynx (Ridewood, 1898; Yager, 1982; Tobias and Kelley, 1985). Previous investigators have noted that the adult male larynx is larger than that of the adult female and has a more complex morphology (Ridewood, 1898; Patterson, 1939). This sex difference in laryngeal morphology is accompanied by differences in vocal behaviors. Male South African clawed frogs, *Xenopus laevis*, use a sex-specific mate call to attract females (Russell, 1954; Wetzel and Kelley, 1983). Adult female *X. laevis* do not mate call (Hannigan and Kelley, 1986). Sex differences in the larynx are, in part, responsible for sex differences in vocal ability (Tobias and Kelley, 1985). The aims of this study were to characterize laryngeal sex differences in *X. laevis* at morphological and cellular levels and to determine when in development these differences appear.

Two possible scenarios for virilization of the larynx were considered. One model, based on observations of the developing mammalian reproductive tract (Jost, 1972), proposes that both male and female embryos develop from initially female or "neutral" anlagen. According to this hypothesis, the larynges of both sexes start out female-like, and the male larynx subsequently diverges during development by acquiring male-specific morphological and cellular characteristics. An

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alternative model is based on the rat bulbocavernosus–levator ani complex, sexually dimorphic skeletal muscles involved in male reproduction. This muscle is absent in adult females. At birth, however, both sexes exhibit the male-specific configuration (Cihak et al., 1970; Breedlove and Arnold, 1983); females lose male-like morphology and cell numbers unless supplied with exogenous androgens. In this second scenario, we might expect juvenile male and female *X. laevis* to have male-like larynges early in development, which degenerate in the female in the absence of endogenous circulating androgens.

We previously demonstrated, using [^{3}H] thymidine autoradiography, that testosterone induces laryngeal chondrogenesis and myogenesis in juvenile (sexually immature) frogs of both sexes but not in other muscle and cartilage (Sassoon et al., 1986). This proliferative response is not observed in other tissues or in sexually mature adults. Thus, one plausible hypothesis for the development of laryngeal sex differences is tissue-specific, androgen-induced muscle and cartilage growth due to male-specific hormone secretion during postmetamorphic development.

For this study, we examined the gross morphology, muscle fiber numbers, DNA content, and rate of cell proliferation in the larynx. Male and female adults and juveniles ranging from 0 to 10 months of postmetamorphic development were examined. In addition, we used testosterone administration, in conjunction with [^{3}H]thymidine administration at four postmetamorphic stages and in adulthood, to determine how the proliferative response to testosterone is regulated during development.

GENERAL MATERIALS AND METHODS

Animals

Animals were obtained from Nasco (Ft. Atkinson, WI). Juvenile frogs had completed premetamorphic development by approximately 2 months after fertilization. Each age group of animals used was from the same ovulation date unless otherwise noted. Zero month postmetamorphic frogs (stage 66; Nieuwkoop and Faber, 1956) were ~0.5–1.0 gm in body weight. Three-month postmetamorphic animals (3-month PM) were ~3.5 gm. Six-month PM animals were ~15–20 gm. Ten-month PM animals were dimorphic in body size; males were smaller (~12–17 gm body weight) and females were larger (~15–20 gm body weight). All operative procedures were performed under cold narcosis. Sex was determined by inspection of gonads following sacrifice and dissection. Adults were at least 2 years postmetamorphic. Males were ~36 gm and females were ~60 gm in body weight. Gonads were inspected to assure that the adults were sexually mature.

Morphology/fiber counts

For morphometric analyses (morphology and muscle fiber counts), animals were perfused transcardially with 0.1 M phosphate-buffered saline (PBS) followed by 1% paraformaldehyde in PBS. Larynges were dissected free of surrounding tissue, weighed, and fixed in 1% paraformaldehyde for 5–24 hr at 4°C. Tissue was postfixed in OsO_4 (1–2 hr) and embedded into Epon (Polysciences). Sections 7μm thick were cut in a plane transverse to the longitudinal axis of the muscle fibers and studied with the aid of a computer-interfaced bit pad and camera lucida for morphometric analysis (Peachey, MorphII System). Six representative fields from right and left dilator laryngis muscles (Ridewood, 1898) were examined, and muscle fibers were counted. The area of the entire muscle for that section was measured, and the total number of fibers was calculated by multiplying the mean number of fibers per field by the total number of fields per muscle. No difference was noted between right and left laryngeal muscles (data not shown). At all stages examined, fiber numbers were greatest in the middle anterior-posterior section(s) through each larynx; these maximal values were therefore used in the present analysis.

We investigated the effects of testosterone on fiber numbers in adult females. For this study, three adult females were ovariectomized and treated for 1 month with testosterone propionate as previously described (Kelley and Pfaff, 1976). The animals were sacrificed, and larynges were prepared as described above. We also assessed the effects of testosterone on cartilage shape and muscle mass for comparison with untreated adult male and female frogs.

An additional study was performed to assess the effects of administration of an antiandrogen on muscle fiber addition. Twenty 0-month PM juveniles received implants of a compressed pellet of Flutamide (Schering Corp., 0.5 mg/gm body weight) into the dorsal lymph sac for 3 months. Animals were

sacrificed and sex was identified by surgical inspection. Three males and three females were prepared for analysis of muscle-fiber numbers as described above and compared to control (non-Flutamide treated) animals from the same fertilization group.

[^{3}H]Thymidine incorporation and DNA determination

Frogs were injected intraperitoneally with [^{3}H]thymidine (NEN, 6.7 mCi/ml, aqueous) at a dose of 1 μCi/mg body weight. Twenty-four hours after nucleotide injection, animals were perfused transcardially with 0.1 M phosphate buffer to remove any residual unincorporated nucleotide. Recent studies from this laboratory have shown that 24 hr is sufficient time for removal of unincorporated thymidine (Gorlick and Kelley, 1986, and unpublished). Larynx and thigh muscle were dissected free of surrounding tissue, placed in 0.1 M TES (0.15M NaCl, 0.02 M Tris-HCl [pH=7.8], 1 mM EDTA) buffer, and stored at −70°C until processed within 2 weeks of sacrifice. Initial studies were performed on entire larynges; additional studies were then carried out separately on laryngeal muscle and cartilage. DNA was extracted according to the method of Atkinson and Just (1975). The tissue was homogenized in a glass/Teflon dounce (Kontes) and DNA was extracted with an equal volume of phenol (Mallincrodt). From 3 to 5 extractions were necessary to eliminate protein contamination. The final aqueous phase was organically extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and precipitated in high salt overnight at −20°C. The precipitate was dried and then resuspended in 150 μl of TES. The pellet was gently washed prior to resuspension to remove residual RNA. DNA content was determined by measuring absorbance at 260 nm on a Hitachi 139 spectrophotometer. Absorbance measurements at 280 nm were routinely performed to assess protein contamination, and measurements were compared with purified bovine DNA (Sigma). For preliminary studies, the nucleotide content of the pellet was determined as predominantly DNA by exposing samples to DNAase or RNAase prior to extraction (data not shown). The amount of [^{3}H]thymidine was determined from scintillation counting of two 50-μl samples of the resuspended pellet. Counts were corrected for efficiency by comparison with a tritium standard (^{3}H-toluene; New England Nuclear).

Data analyses

Results are presented in the text as means (± standard deviation). In figures, variability is indicated with error bars denoting the range of values obtained. The significance of differences between groups was evaluated using the Mann-Whitney U Test (Siegel, 1956).

RESULTS

Adults

General morphology

The larynx of *Xenopus laevis* is composed predominantly of hyaline cartilage and striated muscle (Ridewood, 1898; Patterson, 1939; Sassoon et al., 1986). The interior of the cartilagenous skeleton forms a cavity connecting with the buccal cavity through the glottis (anteriorly) and with the lungs through the bronchi (posteriorly) (see Fig. 1). Sound is produced by the rapid pulling apart of paired arytenoid disks (Yager, 1982), two tightly apposed hard cartilagenous surfaces located anteriorly in the larynx just ventral and posterior to the glottis. The lateral aspects of the laryngeal skeleton support the dilator laryngis muscles (Ridewood, 1898), which insert via a tendon into the arytenoid disks.

As is apparent in Figure 1, the larynges are sexually dimorphic in external appearance. The anterior–posterior length is equivalent for the two sexes, but the male larynx is wider than that of the female. This dimorphism is particularly pronounced in the rostral third of the larynx, where the greater degree of cartilage development in the male imparts a heart-shaped appearance in contrast to the triangular appearance of the female. An additional difference is the insertion of the m. dilator laryngis onto the laryngeal skeleton. As noted by Ridewood (1898), the laryngeal muscles of the male insert onto the posterior pole of the larynx and onto the medial aspect of the cartilage (ventrally). In contrast, the female muscle does not meet at the ventral midline as in the male. The m. dilator laryngis of females is restricted to the lateral surfaces of the laryngeal skeleton.

Cross sections through the anterior–posterior axis of male and female larynges were examined; representative sections are shown schematically in Figure 2. Two prominent lateral cartilagenous rods run along the length of the larynx; these have been previ-

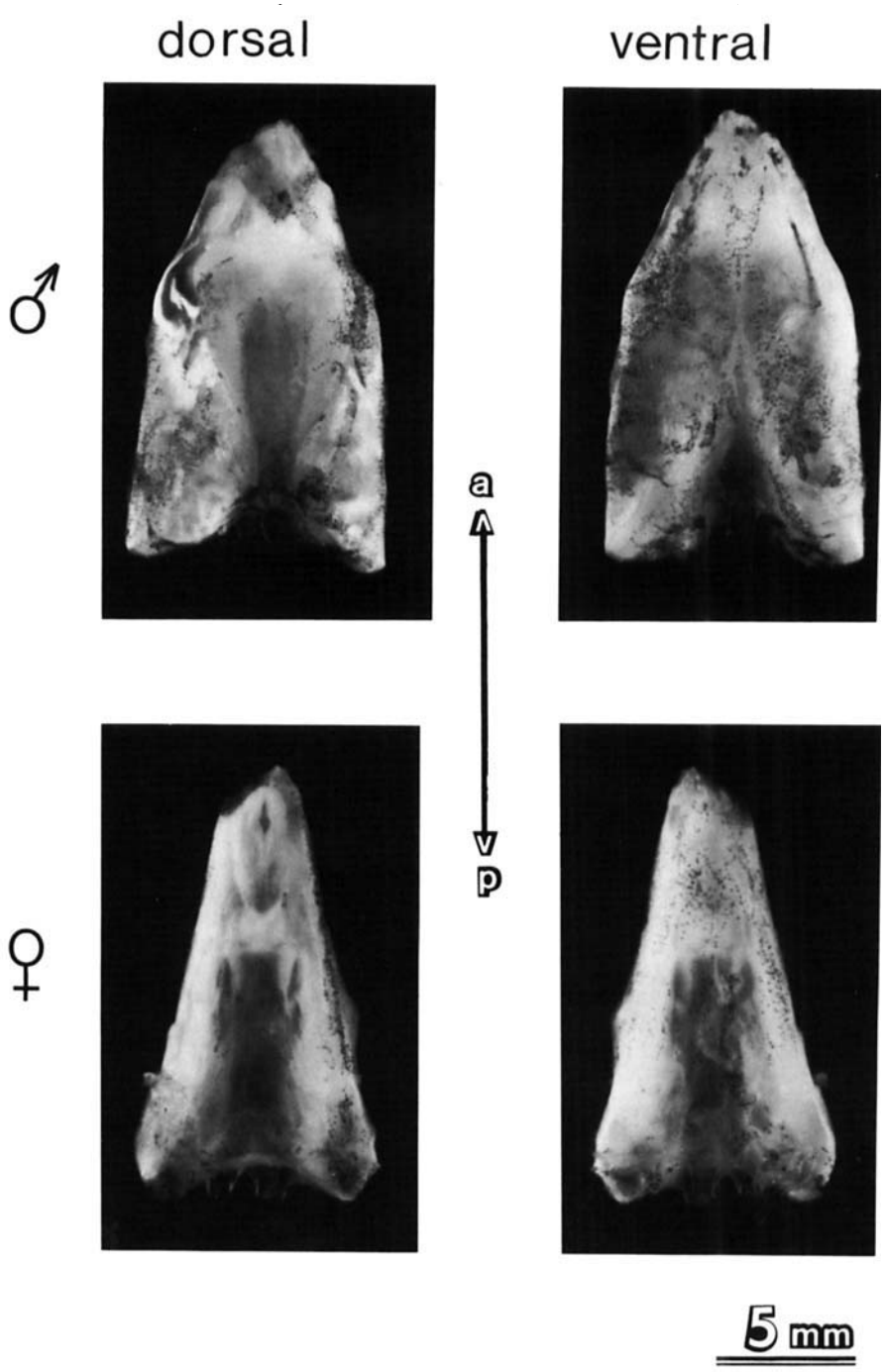

Fig. 1. Photomicrographs of dorsal and ventral views of dissected adult larynges. The male larynx (top row) is larger than the female larynx (bottom row). Enlargement of the cartilage in the anterior third of the male larynx imparts a box-shaped appearence in contrast to the more triangular female morphology. The male dilator laryngis muscles meet at the ventral midline, whereas the female muscles do not. Arrow indicates anterior (a) and posterior (p) poles of the larynx.

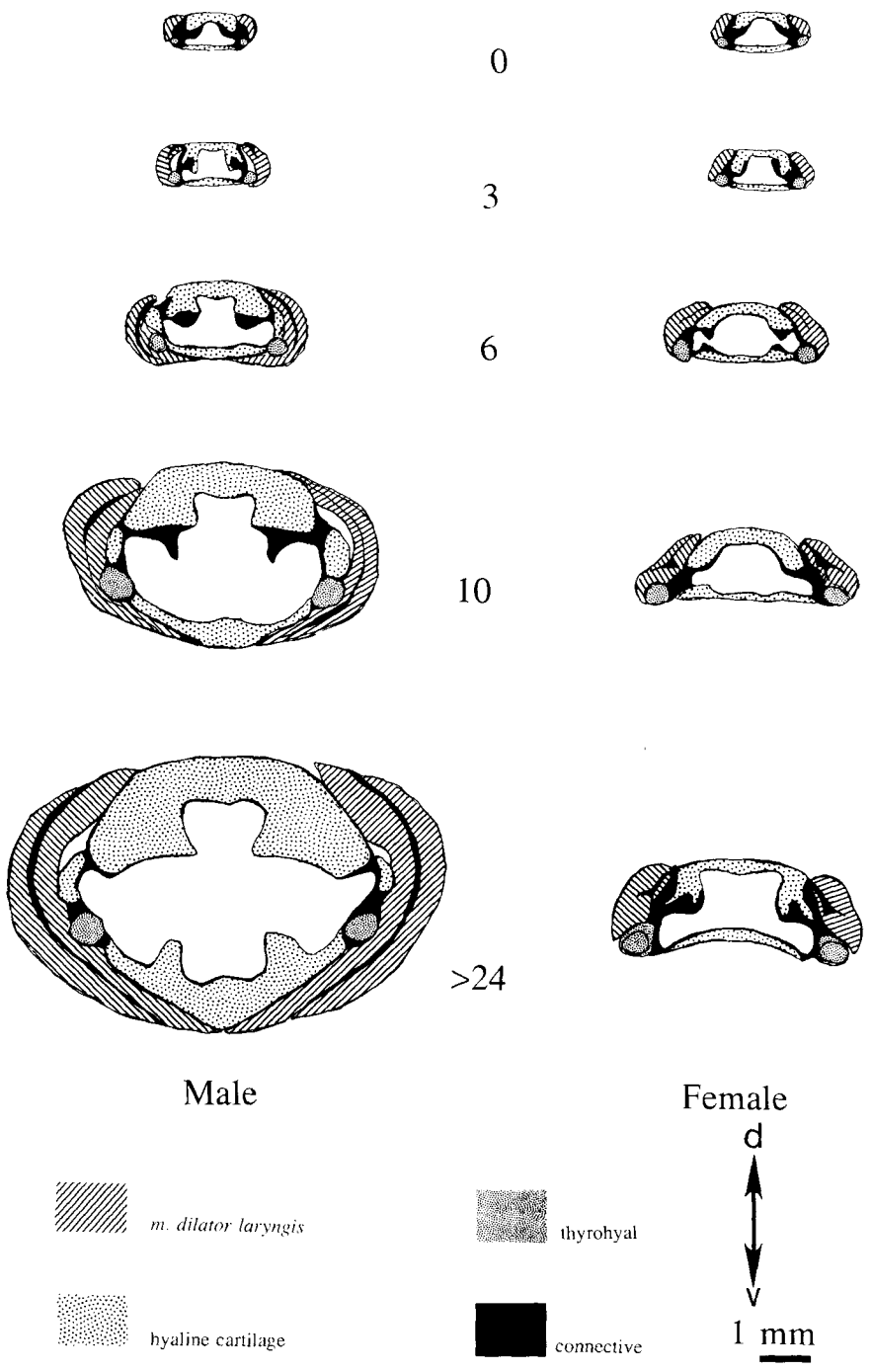

Fig. 2. Drawings of camera lucida projections of cross sections through the middle anterior-posterior axis of male and female larynges at 0, 3, 6, 10, and >24 months of postmetamorphic development. At time 0 PM, no sex difference in morphology is noted and the larynx is feminine in phenotype. Beginning at 6 months PM, sex differences are apparent. The m. dilator laryngis meets at the ventral midline of the male larynx at 6 months, whereas the female muscle is restricted to the lateral margins of the cartilage skeleton. Note the relatively greater proportion of elastin cartilage ("connective" in the female). Arrow indicates dorsal (d) and ventral (v).

ously identified as the *thyrohyals* (Ridewood, 1898). As noted by Ridewood, these structures are more ossified in males than in females. The laryngeal cavity, which is easily compared in cross section, is bounded by hyaline and elastin cartilages. The laryngeal cavity of males is more complex than that of females, consisting of thicker cartilage walls and deeper invaginations. The male dilator laryngis muscles are made up of two leaves that insert into a single tendon, hence are often designated as bipinnate. In contrast, the female dilator laryngis muscles are incompletely septate. The sexual dimorphism of the larynx is clearly reflected in its mass, which is two to three times greater in the male (male > female, $P < 0.05$; male = 503.7 ± 77.5 mg (n = 3); female = 224.0 ± 121.1 mg (n = 3) (Fig. 3). Both muscle and cartilage contribute to this difference in total mass (male muscle = 60 ± 20 mg, cartilage = 440 ± 60; female muscle = 40 ± 10, cartilage = 190 ± 130).

Laryngeal muscle fiber number and DNA content

A previous study of laryngeal muscle (Gray et al, 1985) had shown that the majority of muscle fibers in the male larynx are larger than those of females. Since both the size and the number of muscle fibers could contribute to muscle mass, we determined fiber numbers in the m. dilator laryngis in adult males and females. Counts of right and left muscles were obtained from sections as described above; both male and female fiber numbers were maximal at a section through the middle anterior-posterior region (data not shown). These maximal values obtained from three males and three females were used for comparison. The number of muscle fibers is markedly dimorphic (male > female, $P < 0.05$); males average 31,953 (± 3,969 (n = 3); ~16,000 per side) compared to 4,726 (± 1,833 (n = 3); ~2,400 per side) in the female (see Fig. 4). Thus, a sixfold to sevenfold difference in fiber numbers contributes to the larger muscle mass in the male. The DNA content of three male and three female larynges (muscle and cartilage) was measured. Male laryngeal DNA content is 474 ± 104 μg, compared to 343±73 μg in the female (Fig. 5). The difference in DNA content suggests that males have more cells or cell nuclei than females. The greatest difference between the sexes in DNA amounts is observed in the laryngeal muscle, which has a DNA content of 154 ± 35 μg in the male, compared to 79 ± 21 μg in the female. The cartilage portion of the larynx is less dimorphic, with a DNA

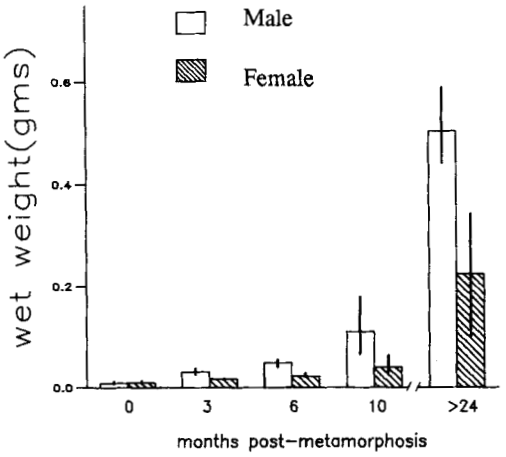

Fig. 3. Changes in laryngeal mass through postmetamorphic development of males and females. Means and ranges (bars) of wet weights from freshly dissected laryngeal tissue are given. For 3-month PM animals and older animals, 3 to 4 animals were measured for each sex. For time 0 PM, 6 males and 11 females were measured.

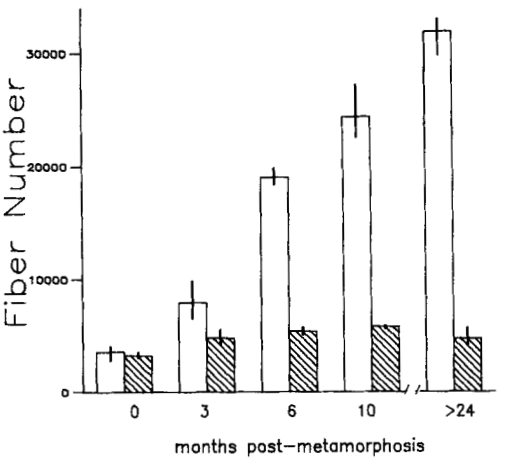

Fig. 4. Changes in laryngeal muscle fiber numbers through postmetamorphic development. Counts represent the mean and ranges (bars) of maximal values for the middle anterior-posterior section through left and right laryngeal muscles from each individual. Three to four larynges for each sex were counted (except for 6-month PM females, n = 2).

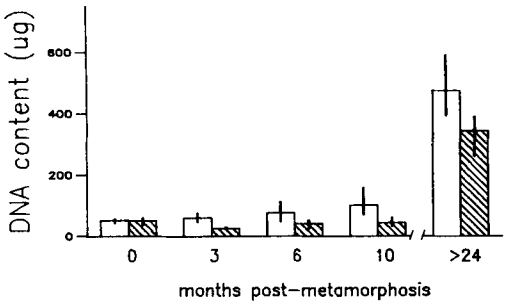

Fig. 5. Increases in laryngeal DNA content of males and females through postmetamorphic development. Means are indicated and error bars represent range of values. Three to five males and females were measured for each stage.

content of 320 ± 80 μg in the male and 263 ± 55 μg in the female.

Postmetamorphic development of laryngeal sex differences

Age-matched, postmetamorphic animals were examined for general laryngeal morphology, weight, muscle fiber numbers, and DNA content. At metamorphosis, no striking sex difference in morphology is apparent although the male larynx appears somewhat more box-shaped than that of the female (Figs. 2, 6). The laryngeal muscle mass has not begun to divide into the two leaves that are characteristic of the adult male. In this respect and in overall form (i.e., triangular shape), the 0-month PM larynx of both sexes is female in appearance. The cavity of the laryngeal skeleton is also similar to the adult female's and does not have any of the complex invaginations characteristic of the adult male. As can be seen in Figure 2, the male larynx undergoes profound changes in morphology over a period of 10 months. These changes include a gradual separation of the dilator laryngis muscle into an inner and outer leaf as well as a thickening of the cartilage and a decrease in elastin cartilage ("connective" cartilage shaded black in Fig. 2). The muscle, which at metamorphosis is confined to the lateral margins of the cartilage skeleton, continues to extend ventrally in the male until the right and left m. dilator laryngis meet at the ventral midline 6 months PM. In contrast, extension of the female laryngeal muscle and cartilage is less marked; the growth of the muscle along the ventral face of the laryngeal skeleton does not occur.

The change in mass during postmetamorphic development is illustrated in Figure 3. At metamorphosis, male and female larynges are not significantly different in mass ($P > 0.5$; male = 8.8 ± 2.5 mg, n = 6; female = 9.9 ± 2.2 mg, n = 11). Both sexes increase in mass over time. The rate of increase in the male is faster (male = 1.1 mg/month) than the rate in the female, which displays a relatively small increase in mass during the first 10 months of postmetamorphic development (female = 0.4 mg/month). Both sexes undergo a fourfold to fivefold increase in mass between 10 months and adulthood (>24 months PM).

Fiber numbers

The adult sex differences in muscle fiber numbers arise, developmentally, by addition of muscle fibers to an initially female complement (see Fig. 4). Females exhibit a modest rise in fiber numbers from 0 to 10 months PM (from 3,260 ± 265 to 6,579 ± 736; 10-month female > 0-month female, $P < 0.001$). No significant difference in fiber numbers is noted between 10-month PM and adult females ($P > 0.5$). In contrast, the number of muscle fibers increases rapidly and continuously during the first 10 months in males.

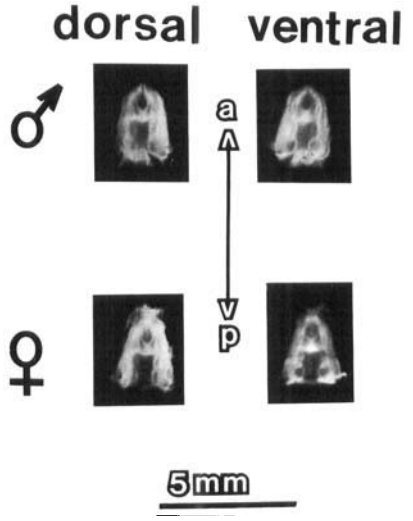

Fig. 6. Photomicrographs of dissected male and female metamorphic larynges (time 0 PM). No sex differences are apparent. The larynges are represented at the same final magnification as for the adults (Fig. 1). Arrow indicates anterior (a) and posterior (p) poles of the larynx.

The number of fibers doubles by 3 months, quadruples by 10 months, and reaches 10 times the number present at metamorphosis by adulthood. The rate of fiber addition increases with age in the male. Between time 0 and 6 months PM, fiber addition proceeds at a rate of ~100 fibers per day, whereas between 6 and 10 months, the rate is ~200 fibers per day. In contrast, the average rate of fiber addition in the female between 0 and 10 months PM is about 10 fibers per day.

DNA content

To assess when changes in laryngeal cell numbers appear in development, DNA content was determined from 3 to 5 postmetamorphic male and female frogs at each stage. At time 0 PM, no dimorphism in DNA content is detectable. As illustrated in Figure 5, the female does not show any net increase in DNA content during the first 10 months of postmetamorphic development, and thus we conclude that the increase in mass observed in the female larynx over time is primarily a result of cell hypertrophy. In fact, a significant decrease in DNA content is observed between 0 and 3 months PM in the female larynx (3-month PM female < time 0 PM female, $P < 0.05$). In contrast, by 10 months, the DNA content of the male larynx has doubled. Both sexes show an approximate threefold increase in DNA content between 10 months PM and adulthood. Thus, addition of new cells or cell nuclei contributes to the large increase in size and mass during this transition.

Cellular proliferation

Age-matched postmetamorphic frogs and adults of each sex were injected with a single dose of radiolabeled thymidine, and incorporation into laryngeal tissue was measured 24 hr later. Total [^{3}H]thymidine incorporation per larynx thus reflects the relative rate and amount of dividing cells at the time of injection. As can be seen in Table 1, there is no significant sex difference in thymidine incorporation at time 0 PM ($P > 0.5$). At 3 and 6 months PM, male laryngeal tissue has a higher level of [^{3}H]thymidine incorporation than female tissue ($P < 0.05$). Males show a gradual increase in thymidine incorporation during development. Females, however, have less [^{3}H]thymidine incorporation at 6 months than at 3 months PM and then show an increase again by 10 months PM.

In order to determine whether the sex-specific patterns of [^{3}H]thymidine incorporation during postmetamorphic development are displayed by nonsexually dimorphic skeletal tissues, we also examined thigh muscles (m. semimembranosus and m. gluteus) from the same animals. Several reports from our laboratory indicate that these muscles are relatively androgen-insensitive and not sexually dimorphic (Segil et al., 1986; Gray et al., 1985; Sassoon et al., 1986). Although large amounts of thigh tissue were used for this study (~0.2–0.5 gm), total incorporation of the radiolabeled nucleotide never exceeded twice the background level of [^{3}H]thymidine incorporation (cpm < 15; data not shown). Thus, as previously reported for 3-week PM animals (Sassoon et al., 1986), laryngeal tissue exhibits a higher rate of cell division during postmetamorphic development than does thigh. In addition, we note that adult larynges, which do not increase in net cellularity, also show a significantly higher level of [^{3}H]thymidine incorporation than does thigh.

TABLE 1. Total counts per minute (cpm) of [^{3}H]thymidine activity per larynx in control (non-hormone-treated) and 4-day testosterone-treated (4-day T-treated) frogs

Months PM	Sex	n	Controls[1]	n	4-day T-treatment[1]	× Induction[2]
0	Male	3	85.1 (70.6)	3	15,670.0 (1,677)	184
0	Female	6	65.3 (30.1)	4	3,357.3 (1,161)	51.43
2–3	Male	3	827.5 (104.9)	3	10,447 (6,260)	12.6
2–3	Female	2	120.3 (n/a)	3	5,311 (4,815)	44.15
6	Male	3	810.3 (714.4)	3	3,406.6 (748.5)	4.2
6	Female	3	83.1 (13.7)	3	515.7 (184.9)	6.2
9–10	Male	3	2,536.7 (592.7)	3	27,766.7 (7,879.2)	10.95
9–10	Female	4	4,107.8 (3,580.6)	3	14,340 (3,020.2)	3.5
>24	Male	3	1,282.4 (243.8)	3	3,142.3 (136.8)	2.45
>24	Female	3	1,091.7 (280.4)	3	2,445.9 (894.3)	2.24

[1]Values are given as means (±SD).
[2]Ratio of hormone-treated total counts over controls.

Effects of sex steroids

Adults. Androgen withdrawal (induced by castration) for 6–9 months does not affect male laryngeal mass or morphology (Segil et al., 1986), nor does it affect the metabolic properties of laryngeal muscle fibers (Gray et al., 1985; Sassoon et al., 1986). In contrast, in adult females, treatment with testosterone for a period of 1 month results in a dramatic increase in cartilage and muscle mass as well as a change in overall laryngeal morphology (see Fig. 7). This response was therefore examined in more detail.

Adult females were ovariectomized and implanted with a pellet of testosterone propionate. One month later, animals were sacrificed, larynges were removed, and muscle fiber numbers were counted. As illustrated in Figure 6, muscle and cartilage cross-sectional area is larger following testosterone treatment. Muscle fiber numbers (5,413 ± 1,122, n = 3), however, were not signifi-

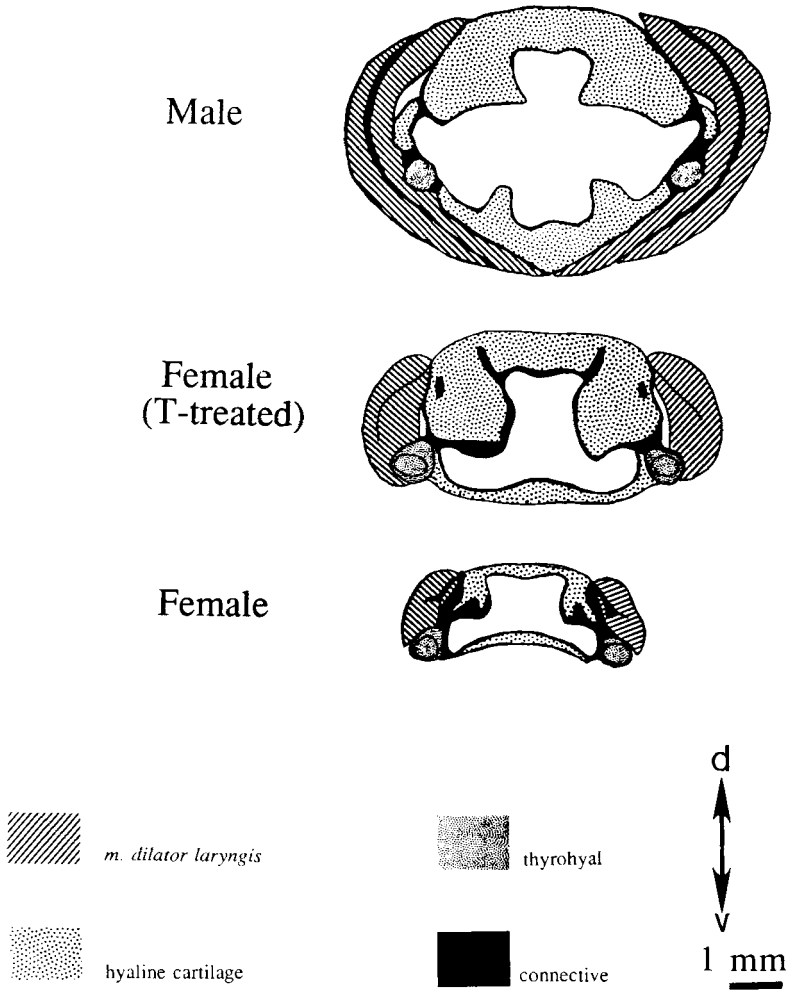

Fig. 7. Drawings of camera lucida projections of cross sections through an adult male (top), female (bottom), and testosterone-treated female (middle) larynx. Section level is from the middle anterior–posterior axis of the larynx. Note that although the female larynx increases in cartilage and muscle area following 1 month of hormone treatment, the morphology of the female larynx is not completely masculinized. For example, the dilator laryngis muscles remain incompletely septate and do not meet at the ventral midline. Arrow indicates dorsal (d) and ventral (v).

cantly different than numbers for normal females ($P > 0.5$; 4,726 ± 1,833, n = 3).

Juveniles. We tested the hypothesis that androgens direct the development of male-specific laryngeal characteristics. Since hormone titers during postmetamorphic development have not been described, our initial approach involved the administration of large doses of testosterone propionate and assays of effects on muscle fiber numbers. Treatment of 0-, 1-, or 2-month PM males and females for periods longer than 5 weeks resulted in high mortality. The larynges, although greatly increased in cartilage and muscle mass, showed no increase in fiber numbers, and in some cases fiber numbers actually decreased (data not shown). As observed with androgen-treated adult females, the cartilage is masculinized to some extent following continuous androgen treatment.

One explanation for the high mortality and rapid laryngeal enlargement observed in response to exogenously administered testosterone in juveniles is that doses administered far exceeded physiological levels. We chose to interfere with endogenous androgen by administering an antiandrogen, Flutamide. In rats, this compound is metabolized to an active androgen antagonist that inhibits development of secondary sexual characteristics; in females, no obvious effects are observed (Neri et al., 1972; Breedlove and Arnold, 1983). Metamorphic (time 0 PM) male and female juveniles were treated continuously with Flutamide for 3 months, at which time larynges were removed and laryngeal muscle fiber numbers were counted. Male and female larynges from sibling control (non-Flutamide-treated) animals were also compared to assure that the twofold sex difference in fiber numbers at 3 months previously observed (Fig. 4) was achieved under laboratory rearing conditions. The results from this study are presented in Figure 8. Control male values, as expected, were approximately two times higher than that of control females (males = 8,090 ± 1,702, females = 4,451 ± 308; males > females, $P < 0.05$). Laryngeal muscle-fiber numbers of Flutamide-treated males were less than those of control males, and were not significantly different from those of normal females (Fig. 8). Flutamide treatment of females had no effect on fiber numbers. Thus, we conclude from this study that androgens present during postmetamorphic development are required for the addition of laryngeal muscle fibers in the male.

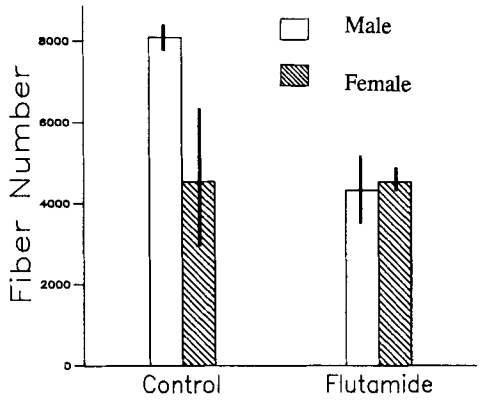

Fig. 8. Laryngeal muscle fiber number in male and female 3-month PM juveniles treated since metamorphosis with the antiandrogen Flutamide. Note that the control animals are dimorphic in muscle fiber numbers, whereas the fiber numbers in Flutamide-treated males is equal to the normal female value at 3 months. Values are given as means (n = 3 for each group). Error bars represent the range of values.

Testosterone-induced cell proliferation

We have previously reported, using [^{3}H]thymidine autoradiography, that exogenously administered testosterone results in a burst of laryngeal myoblastic and chondroblastic cell proliferation in 3-week PM frogs (Sassoon et al., 1986). Adult frogs do not exhibit this response. We wished to determine how the proliferative response to testosterone is regulated during PM development. We implanted 0-, 3-, 6-, and 10-month PM and adult male and female frogs with testosterone. Four days following hormone pellet implantation, animals received a single injection of [^{3}H]thymidine and were sacrificed 24 hr later. [^{3}H]thymidine incorporation was measured as described above and results were compared to outcomes for age-matched controls. Total [^{3}H]thymidine incorporation, presented as cpm/larynx, indicates the amount and rate of cellular proliferation in response to testosterone administration. Males exhibit a higher amount of thymidine incorporation per larynx in response to testosterone treatment than do females during the postmetamorphic stages examined (Table 1).

We then compared these results to our pre-

TABLE 2. Mass of larynges (wet weight in grams) from control (non-hormone-treated) and 4-day testosterone-treated (4-day T-treated) frogs

Months PM	Sex	n	Controls[1]	n	4-day T-treatment[1]	× Induction[2]
0	Male	6	0.0088 (0.0025)	6	0.0200 (0.0035)	2.27
0	Female	11	0.0099 (0.0022)	5	0.0100 (0.0018)	1.01
2–3	Male	4	0.0308 (0.0080)	3	0.0300 (0.0100)	0.97
2–3	Female	3	0.0168 (0.0017)	4	0.0200 (0.0036)	1.24
6	Male	3	0.0490 (0.0089)	4	0.0500 (0.0100)	1.02
6	Female	3	0.0220 (0.0089)	3	0.0300 (0.0031)	1.21
9–10	Male	3	0.1100 (0.0610)	3	0.2000 (0.0800)	1.85
9–10	Female	3	0.0423 (0.0189)	3	0.0400 (0.0100)	0.95
>24	Male	3	0.5037 (0.0775)	4	0.5000 (0.0600)	0.99
>24	Female	3	0.2240 (0.1211)	4	0.2400 (0.0400)	1.07

[1]Values are given as means (±SD).
[2]Ratio of hormone-treated total mass over controls.

vious study with age-matched juveniles by determining the ratio of [^{3}H]thymidine incorporation in hormone-treated larynges and nonhormone-treated larynges (Table 2). At metamorphosis, testosterone induces 184 times more thymidine incorporation in males than is observed in controls. This induction decreases to 12× at 3 months and 4× at 6 months PM. Thus, the first 6 months of postmetamorphic development in males are marked by a decline in the proliferative response to testosterone. At 10 months, however, a 10-fold increase in testosterone-induced thymidine incorporation in males is observed compared to controls. Time 0 PM females are less responsive to testosterone than males (females = 51×; males = 184×). At 3 months PM, testosterone induction is 44× and then decreases to 6× at 6 months PM and 4× at 10 months PM. Hormone-treated adult males and females show a twofold increase in thymidine incorporation compared to adult controls.

The laryngeal DNA content of testosterone-treated larynges at all stages was determined (Table 3). DNA content increases significantly in males and females as a result of testosterone treatment at 0, 3, and 6 months PM, but not at 10 months PM or in adults. Thus, the pattern of testosterone-induced thymidine incorporation differs for the sexes. Males show marked testosterone-induced proliferation at metamorphosis which declines (except for a burst at 10 months PM) thereafter. In females, testosterone-induced proliferation is less marked than in males at metamorphosis but stays elevated at 3 months and then falls to adult values thereafter (no burst at 10 months). Thus, testosterone, administered early in development, results in a more rapid change in the number of cells or cell nuclei in the larynx.

TABLE 3. DNA content (micrograms) per larynx in control (non-hormone-treated) and 4-day testosterone-treated (4-day T-treated) frogs

Months PM	Sex	n	Controls[1]	n	4-day T-treatment[1]	× Induction[2]
0	Male	4	51.05 (6.66)	4	83.34 (60.43)	1.63
0	Female	4	49.99 (10.44)	4	71.28 (18.88)	1.43
2–3	Male	3	60.00 (15.00)	3	73.67 (27.21)	1.23
2–3	Female	3	25.00 (4.36)	3	30.33 (7.51)	1.21
6	Male	3	75 (34.4)	3	106.25 (12.15)	1.42
6	Female	3	40.5 (15.98)	3	62.5 (8.66)	1.54
9–10	Male	3	100.3 (50.6)	3	110 (22.91)	1.10
9–10	Female	4	42.75 (12.65)	3	50 (17.32)	1.17
>24	Male	3	473.67 (103.92)	4	344.7 (38.1)	—
>24	Female	3	342.6 (72.64)	4	258.73 (66.56)	—

[1]Values are given as means (±SD).
[2]Ratio of hormone-treated total DNA content over controls.

DISCUSSION

Sexual dimorphism of the larynx

The sexual dimorphism of the vocal organ in the South African clawed frog, *Xenopus laevis* was first recognized at the turn of the century by Ridewood (1898). He noted that despite its more complex appearance, the male larynx has the same structural components found in the female larynx. Although several hyobranchial muscles and cartilages are intimately associated with the larynx, only the intrinsic m. dilator laryngis and cartilagenous skeleton were noted to be sexually dimorphic. We observe that the mass of the male larynx is at least twice that of the female. Both muscle and cartilage contribute to this difference in total mass. As observed also by Ridewood, we find that the structure of the male and female larynx consists of a cartilagenous skeleton that forms a box supporting a pair of intrinsic muscles (m. dilator laryngis) that insert at the anterior and posterior ends. The female larynx is triangular, and the muscle is restricted to the lateral margins of the laryngeal skeleton. In contrast, the male laryngeal skeleton is more box-like. The ventral surface in the male is almost completely covered by the larger paired laryngeal muscles.

Our results suggest that sex differences in size and mass of the larynx can be accounted for, in part, by a higher DNA content in the male cartilage and muscle. Assuming that both male and female cartilage have the same DNA content per cell nucleus, we estimate that the male has about 20% more cells in the cartilage skeleton. In the muscle tissue, we estimate that males possess about 200% more nuclei than females. Counts of laryngeal muscle fibers reveal that males have 6 to 7 times more fiber than females. Muscle tissue consists of both muscle and nonmuscle cell types (i.e., fibroblasts, pericytes, endothelial cells). In addition, muscle cell nuclei are present in both myofibers, which are multinucleated cells, and in single-celled myogenic or "satellite" cells (Mauro, 1961). The number of nuclei per individual muscle fiber in males and females has not been established. Taken together, these data indicate that there are fewer nuclei in each muscle fiber in the male than there are in a female muscle fiber.

Laryngeal development

Though smaller in overall size, the larynx of both sexes at metamorphosis is morphologically similar to that of the adult female. Our observations indicate that the male larynx develops as a result of masculinization of an initially feminine phenotype during the first 10 months after metamorphosis. As can be seen from cross sections of the larynx in developing males (Fig. 2), the process of virilization involves dramatic changes in the laryngeal skeleton and muscle shape. In particular, the male laryngeal muscle becomes divided into an inner and outer leaf; in contrast, the female muscle never becomes completely separated. Unlike the male, the female larynx appears only to increase in size and mass without a concomitant change in overall shape.

Both male and female laryngeal muscle have approximately 4,000 muscle fibers at metamorphosis, the same fiber numbers as the adult female larynx. Thus, as was the case for laryngeal shape, both male and female have the feminine phenotype for muscle fiber numbers early in development. The male continues to add muscle fibers during the next 10 months following metamorphosis and to increase in muscle mass. The female shows a very modest increase in fiber numbers such that by 10 months PM, the female larynx has significantly more fibers than both time 0 PM and adult frogs. It is possible that the developmental program of the male larynx is expressed weakly in the female. The decrease in fiber numbers back to adult values may indicate muscle cell death after the 10-month PM stage.

We have attempted to assess the relative contribution of DNA synthesis and addition of new cells (hyperplasia) and the contribution of growth of existing cells (hypertrophy) to increases in laryngeal mass during male and female development. Comparison of changes in [^{3}H]thymidine incorporation, DNA content, and mass during development suggest that the growth process differs for males and females. Taken together, our data suggest that ongoing cellular proliferation during the first 6 months of postmetamorphic development establishes the greater cell numbers of the male larynx. In females, there is little proliferation, and DNA accumulation is modest. At 10 and >24 months after metamorphosis, the rate of thymidine incorporation is much greater for both sexes than at earlier stages (Table 1); between 10 months PM and adulthood, there is substantial laryngeal DNA and mass increase in both sexes (Tables 2 and 3). In the male, this

growth completes the development of an already masculinized larynx. In the female, this growth amplifies the existing female phenotype.

The development of the male larynx is unusual in that an appreciable number of muscle fibers are elaborated over a protracted period (~10 months) after metamorphosis. It is generally observed that vertebrate muscle experiences little, if any, muscle fiber addition postnatally (Ontell and Dunn, 1978; Ontell, 1979; Gollnick et al., 1981). In this respect, thigh muscle may be more similar to other vertebrate muscle than is larynx, since it exhibits little, if any, cellular proliferation after metamorphosis. The capacity to form additional muscle fibers late in development suggests that laryngeal muscle development is governed by different mechanisms than those present in other skeletal muscles.

A recent histochemical study of muscle fiber types in *X. laevis* (Gray et al, 1985) revealed a striking dimorphism in male and female laryngeal muscle but not in thigh. Administration of androgen to juvenile males and females will masculinize laryngeal muscle fibers but does not affect thigh (Gray et al., 1985). Adult fiber type is less affected by androgen. We report here that administration of an androgen antagonist, Flutamide, inhibits the addition of muscle fibers after metamorphosis. In addition, testosterone stimulates proliferation in the larynx; our previous studies indicated that testosterone-induced myoblast cell division results in the incorporation of the new myonuclei into myofibers (Sassoon et al., 1986). Taken together, these data indicate that laryngeal muscle is highly sensitive to androgens and that androgens play a key role in the laryngeal development.

Role of testosterone in laryngeal development

A previous study using [^{3}H]thymidine autoradiography revealed that exogenous testosterone administration to sexually immature (~1-month PM) male and female frogs induced rapid chondrogenesis and myogenesis in the larynx (Sassoon et al., 1986). This response, characterized by incorporation of thymidine into 30% of all muscle tissue cell nuclei, is not observed in other somatic musculature (i.e., thigh < 1%), nor is this response observed in adult larynx (< 1%). For this study, we used testosterone administration followed by [^{3}H]thymidine injection at several stages during postmetamorphic development in an effort to determine how the proliferative capacity of the larynx is regulated developmentally. In contrast to our autoradiographic observations with the same experimental paradigm, we observed a testosterone-induced increase in thymidine incorporation at all stages including adulthood. We do not, however, observe any significant uptake in thigh muscle (m. gluteus and m. semimembranosus) at any stage under any conditions. One possible explanation for our inability to observe proliferating cells in adult larynx, by means of autoradiography, is that the number of myogenic cells relative to the entire muscle is very small in the adult larynx. Actively proliferating cells make up a much larger proportion of cells in the developing larynx.

We propose from these data that masculinization of the larynx is largely established during the first 6 months of postmetamorphic development. One crucial feature of the juvenile larynx is the relatively high proportion of mitotically competent, testosterone-responsive cells. As these cells decrease in either relative or absolute numbers, the degree of masculinization possible (i.e., response to androgen) decreases. It is likely that, in females, the number of laryngeal cells that can be stimulated to proliferate by testosterone decreases during the first 6 months. Support for this hypothesis comes from several lines of evidence. First, the amount of DNA in the female larynx is lower at 3, 6, and 10 months than it is at metamorphosis (Table 3). Second, preliminary in vitro studies indicate that the number of myogenic cells that can be liberated from the female larynx is considerably less at 6 months than at 0 months PM (Sassoon, unpublished). The loss of androgen-sensitive proliferating cells in females may be particularly marked after 3 months, because testosterone-induced proliferation is quite high until then but falls abruptly afterwards (Table 1). In males, the number of androgen-responsive mitotic cells is maintained during early development, most probably owing to endogenous androgen secretion during this time. The laryngeal DNA increases from 0 to 10 months PM (Table 3). If endogenous androgen in males has already induced the hormone-sensitive cells in the larynx to proliferate, fewer cells may be available for induction 3 or 6 months after metamorphosis than in females, accounting for the decline in testosterone induction of thymidine incorporation during this period

(Table 1) when DNA content is rising (Table 3). By adulthood, both male and female larynges have lost the capacity to increase DNA content and show only a minor induction of proliferation in response to testosterone (Table 3). In vitro studies, assessing the number of single cells liberated from adult and juvenile larynges, indicate that the adult has only a minute fraction of myoblasts and chondroblasts present in time 0 PM larynges (Sassoon, unpublished).

What accounts for androgen sensitivity of the larynx? High levels of intracellular androgen-binding proteins (receptors) are characteristic of androgen-responsive tissues. We have recently established that laryngeal muscle has very high levels of androgen receptor during postmetamorphic development (Sassoon et al., 1985) and that high levels are maintained relative to other somatic musculature (i.e., thigh) in the adult (Segil *et al.*, 1983). Biochemical analysis of laryngeal androgen receptor levels indicates that 10-fold higher levels of receptor are present in time 0 PM larynges than in adult males. Androgen receptor values decline gradually in females over the next 6 months but are maintained at high levels in males. By 10 months, both sexes have experienced a marked decrease in receptor levels, resulting in values comparable to those observed in adults. We suggest that high androgen receptor levels may underlie the sensitivity of laryngeal tissue to testosterone. Further, sex-specific patterns of hormone receptor regulation may underlie the dimorphic development of the larynx. Thus, the decrease in receptor levels that occurs following metamorphosis in females may preclude the cellular processes that give rise to a male larynx. This hypothesis is consistent with the observation that male laryngeal tissue incorporates more [^{3}H]thymidine than does female laryngeal tissue in response to testosterone during development. Indeed, at metamorphosis, the male larynx has a more marked response to testosterone than does the female, which may indicate hormone exposure prior to metamorphosis in the male.

Several lines of evidence from this study and others (see also Hannigan and Kelley, 1986; Sassoon et al., 1986) strongly indicate that androgen secretion in the male is responsible for masculinization of the larynx during development. What cellular mechanisms are particularly responsible for the masculinization of muscle fiber numbers? We have already suggested that a decrease in the proportion of mitotically competent, androgen-responsive cells could account for the decrease in the proliferative response that occurs by adulthood. This scenario could also account for the inability of the adult female larynx to add muscle fibers or express the male pattern of muscle fiber types following prolonged androgen treatment (Gray et al., 1985). Muscle differentiates by a progression from myoblast to myotube to mature myofiber. It is possible that androgen receptor proteins, highly expressed by mitotically competent myoblasts, are more weakly expressed in their differential progeny. One prediction from this hypothesis would be that androgen stimulation, which results in muscle formation, also causes a decrease in androgen-receptor expression. We have tested this hypothesis by administration of high doses of dihydrotestosterone to juvenile frogs (Kelley et al., Submitted). This treatment down-regulates receptor levels to levels observed in adult laryngeal tissue. The pattern of androgen secretion during postmetamorphic development has been recently established (Lambdin and Kelley, 1986). Androgen secretion is minimal at time 0 PM and is sexually monomorphic. Levels are dimorphic by 2 months PM and increase steadily until 6 months, at which point adult levels are achieved in males. We note that androgen secretion is not restricted to the male in *Xenopus*. In particular, levels in the female rise slowly until 10 months PM; this coincides with an increase secretion in laryngeal mass and DNA content and also coincides with a decrease in androgen receptor levels (Sassoon et al., 1985).

The development of the larynx can be compared to that of another sexually dimorphic muscle, the m. levator ani of male rats. The levator ani muscle has a male-like configuration at birth in both sexes, and later disappears in the female. Cihak and colleagues (1970) observed that the muscle undergoes hypertrophy in the postnatal male rat and cell death in the postnatal female. An aim of this study was to determine whether a similar program of initial male morphology characterized the development of the larynx of *X. laevis*. We observe that the larynx is sexually monomorphic at time 0 PM with regard to total mass, DNA content, and muscle fiber numbers. The general shape of the cartilage and muscle mass is reminiscent of the adult female. In addition, the laryngeal muscle

fiber numbers is female-like. Following metamorphosis, the male larynx gradually undergoes a change in cartilage and muscle morphology, as well as a substantial rise in muscle fiber numbers. Thus, we conclude that the larynx does not follow the same pattern of development as that seen in the m. levator ani of rats, but instead follows a pattern of development more similar to that observed in the reproductive tract of mammals–i.e., an initial female-like configuration in both sexes.

Another sexually dimorphic set of muscles that has been examined developmentally is the temporal and masseter muscles of the male guinea pig. Kochakian et al. (1964) observed that castration soon after birth retards, but does not prevent, the accumulation of DNA to adult levels. These authors also observed that testosterone administration greatly accelerates RNA accumulation and results in a slight increase in DNA. In these experiments, castration was not performed prior to the perinatal pulse of androgen that is characteristic of rodent development. This pulse occurs pre-natally in guinea pigs (Feder, 1981). In any event, these results suggest that a third mechanism of dimorphic muscle development may exist in vertebrates that involves the establishment of a dimorphic program by means of a brief hormonal trigger (e.g., perinatal) that can proceed even in the absence of further testosterone exposure.

The larynx of *X. laevis* is the effector organ for the male-specific vocalization or mate call (see Kelley, 1986, for review). This behavior is observed in sexually mature males and cannot be elicited in adult females under a number of conditions including exogenous testosterone treatment (Hannigan and Kelley, 1986). What factor constrains the female from mate calling? Work from this laboratory has determined that males possess more neurons than females (Hannigan and Kelley, 1981) and that motor neuron somal size and dendritic extent are greater in males (Hannigan and Kelley, 1983; Kelley and Fenstemaker, 1983); certain connections of the CNS vocal "circuit" are more robust in males (Wetzel et al., 1985). Hormone treatment of adult females does not increase motor-neuron numbers or dendritic extent but does increase neuron size. Thus, sex differences in cell numbers in the CNS may restrict mate calling in the female. The present study indicates that a similar situation exists in the periphery. We report here that muscle fibers are more numerous in the male and, although muscle mass increases in the adult female in response to testosterone, muscle fiber numbers remain unaffected. Thus, limitations in both the CNS and periphery may preclude the female from producing male-specific vocalizations.

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