Thyroid Hormone Controls the Onset of Androgen Sensitivity in the Developing Larynx of *Xenopus laevis*

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Gonadal differentiation, the onset of androgen-stimulated laryngeal growth and the genesis of a sex difference in laryngeal innervation, all temporally coincide with thyroid hormone (TH)-induced metamorphosis in *Xenopus laevis.* To explore the role TH plays in the ontogeny of the *Xenopus* androgen-sensitive vocal neuromuscular system, we examined gonadal and laryngeal development in tadpoles in which metamorphosis had been blocked by treatment with the thyroxine synthesis inhibitor propylthiouracil (PTU). PTU treatment did not arrest gonadal differentiation. Testes from PTU-treated male tadpoles had seminiferous tubules and advanced stage male germ cells, while in females stage 1 oocytes were present. In contrast to the gonads, PTU did block morphological development of the larynx. Tadpoles treated with PTU for 50 or 100 days had larynges which structurally resembled those of stage 54 control tadpoles. PTU-treated animals did not exhibit the extensive development of the laryngeal cartilage seen in untreated animals. Laryngeal cartilages of hypothyroid tadpoles exhibited low density and minimal patterning of chondrocytes; the complex lumen and marked expansion of the dilator muscles characteristic of 50- and 100-day untreated animals were absent. Laryngeal growth evoked by exposure to exogenous androgen (dihydrotestosterone) was entirely prevented by PTU treatment. Hypothyroid tadpoles did not exhibit the decline in laryngeal nerve axon number characteristic of age-matched controls, nor were laryngeal nerve axon numbers sexually dimorphic. PTU treatment also interfered with the myelination of laryngeal axons. We conclude that while gonadal differentiation is independent of TH, androgen sensitive laryngeal development and sexually dimorphic laryngeal innervation require exposure to secreted TH.   © 1996 Academic Press, Inc.

INTRODUCTION

Amphibian metamorphosis is a dramatic instance of endocrine-directed vertebrate development. During this transformation, secreted thyroid hormone (TH) induces morphological and biochemical modification of larval tissues and growth of nascent structures, resulting in the juvenile form (reviewed by Dodd and Dodd, 1976). The value of metamorphosis as a model in developmental research is reflected in the wealth of investigation exploring this phenomenon; these studies include examinations of tissue hormone sensitivity (Maclean and Turner, 1976; Schönenburger and Escher, 1988; Galton, 1992), hormone receptor expression

(Yaoita and Brown, 1990; Yaoita *et al.,* 1990; Kawahara *et al.,* 1991), interaction of hormones in development (Kawahara *et al.,* 1989; Gray and Janssens, 1990; Buckbinder and Brown, 1993a, b), and endocrine control of gene expression (Kanamori and Brown, 1992; reviewed in Tata, 1993). Anuran metamorphosis offers unique advantages as an experimental system because larval development can be readily manipulated with exogenous TH or anti-thyroid agents (Dodd and Dodd, 1976).

Here we examine the interaction of TH with another dramatic developmental program under endocrine control, androgen-directed masculinization. In the clawed frog, *Xenopus laevis,* androgen secretion is responsible for masculinization of the laryngeal neuromuscular system that subserves the male's ability to produce courtship songs (reviewed in Kelley, 1996). Most androgen-dependent secondary sex characteristics of the *Xenopus* mate calling system develop after metamorphosis is complete. However, a sex difference in the number of axons innervating laryngeal muscle arises during

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metamorphic climax of late tadpole development, between stages 59 and 62 (Kelley and Dennison, 1990; Robertson *et al.,* 1994). The temporal coincidence of metamorphosis and sex differences in axon numbers suggests that thyroid hormone secretion might contribute to the onset of androgen-directed, sexually differentiated laryngeal neuromuscular development.

Exposure to supraphysiological levels of androgen is lethal both during early postmetamorphic stages (Sassoon and Kelley, 1986) and in late tadpole stages (Robertson *et al.,* 1994), presumably because induced laryngeal hypertrophy compromises cardiovascular and respiratory function. Significantly, this dramatic androgen effect can only be obtained in tadpoles that have reached metamorphic climax, the period corresponding to peak circulating TH levels in larval *Xenopus* (Leloup and Buscaglia, 1977). Thus, observations in androgen-treated animals indicate that development of laryngeal androgen sensitivity might be linked to TH secretion (as is the case for hepatic estrogen-stimulated vitellogenin synthesis, Kawahara *et al.,* 1987, 1989).

However, during metamorphosis the gonads also become sexually differentiated in morphology (reviewed in Witschi, 1971; Kelley, 1996), raising the possibility that TH could exert its effects on the larynx by controlling the differentiation (and steroidogenic capacity) of the testes. Well differentiated testes and ovaries have been described in thyroidectomized *Rana catesbiana* and *Rana sylvanica* tadpoles (Allen, 1918; Hoskins and Hoskins, 1919) and in anurans rendered hypothyroid by chemical means (Hsu *et al.,* 1974). If, in *Xenopus,* the development of testes and ovaries is similarly TH independent, the onset of laryngeal androgen sensitivity and gonadal differentiation might be regulated by different developmental mechanisms.

The aims of this study were thus to determine if TH is required for gonadal differentiation and the emergence of a sex difference in laryngeal innervation, events which temporally coincide with metamorphosis, as well as for androgen-directed laryngeal growth and hyperinnervation. We blocked metamorphosis at tadpole stage 54 by inducing hypothyroidism using an anti-thyroid compound, propylthiouracil (PTU); some tadpoles were simultaneously treated with both androgen (dihydrotestosterone; DHT) and PTU. Laryngeal and gonadal morphology and fine structure of hypothyroid tadpoles were compared to untreated, normally developing animals (age-matched controls) and untreated stage 54 tadpoles (stage-matched controls). Results of the present study have been presented in abstract form (Robertson and Kelley, 1992).

METHODS

Rationale

Embryonic and larval development of *X. laevis* has been divided into 66 stages based on morphological characteristics (Nieuwkoop and Faber, 1956). Stage 1 represents fertilization of the egg; embryonic development then proceeds to hatching at stages 35/36 (50 hr postfertilization or PF). Larval or tadpole development (stages 37 through 66) has been further classified according to metamorphic status of the tadpole (Dent, 1968). The period from stage 37 to stage 53 (24 days PF) is termed premetamorphosis; stage 54 marks the start of prometamorphosis or TH-directed metamorphosis. Forelimbs emerge at stage 58 (44 days PF), and metamorphic climax corresponds to peak plasma TH levels and occurs at stages 59–62. Circulating TH then decreases and metamorphosis from tadpole to froglet is complete at stage 66 (58 days PF).

Development of TH-deficient *Xenopus* tadpoles is arrested at stage 54, the terminal pre-TH-dependent state (Leloup and Buscaglia, 1977); hypothyroid tadpoles continue to grow but body morphology does not progress beyond stage 54. Tissues or characteristics of hypothyroid tadpoles can thus be compared to untreated, normally developing animals (age-matched controls) and to untreated stage 54 animals (stage-matched controls). If development is dependent on TH, then tissue from a hypothyroid tadpole would be expected to resemble that of an untreated stage 54 tadpole. Conversely, if TH is not needed for development to progress, tissue from a hypothyroid animal should share characteristics with that of an untreated, age-matched animal.

Experimental Protocol

X. laevis tadpoles (stages 45–48) were obtained from a commercial supplier (Nasco, Ft. Atkinson, WI) and maintained in the laboratory at ~25°C. Established morphological parameters (Nieuwkoop and Faber, 1956) were used to stage tadpoles; postmetamorphic (PM) animals were staged according to the criteria of Tobias *et al.* (1991). Animals were maintained in Novaqua (Kordon, Heyward, CA)-treated filtered tap water in polycarbonate tanks under a 12L:12D photoperiod. Tadpoles were fed nettle powder daily; after metamorphosis, frogs were instead fed a commercial brittle diet (Nasco) twice weekly.

Tadpoles reaching stage 50 over a 5-consecutive-day period were randomly assigned to one of three experimental groups: 0.01% 6-*n*-propyl-2-thiouracil (PTU, Sigma), 0.01% PTU and 0.025 mg/liter dihydrotestosterone (5α-androstan-17β-ol-3-one; DHT, Sigma), or were reared as untreated controls. DHT was solubilized in ethanol (50 μl per liter of tank water); to control for alcohol effects, the PTU only and control groups also received an equal volume of ethanol. Water was changed and treatments renewed in all tanks three times weekly. Androgen treatment alone at the dose employed here causes marked laryngeal hypertrophy and mortality of prometamorphic tadpoles (Robertson *et al.,* 1994); animals from this previous study were available for comparison. One to 2 weeks after the start of treatment, several stage 54 animals from the control group were killed and their gonads and larynges were processed and analyzed (see below). Fifty and 100 days after treatments began, animals from all three experimental groups were sacrificed. At the 50-day point, untreated control animals were just completing metamorphosis (i.e., were at tadpole stage 66); at 100 days, control frogs were approximately 7 weeks postmetamorphosis (i.e., were at stage PM1).

For the purposes of assessing longer-term effects of hypothyroidism, a separate group of tadpoles (3 males and 1 female) maintained on 0.01% PTU treatment for 1 year was also sacrificed at the same time as the 100-day PTU group. Tadpoles treated with PTU for a year had not received vehicle alcohol and their water had been

changed weekly. At the time of sacrifice, these tadpoles were judged to be at stage 54 according to external morphological criteria.

At sacrifice, animals were anesthetized via cold narcosis and immersion in a 0.1% solution of MS222 (ethyl *m*-aminobenzoate, methanesulfonic acid, Aldrich) and weighed. For frogs and larger tadpoles, the larynx and gonads were quickly removed and placed in cold fixative (1% paraformaldehyde, 1% glutaraldehyde in 0.1 *M* phosphate buffer, pH 7.2). Tadpole gonads were removed and processed attached to the larger interrenal glands. For stage 54 control tadpoles, larynges and gonads were fixed and processed *in situ* after exposing the abdominal cavity and removing the intestine, the tail, and the anterior region of the head. After several hours of fixation at 4°C, tissues were rinsed in 0.1 *M* phosphate buffer and postfixed for 2 hr in 1% osmium tetroxide (in 0.1 *M* phosphate buffer, pH 7.2). Following dehydration in a graded alcohol/propylene oxide series, gonads and larynges were embedded in LX112 medium (Ladd, Burlington, VT).

Histology and Ultrastructure

Larynges were compared by examining semithin (1–2 μm) serial sections from the anterior–posterior axis using light microscopy. In addition, the number of axons in the laryngeal nerve was determined for the stage 54 control group, the 100-day control, PTU- and PTU/DHT-treated groups, and for the 1-year PTU-treated animals. Thin (~0.08 μm) cross sections of the left or right laryngeal nerve were cut from the caudal end of larynges, stained with uranyl acetate and lead citrate, and examined using a JEOL 1200EX transmission electron microscope. Since there is no significant difference in axon numbers from the left or right laryngeal nerve of an animal, and since multiple sections from a single nerve evince no significant variation in axon counts (Kelley and Dennison, 1990), myelinated and unmyelinated axons were counted on photomicrographs of a single laryngeal nerve section per animal. High-magnification (at least 20,000×) montages of tadpole nerves were prepared to ensure accurate counts of small unmyelinated axons. For this study, myelinated axons were defined as those with at least two complete layers of glial cell ensheathment. Because the preexit fasiculation of the laryngeal nerve is not complete in stage 54 tadpoles, intramuscular axons surrounding the laryngeal nerve were counted and added to the axon totals for the more compact and well-fasiculated laryngeal nerve proper of these animals.

The number of axons in the laryngeal nerves of PM1 frogs were determined using low-magnification (1000×) transmission electron microscopy (TEM) photomicrographs. Frog laryngeal nerves contain small numbers of unmyelinated sensory efferent axons from the glottis (Kelley and Dennison, 1990); these were not included in axon counts of PM1 frog laryngeal nerves.

Gonads from animals in each treatment group were also serially semithin sectioned and examined by light microscopy. While osmication typically imparted sufficient contrast to tissues for light microscopy, some gonad and larynx semithin sections were also stained with a 0.6% toluidine blue solution. Gonads from animals in all groups were, in addition, thin sectioned in order to examine fine structure using TEM. Gonadal histology and gamete ultrastructure in PTU and PTU–DHT-treated groups were compared to previous results on testicular and ovarian development and gametogenesis in *Xenopus* (Nieuwkoop and Faber, 1956; Witschi, 1971; Al-Mukhtar and Webb, 1971; Dumont, 1972; Reed and Stanley, 1972; Merchant-Larios and Villapando, 1981; Iwasawa and Yamaguchi, 1984; Villapando and Merchant-Larios, 1990; Kelley, 1996).

Statistical Analysis

Axon numbers from stage 54 controls (in which sex cannot be determined, see below) were compared to laryngeal nerve axon values from males and females in the 100-day PTU- and PTU/DHT-treated and 100-day untreated groups using a one way ANOVA, followed by Tukey–Kramer pairwise comparison tests to identify significant differences ($P < 0.05$, two-tailed) between sexes or treatment groups. As is the case for normal development (Kelley and Dennison, 1990), variability in axon number was greater in tadpoles than in postmetamorphic frogs. However, a one-way ANOVA is sufficiently robust to accommodate some intergroup differences in variance. Ratios of myelinated axons to total axons were first arcsin transformed (Snedecor, 1989) and then subjected to the above statistical analysis.

RESULTS AND SPECIFIC DISCUSSION

General Development and Growth

Exposure of premetamorphic *X. laevis* tadpoles to 0.01% PTU resulted in a consistent arrest of general morphological development at stage 54; similar results have been obtained in other laboratories (Maclean and Turner, 1976; Schönenberger and Escher, 1988). During tadpole development, circulating TH levels typically begin to rise at stage 54 (Leloup and Buscaglia, 1977) initiating prometamorphosis; PTU inhibition of thyroxine synthesis thus blocks prometamorphosis. Preventing TH production perturbs the thyroid–pituitary negative feedback loop, and we did observe enlarged thyroid glands in PTU-treated animals. Thyroid hypertrophy, however, did not appear to impede laryngeal growth.

Although development is arrested at stage 54, PTU-treated tadpoles continued to grow for the next 50 to 100 days (Table 1). However, the body weights of 1-year PTU-treated tadpoles did not exceed those of tadpoles treated for only 100 days. This apparent ceiling to growth could represent a physiological constraint or simply reflect limitations of rearing conditions.

The development and growth of tadpoles simultaneously treated with PTU and DHT could not be distinguished from growth and development of animals treated with PTU alone. Untreated control animals developed and grew at rates consistent with published observations (Nieuwkoop and Faber, 1956; Tobias *et al.,* 1991). Induction of hypothyroidism by treatment with methimazole has been reported to masculinize *R. catesbeiana* tadpole ovaries (Hsu *et al.,* 1974); sex transformation appears to result from a direct chemical effect of methimazole on the ovary. We, however, observed no indication of gonadal sex transformation in PTU-treated *Xenopus* tadpoles.

Gonadal Development in Control Animals

At stage 54, the gonads of tadpoles do not appear sexually differentiated with respect to overall morphology, cellular composition or fine structure (Fig. 1). In the most advanced

TABLE 1

Mean Body Weights (in grams) of Control and Metamorphically Blocked Animals Examined in This Study

	Females	?[a]	Male
Stage 54 controls		0.2 (0.01)	
		$n = 5$	
50-Day control	nd[b]		nd
50-Day PTU	1.3 (0.3)		1.2 (0.3)
	$n = 4$		$n = 4$
100-Day control	3.1 (0.4)		4.5 (0.7)
	$n = 3$		$n = 2$
100-Day PTU	2.8 (0.8)		2.5 (0.2)
	$n = 4$		$n = 4$
100-Day PTU/DHT	2.6 (0.4)		2.7 (0.2)
	$n = 3$		$n = 4$
1 Year PTU	2.7		2.2 (0.6)
	$n = 1$		$n = 3$

Note. Standard errors (SEM) are in parentheses and numbers of animals (*n*) in each group are listed.

[a] Sex cannot be determined in untreated stage 54 tadpoles because the gonads are not morphologically differentiated.

[b] nd, not determined.

animals at this stage, a distinct central medulla is distinguishable from an outer cortical cell layer (Fig. 1a). The relatively few large primordial germ cells present are localized to the cortex, usually in the distal region (Fig. 1b).

In contrast, the ovaries and testes of 50-day control animals (stage 66 tadpoles) are clearly differentiated (Figs. 2a and 2b). Relative to stage 54 tadpoles, stage 66 gonads are enlarged overall. Stage 66 female gonads are readily identifiable by a prominent ovarian cavity (Fig. 2a). By this stage, the ovarian medulla is reduced to an epithelium lining the central cavity. Germ cells (oogonia and primordial germ cells) are larger and more numerous than at stage 54 and remain within the expanded cortex of the ovary. At stage 66, testes are elliptical in cross section; the medulla has expanded markedly, with the cortex occupying a narrow outer margin (Fig. 2b). Germ cells (spermatogonia and primordial germ cells) are now located within the testicular medulla and are numerous. In some cases, medullary cells are arranged around single or small groups of germ cells, possibly reflecting early stages in formation of seminiferous tubules. In both sexes at stage 66, germ cells are in the earliest stages of gametogenesis.

Further gonadal differentiation and growth are evident in 100-day control animals (PM1 frogs; Figs. 3a and 3b). Increased size and morphological differentiation of the gonads make it possible to sex PM1 frogs by visual inspection. Ovaries of PM1 females are convoluted and large numbers of various sized oocytes are apparent in cross sections (Fig. 3a). All oocytes appeared to be stage 1 (less than 350 μm in diameter; Dumont, 1972). A large nucleus, nucleoli, and a mitochondrial mass (Balbioni body) are common features

of larger oocytes. However, there is no histological or ultrastructural indication of yolk droplets or cortical granules in the ooplasm at this stage; in addition, few microvilli extend from the oocyte surface. A tripartite cell layer (epithelium, theca, follicular epithelium) surrounds the oocytes in ovaries of PM1 frogs. The testes of PM1 frogs are densely packed with seminiferous tubules (Fig. 3b). Clusters or nests of germ cells are radially arranged around the central lumen of each tubule; cells within each nest appear to be at a similar stage of gameteogenesis. Relatively few mature germ cells (spermatids or spermatozoa, Reed and Stanley, 1972) can be identified in the PM1 frog testes; the majority of cells are either spermatocytes or are at an earlier stage of development.

Our observations that control stage 54 tadpoles have undifferentiated gonads while stage 66 tadpoles have morphologically discernible testes and ovaries are consistent with previous reports that the testes and ovaries of *Xenopus* differentiate histologically or ultrastructurally at stage 56 (Nieuwkoop and Faber, 1956; Zust and Dixon, 1977; Merchant-Larios and Villapando, 1981; Iwasawa and Yamaguchi, 1984; Villapando and Merchant-Larios, 1990).

Gonadal Development in PTU-Treated Animals

Gonads of PTU-treated animals were more similar to age-matched than to stage-matched controls. Despite being developmentally blocked at stage 54, tadpoles treated with PTU for 50 days had well-developed gonads that had clearly differentiated beyond the point seen in any control stage 54 tadpole gonads (Figs. 2c and 2d). Development of ovaries and testes in the 50-day PTU-treated tadpoles appeared to have progressed to an extent closely resembling age-matched control animals (i.e., stage 66). That is, ovaries from animals treated with PTU for 50 days were characterized by an ovarian cavity and proliferation of germ cells in the enlarged cortex (Fig. 2c). Testes from 50-day PTU-treated males had a reduced marginal cortex and an expanded medulla with numerous individual or clustered immature germ cells (Fig. 2d). The major difference between gonads of control and hypothyroid animals at 50 days was that the testes of PTU males had lower germ cell density and more connective tissue-like cells in the medulla.

Ovaries and testes of 100-day PTU-treated tadpoles were larger and more differentiated than those of 50-day PTU-treated animals (Figs. 3c and 3d). Ovaries from the 100-day PTU group contained stage 1 oocytes (Fig. 3c). As in 100-day controls (PM1 frogs), testes of 100-day PTU-treated tadpoles had seminiferous tubules with nests of germ cells (Fig. 3d); some spermatids were observed in these testes, but most germ cells were less mature. Testes of 100-day PTU tadpoles were, however, smaller than those of PM1 frogs and continued to exhibit lower germ cell density. At 100 days, the PTU-treated animals' testes
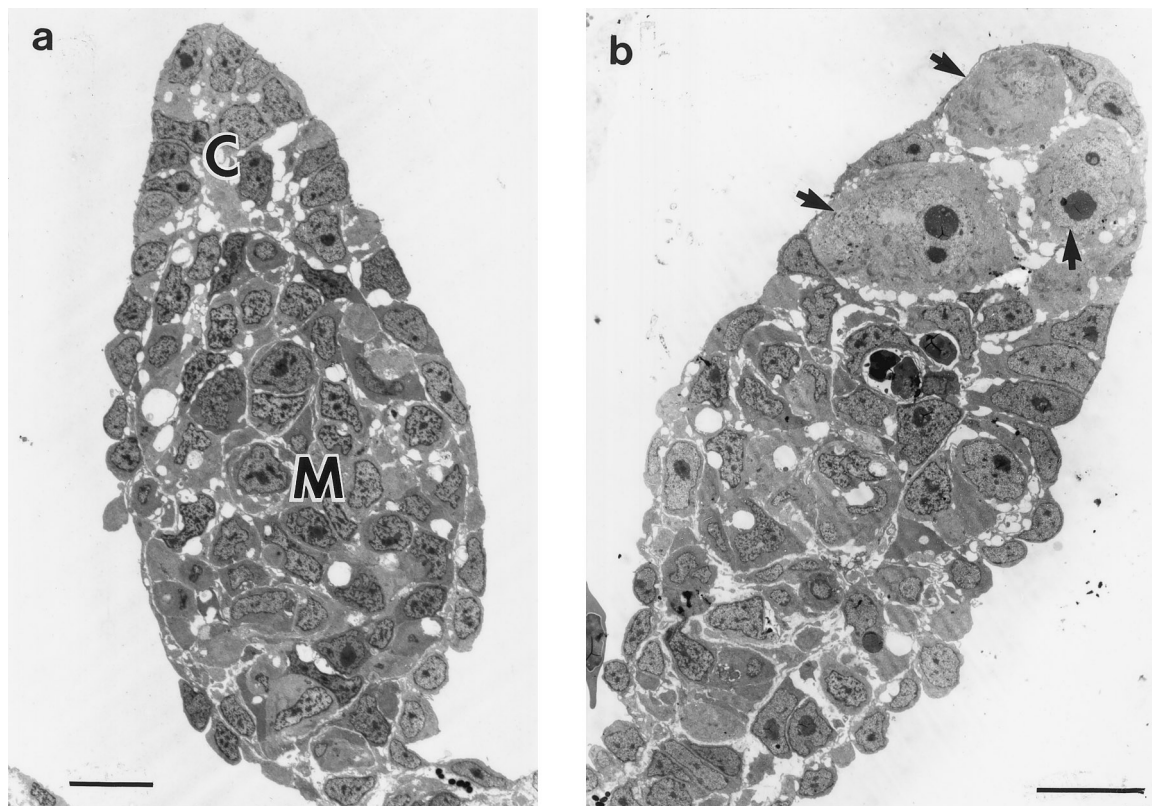
FIG. 1. Transmission electron micrographs of whole cross-sectioned gonads from control stage 54 tadpoles. Scale bars, 10 μm. (a) Cells of gonad are arranged in distinct central medulla (M) and peripheral cortex (C) regions. Corticomedullary organization was the most advanced structural feature found in control stage 54 gonads; at this level of morphological development, gonads cannot be sexually differentiated. (b) Several large primordial germ cells (arrows) are located in the distal cortex of a sexually undifferentiated stage 54 tadpole gonad. These cells are characterized by their large size, electron-lucent cytoplasm, and irregularly shaped nucleus with prominent nucleoli.

were extended, tapered structures typical of tadpole testes while the age-matched PM1 male gonads had an adult-like kidney bean shape. The ovaries of 100-day PTU-treated tadpoles were also smaller and not as highly convoluted as ovaries of PM 1 frogs. Testicular and ovarian development in tadpoles simultaneously treated with PTU and DHT for 50 and 100 days was identical to that of PTU-only-treated animals (not shown).

Gonads of 1-year PTU-treated tadpoles were similar in size and gross morphology to those of 100-day PTU-treated animals. In Fig. 4a, a stage 1 oocyte from a PTU-treated female tadpole is illustrated. The oocyte lacks yolk deposits in the cytoplasm and is surrounded by epithelial and connective tissue layers. Ovaries of the 1-year PTU-treated female examined had some larger oocytes than those seen in 100-day-treated females, but all oocytes still appeared to be stage 1. After 1 year of PTU treatment, male germ cells in various stages of differentiation are present in the testis (Fig. 4b), with the density of germ cells and frequency of mature gametes increased over that in 100-day PTU-treated tadpoles. A central nucleus and outer mitochondria are seen in cells sectioned through the midpiece, while sectioned tails clearly exhibit the 9×2 tubular arrangement of the axoneme (Fig.

FIG. 2. Cross sections of gonads from 50-day control (a, b) and 50-day PTU-treated animals (c, d) illustrate TH-independent differentiation of *Xenopus* gonads. Control animals are at the end of metamorphosis (tadpole stage 66), while PTU animals are morphologically blocked at tadpole stage 54. Ovaries (shown in a and c) have prominent central cavities lined by a thin epithelium of medullary origin. The expanded cortex (C) contains the oogonia. Testes (b and d) have an expanded medulla (M) containing germ cells; the cortex has been reduced to a narrow outer margin. Scale bar, 50 μm.
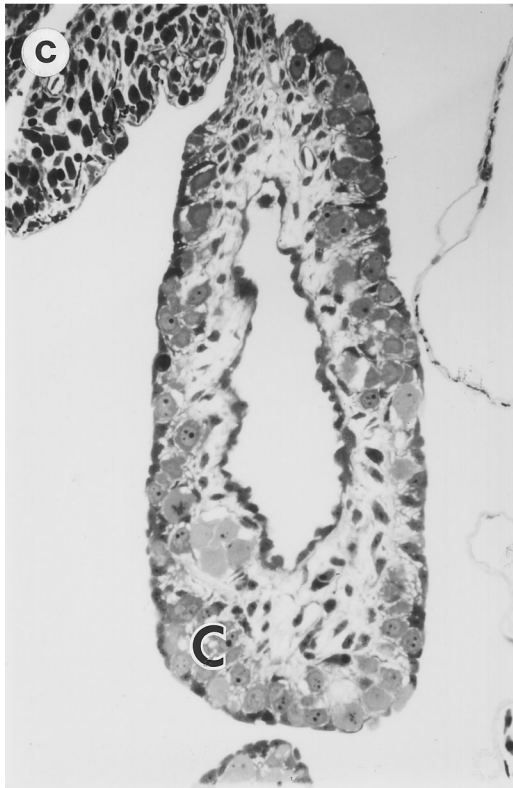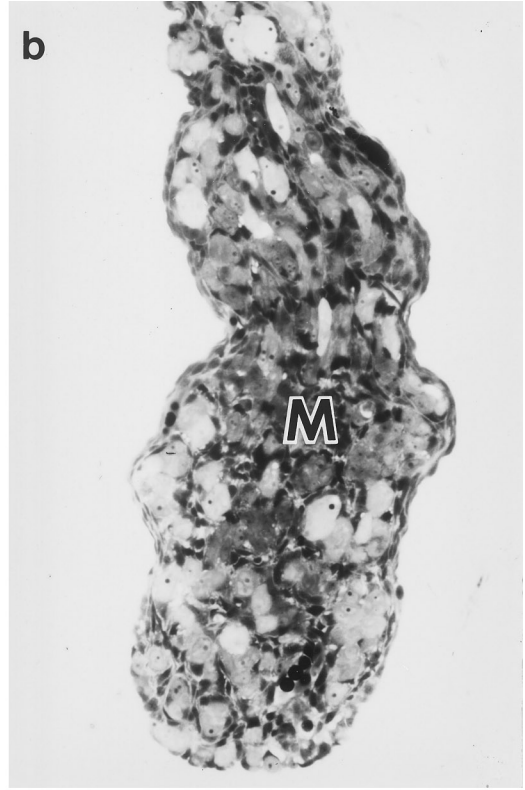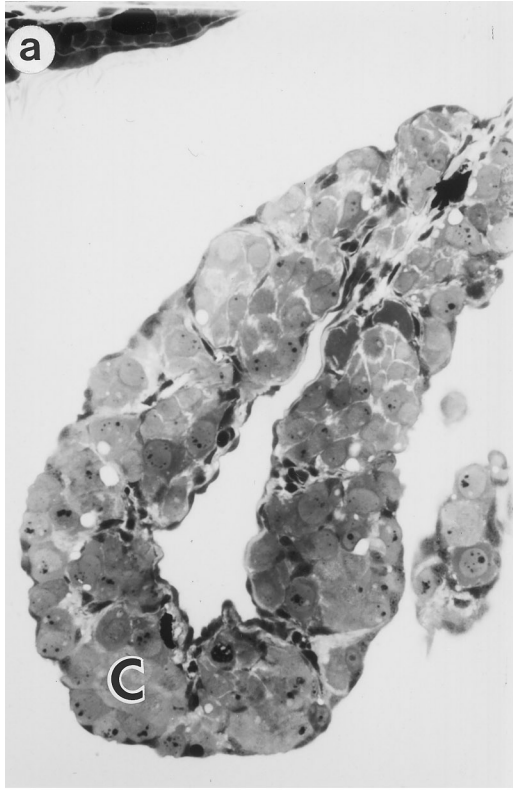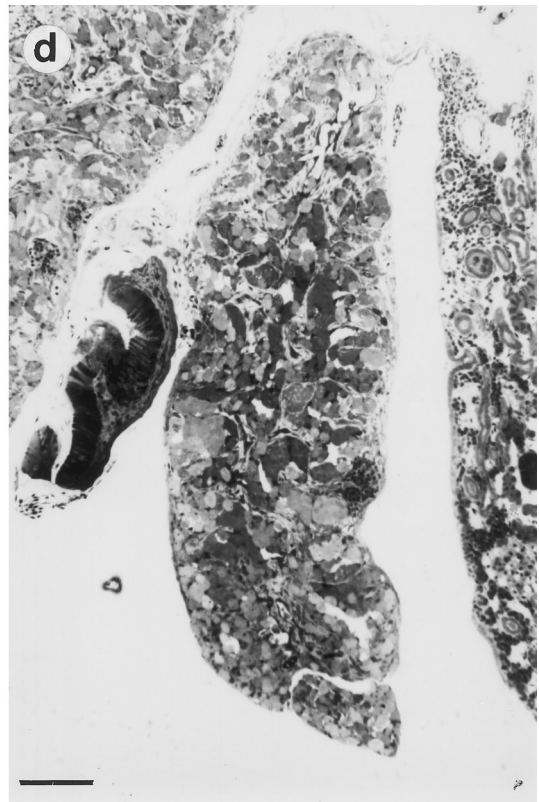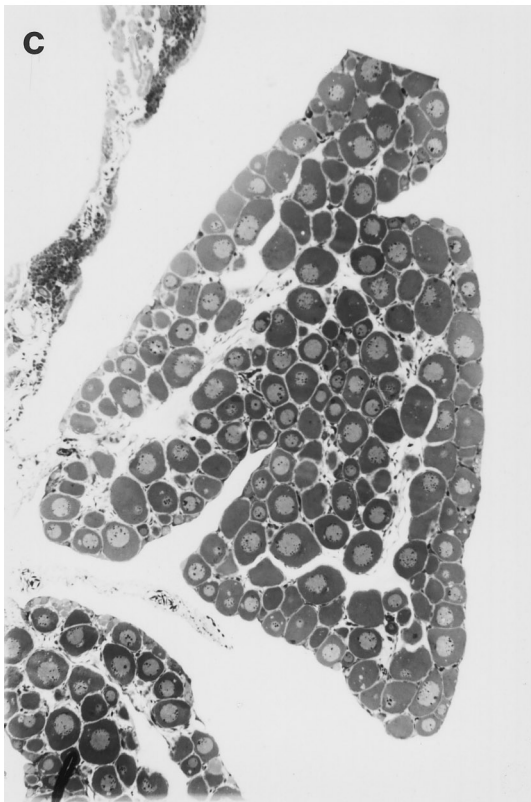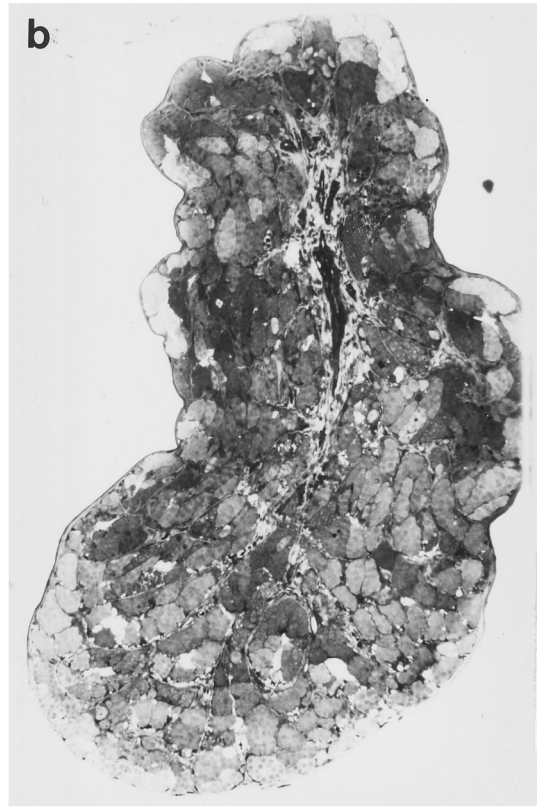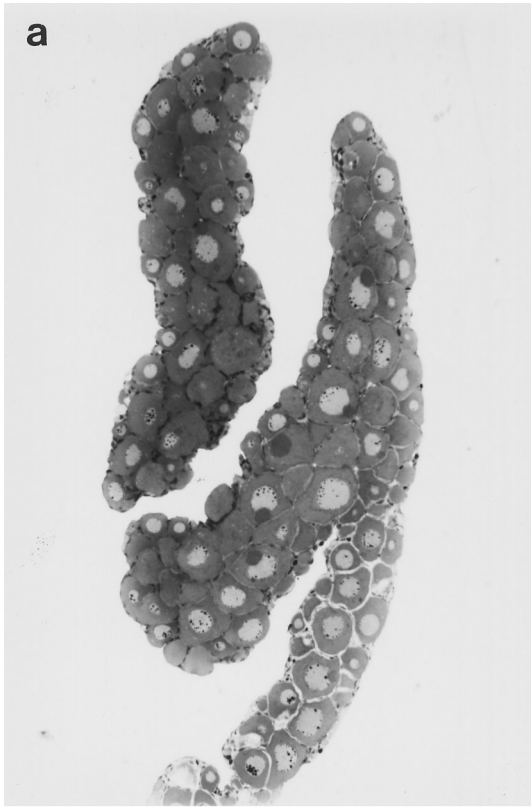
4c). Longitudinal section of the head and midpiece of a spermatid reveals highly condensed nuclear material (Fig. 4d).

For comparison, 1-year postmetamorphic frogs routinely attain reproductive maturity in our laboratory (Kelley, 1996); in mature frogs, ovaries contain large numbers of stage 6 (> 1 mm diameter) oocytes, while adult testes can approach a centimeter in diameter.

We conclude that the initial stages of sexual differentiation of the gonads of *X. laevis,* although they occur at metamorphic climax, are independent of TH secretion. However, events associated with later stages of gonadal differentiation, such as yolk deposition in oocytes and attainment of characteristic adult gonad morphology, do not occur in hypothyroid animals.

Laryngeal Development in Control Animals

The larynx in adult *X. laevis* communicates with the buccal cavity via the glottis anteriorly and with the lungs via paired tracheae posteriorly. The vocal organ is composed of a cartilagenous skeleton surrounding a central, air-filled lumen and flanked bilaterally by bipennate muscles which insert, via tendons, onto the sound producing arytenoid disks (Fischer and Kelley, 1991). In the adult, paired rods of ossified cartilage, the thyohyrals, extend from anterior to posterior within the hyaline cartilage lateral to the central lumen.

Several major structural features of the adult larynx are already present at stage 54 (Fig. 5a). The glottis, located anterior and dorsal to the larynx proper, communicates with the more posterior, rounded central lumen of the cartilagenous skeleton. Two pairs of laryngeal muscles are also readily discernible at stage 54. The constrictor muscles flank the lateral aspect of the glottis for its full anterior–posterior length. The dilator muscles project posteriorly from the middle of the glottis to the caudal end of the larynx. The most differentiated laryngeal cartilage at stage 54 is that adjacent to the glottis; chondrocytes were most dense in this anterior and dorsal region. However, even in this area, little patterning of chondrocytes is present. Chondrocytes were less dense in other regions of the larynx, with a notable paucity of cells in the posterior ventral larynx (see Fig. 5a).

By stage 66 (50-day controls), the larynx has undergone significant growth and differentiation (Fig. 5b). The glottis and constrictor muscles occupy a smaller proportion of the total larynx length, relative to stage 54, while the dilator muscle has increased dramatically in length and diameter.

The cartilagenous skeleton of the larynx is well formed; variations in chondrocyte density and orientation contribute to the complex morphology of the laryngeal cartilages. The thyohyrals, not evident within the larynx at stage 54, are present by stage 66. The lumen is no longer simply rounded but instead exhibits a series of complex involutions along its length. Continued laryngeal growth and differentiation are evident in 100-day control animals (PM1 frogs). The musculature, cartilage, and lumen of the larynx continue to enlarge (Fig. 5d). Additionally, the paired arytenoid discs at the anterior end of the larynx are well differentiated in the larynges of PM1 frogs. In confirmation of previous observations (Sassoon and Kelley, 1986; Marin *et al.,* 1990), no gross sex differences in laryngeal morphology are present at stage 66 or PM1.

Laryngeal Development of PTU-Treated Animals

In contrast to gonadal development, larynges of PTU-treated males were more similar to stage-matched than to age-matched controls; 50- and 100-day PTU-treated tadpole larynges structurally resembled those of stage 54 control animals (compare Figs. 5c and 5e to 5a). As in stage 54 controls, the glottis and constrictor muscles constituted a significant portion of the total length of the larynx in hypothyroid animals. Cartilage appeared best formed along the glottis, but even here chondrocyte density was low and minimal patterning of cells was observed; chondrocytes were most sparse in the posterior ventral larynx region of PTU-treated animals. PTU-treated tadpoles (Figs. 5c and 5e) did not exhibit the extensive development of the laryngeal cartilage seen in contemporaneous untreated animals (Figs. 5b and 5d); elaborate patterning of dense chondrocytes and formation of the thyohyrals and arytenoid discs were not present in larynges from hypothyroid animals. The lumen of the larynx in hypothyroid tadpoles also had a simple circular or oval cross-sectional shape; the complex lumen shapes and marked expansion of the dilator muscles characteristic of 50 (Fig. 5b)- and 100-day (Fig. 5d) untreated animals were not seen in PTU-treated animals (Figs. 5c and 5e). Unlike control animals, no progression of laryngeal development was noted between the 50- and 100-day PTU-treated tadpoles (compare Fig. 5c to 5e). Thus, the larynx of hypothyroid tadpoles appears to be developmentally arrested at stage 54.

The only major differences noted between control stage 54 tadpoles and 50- and 100-day PTU-treated animals were an increase in laryngeal size and modest increases

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FIG. 3. Cross sections of gonads from 100-day control (a, b) and 100-day PTU-treated tadpoles (c, d) illustrate the continued development of gonads in hypothyroid tadpoles. Control animals are stage PM1 frogs; PTU tadpoles remain blocked at stage 54. Stage 1 oocytes with large nuclei can be clearly seen in sections through the convoluted ovaries (a and c). Testes (b and d) are characterized by seminiferous tubules packed with nests of male germ cells. Scale bar, 100 μm.
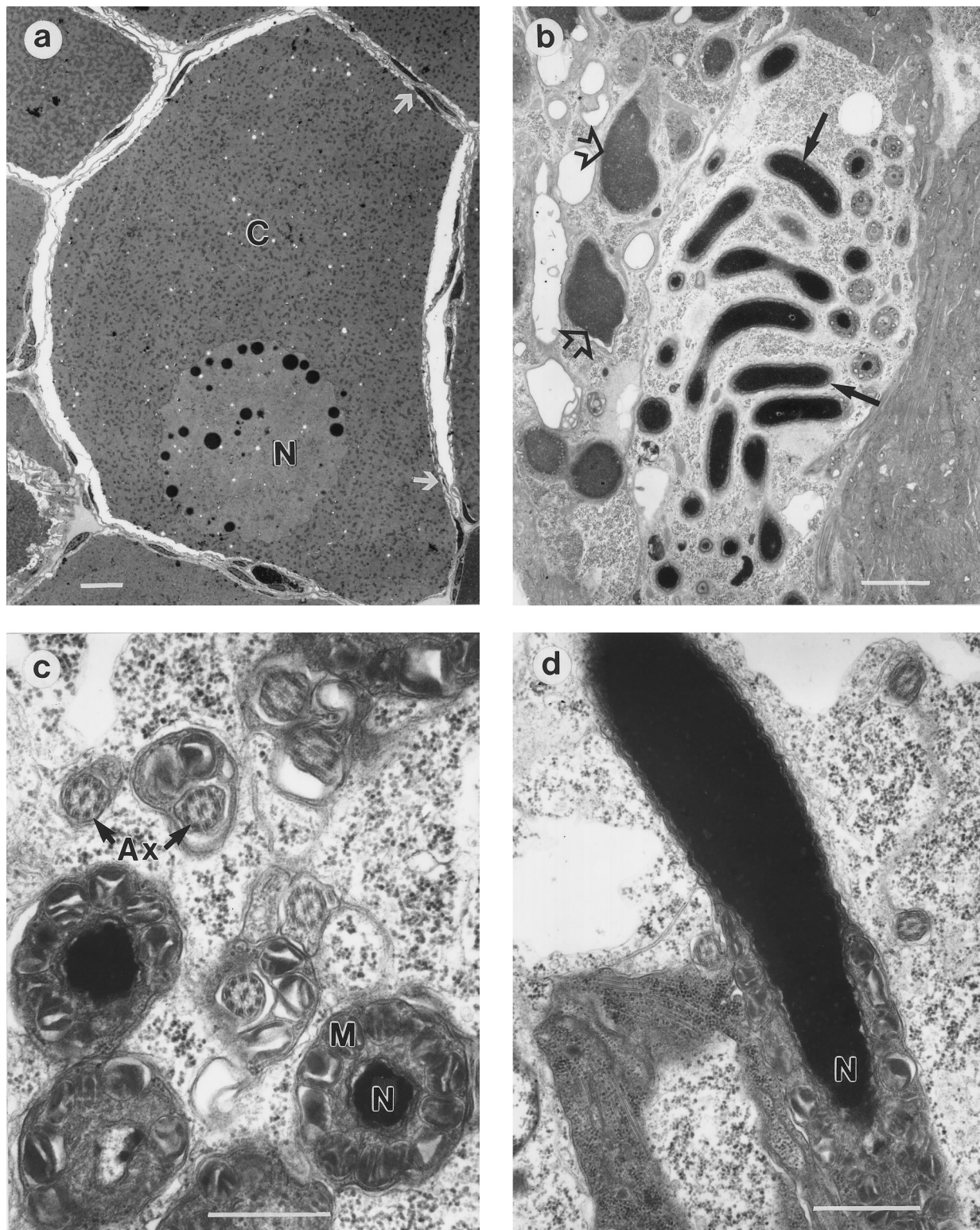
FIG. 4.   Electron micrographs of developing germ cells in testes and ovaries from metamorphically blocked, PTU-treated tadpoles. (a) Stage 1 oocyte from a PTU-treated female tadpole has nucleus (N) with many nucleoli. Oocyte lacks yolk deposits in the cytoplasm (C) and is surrounded by epithelial and connective tissue layers (arrows). Bar, 10 μm. (b) Low-magnification image of male germ cells in

in muscle mass (Fig. 5a versus 5c and 5e). The greater size of PTU-treated tadpole larynges compared to stage 54 control larynges thus accompanies the larger body size of the hypothyroid animals (Table 1). The increased muscle mass of hypothyroid tadpoles was minimal when compared to the increased mass of untreated animals of the same age. No further growth or development of the larynx was observed in animals maintained in PTU for 1 year (not shown).

Laryngeal Development of PTU- and DHT-Treated Animals

Exogenous androgen stimulates growth and morphological changes in larynges of tadpoles during metamorphic climax (Robertson *et al.,* 1994), suggesting that the larynx is sensitive to androgen at this time. If TH is essential for androgen sensitivity, we should be able to block the effects of androgen by treating tadpoles with PTU. Thus, a group of tadpoles received combined PTU and DHT (0.025 μg/liter) treatment. The larynges of tadpoles treated for 50 and 100 days with both PTU and DHT were identical to larynges of animals contemporaneously treated with PTU alone (Fig. 5f). We conclude that TH secretion is necessary for development of androgen sensitivity in the tadpole larynx.

Laryngeal Innervation

Adult male *X. laevis* have more laryngeal nerve axons than females; this dimorphism is first seen at tadpole stage 62 and persists into postmetamorphic development (Kelley and Dennison, 1990). Axon number in males is decreased by antiandrogen treatment and axon number in females is increased by exogenous androgen (Robertson *et al.,* 1994). The sexual dimorphism in laryngeal innervation thus depends on androgen; we wished to determine whether sexually differentiated innervation also depends on exposure to thyroxine.

Stage 54 control tadpoles have undifferentiated gonads and cannot be sexed (see above); stage 54 laryngeal axon numbers from this study (Table 2) are similar to values reported previously (Kelley and Dennison, 1990). In the 100-day untreated control group (PM1 frogs), males had significantly more ($P < 0.001$) laryngeal nerve axons than females; numbers are again very similar to the previous report (Kelley and Dennison, 1990). However, in tadpoles treated with PTU for 100 days, there is no sex difference in axon numbers ($P > 0.05$). We conclude that develop-

ment of a sex difference in laryngeal nerve axon number is TH dependent. Laryngeal nerve axon numbers of 100-day PTU/DHT-treated male and female tadpoles also were not significantly different from those treated with PTU alone ($P > 0.05$). Exposure to androgen during late stages of tadpole development had previously been shown to increase axon numbers in both males and females (Robertson *et al.,* 1994). We thus also conclude that without TH, laryngeal innervation cannot be influenced by exposure to androgen.

Moreover, the ontogentic decrease in axon numbers that normally occurs between stage 54 and PM1 (100 days) is blocked in hypothyroid tadpoles of both sexes. The 100-day untreated control animals (PM stage 1 frogs) had significantly fewer laryngeal nerve axons than stage 54 untreated control tadpoles ($P < 0.05$; Table 2). By contrast, axon numbers in the 100-day PTU-only or PTU/DHT-treated groups remained at or above the values of stage 54 controls (Table 2). We conclude that TH plays a role in the characteristic pattern of axon loss seen in the laryngeal nerve of late-stage tadpoles and juvenile frogs, a result that has also been obtained for the oculomotor nerve in *X. laevis* (Schönenberger and Escher, 1988).

Myelination of laryngeal nerve axons normally occurs between stages 54 and 66 in both sexes (Kelley and Dennison, 1990). In agreement with previous observations, less than 1% of the laryngeal nerve axons of control stage 54 tadpoles were myelinated in the present study (Table 2 and Fig. 6a). After 100 days of PTU treatment (Fig. 6c), the percentage of myelinated axons in the laryngeal nerve was significantly ($P < 0.001$) larger in both males (4.8%) and females (5%); there was no significant difference in myelination between sexes. However, in untreated 100-day male and female controls (PM1 frogs; Fig. 6b), all the laryngeal nerve axons were myelinated (as in Kelley and Dennison, 1990). Hypothyroidism thus severely reduces but does not wholly prevent the increase in laryngeal nerve axon myelination associated with metamorphosis. The percentage of myelinated axons present after 1 year did not differ from values obtained after 100 days (Table 2: the mean for three males was 5.0% ; the single female had 4.6 % myelinated axons).

In both sexes exposure to high doses of DHT induces precocious myelination of laryngeal axons (Robertson *et al.,* 1994). In this study, simultaneous PTU and DHT treatment for 100 days resulted in a small increase in percent of myelinated axons over PTU-only treatment (Fig. 6d vs 6c); male 100-day PTU- and DHT-treated tad-

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different stages of maturation. More mature spermatids (closed arrows) have greater condensation of nuclear material than less advanced cells (open arrows). Bar, 4 μm. (c) High magnification of cross-sectioned spermatids from a PTU-treated tadpole testis. Central nucleus (N) and outer mitochondria (M) are seen in cells sectioned through the midpiece, while sectioned tails clearly exhibit the 9×2 tubular arrangement of the axoneme (Ax). Bar, 1 μm. (d) Longitudinal section of the head and midpiece of a spermatid; note the highly condensed nuclear material. Bar, 1 μm.
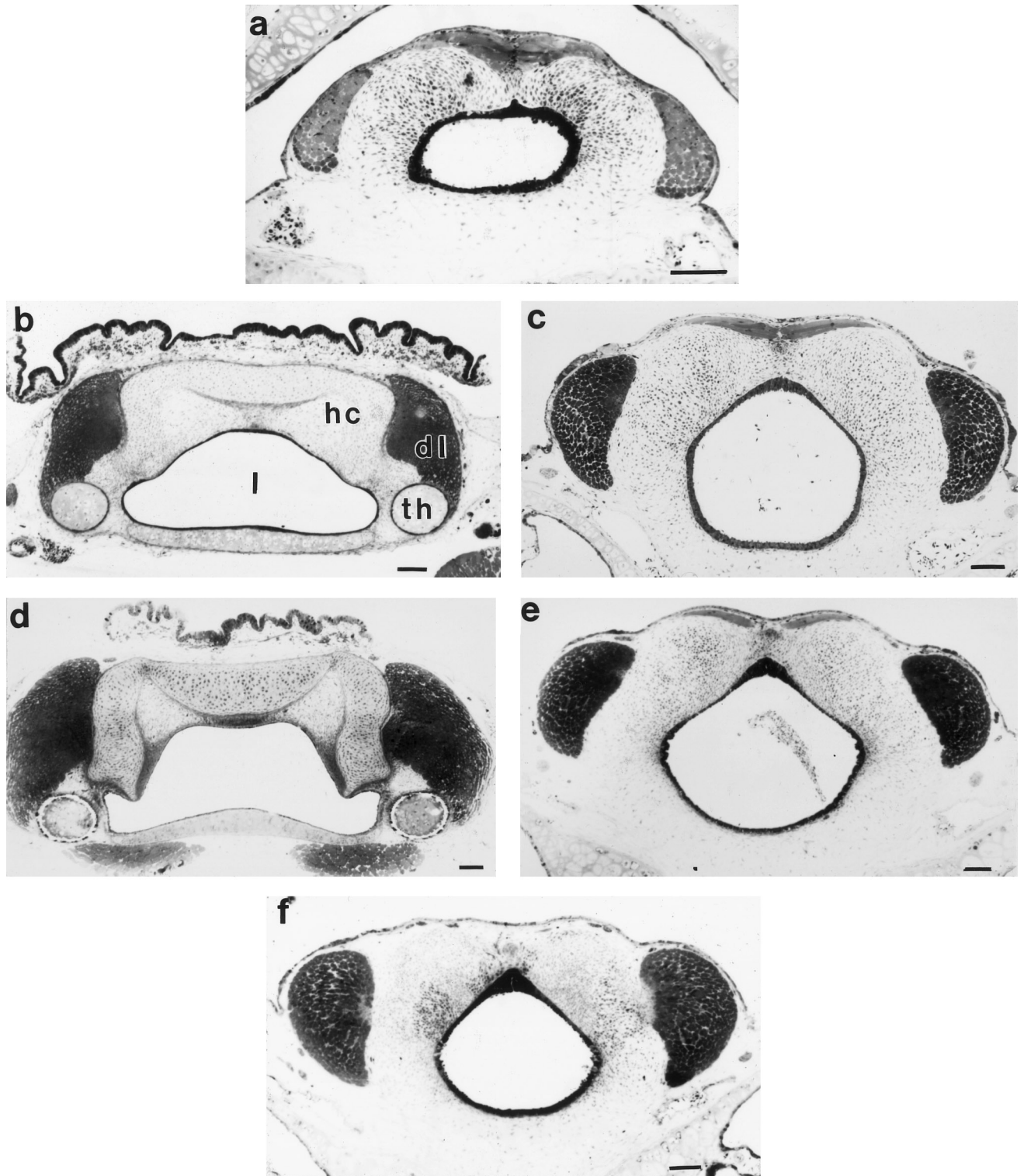
FIG. 5. Transverse sectioned larynges of control and PTU- and DHT–PTU-treated animals; sections are from corresponding posterior regions of each larynx. Dorsal side is at the top of all micrographs. (a) Larynx of a stage 54 tadpole. (b) Larynx of a control tadpole 50 days after the onset of vehicle treatment (tadpole stage 66). (c) Larynx of a tadpole 50 days after the onset of PTU treatment. (d) Larynx of a

TABLE 2

Total Axon Number (Top Row) and Percent Myelinated Axons (Middle Row) in the Laryngeal Nerve of Various Hypothyroid and Control Groups Examined in This Study

	Stage 54	Control 100	PTU 100	PTU/DHT 100	PTU 1 year
Sex unknown	637 (68) 0.2 (0.008) $n = 5$				
Females		295 (10.1) 100 $n = 3$	774 (70) 5.0 (0.7) $n = 4$	831 (70) 7.5 (0.88) $n = 3$	985 4.6 $n = 1$
Males		544 (13) 100 $n = 2$	771 (57) 4.8 (0.11) $n = 4$	875 (44) 9.8 (1.0) $n = 4$	834 (45) 5.0 (0.19) $n = 3$

Note. Data presented are means of number of individuals indicated (*n*; bottom row), with SEM in parentheses. Percent myelination was calculated: (myelinated axons ÷ total axons) × 100.

poles had 9.5% myelinated axons and females 7.5% myelination, with again no sex difference in myelination. The increase was significant ($P < 0.001$) only in the case of PTU males versus PTU/DHT males. Thus androgen treatment can potentiate myelination of laryngeal axons even in the presence of PTU; the effect is somewhat less than that reported previously for DHT treatment alone (~20% myelinated axons; Robertson *et al.,* 1994). Myelination of laryngeal nerve axons in DHT-treated hypothyroid animals is still, however, quite limited in comparison to the 100% myelination of age-matched controls (compare Fig. 6d vs 6b).

DISCUSSION

When thyroxine synthesis is prevented, the typical amphibian progression from the larval to the juvenile form is blocked. As we show here, this blockade includes: (i) the development of the vocal organ, (ii) the acquisition of a laryngeal growth response to exogenous androgen, and (iii) the sexual differentiation of laryngeal innervation, which is dependent upon androgen secretion in males (Robertson *et al.,* 1994).

During metamorphosis, both the gonads and the larynx become sexually differentiated. Since the gonads secrete androgenic steroids known to affect laryngeal masculinization, TH could exert its effects on the larynx simply by controlling the differentiation (and steroidogenic capac-

ity) of the testes. The results of this study rule out this particular interpretation; the gonads develop even in the presence of PTU and the larynx does not respond to testicular hormones (endogenous or exogenous) if PTU is present. The presents results do not, however, demonstrate that TH effects on the larynx are cell autonomous; TH influences on the secretion of other gonadal hormones or on other tissues, for example motor neurons innervating the larynx, could be required for masculinization to proceed.

Sexual differentiation of laryngeal innervation, a process which we have shown to be sensitive to exposure to exogenous androgen (Robertson *et al.,* 1994), is not seen in PTU-treated animals. Furthermore, PTU-treated tadpoles survive, apparently unaffected, for up to 100 days in a DHT solution that would normally induce laryngeal hypertrophy and kill all tadpoles within 20 days (Robertson *et al.,* 1994). We conclude that exposure to TH is required for sensitivity to androgenic steroids. It then follows that the sensitive period for androgen action on the larynx is opened by thyroxine secretion during prometamorphosis.

TH Effects on Neuronal Differentiation

Independent of its ability to confer sensitivity to gonadal steroids, TH secretion is responsible for a number of key features in neural development (Harris, 1990); for example, thyroxine secretion is responsible for oculomo-

control frog 100 days after the onset of vehicle treatment (PM1 frog). (e) Larynx of an experimental animal 100 days after the onset of PTU treatment. (f) Larynx of an experimental animal 100 days after the onset of DHT-PTU treatment. Refer to text for detailed descriptions. Abbreviations: dl, laryngeal dilator muscle; l, lumen; th, thyohyral cartilage; hc, hyaline cartilage. Thyohyrals are not evident within the larynx at stage 54. Scale bars, 100 μm.
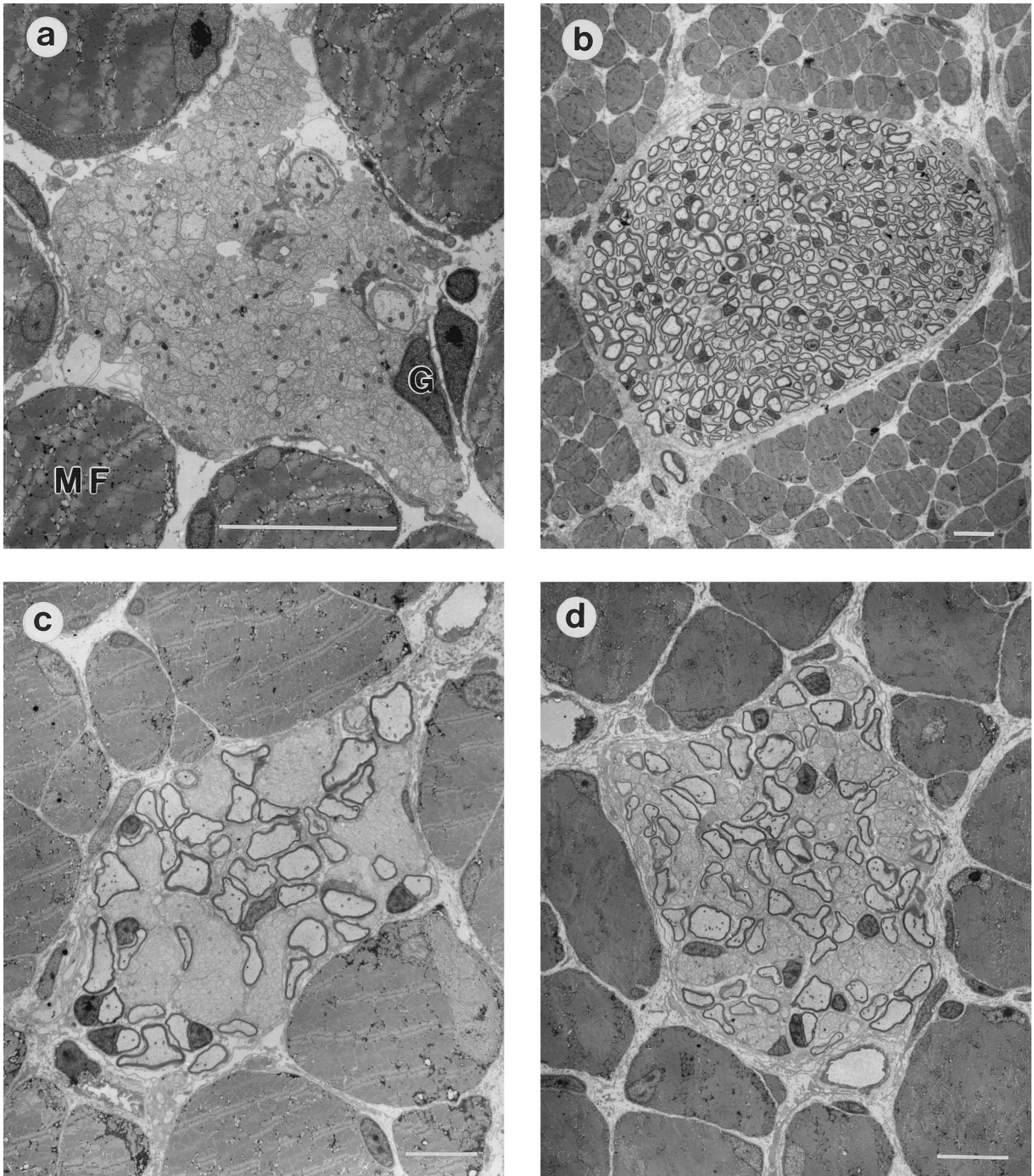
FIG. 6.   Electron micrographs of cross-sectioned laryngeal nerves from animals in various treatment groups. (a) Laryngeal nerve of control stage 54 tadpole is composed entirely of small unmyelinated axons. Also seen are glial cells (G) and muscle fibers (MF). (b) A 100-day control animal (PM1 frog). By 7 weeks postmetamorphosis, 100% of the laryngeal motor axons are myelinated. (c) In a 100-day PTU-treated tadpole, only a small percentage of the laryngeal nerve axons are myelinated. (d) A 100-day combined PTU/DHT-treated tadpole has a small increase in laryngeal nerve myelination compared to PTU-only-treated animals (Fig. 3c), but still far less myelination than age-matched controls (Fig. 3b). Bars, 10 μm.

tor nerve myelination in *X. laevis* (Schönenberger and Escher, 1988). In our study, hypothyroid tadpoles had a higher percentage of myelinated laryngeal nerve axons than stage-matched controls, but much lower percent myelination than age-matched animals. Thus, TH appears to have a role in the increased myelination seen in the laryngeal nerve during metamorphosis (Kelley and Dennison, 1990).

Thyroxine also plays a role in control of cell number in the developing nervous system. In the *Xenopus* olfactory nerve, PTU treatment blocks the axon addition seen in untreated, age-matched controls (Burd, 1992). Thyroxine secretion also initiates neuron loss in motor systems (Prestige and Wilson, 1972). TH secretion has been shown to control axon numbers in the oculomotor nerve of *X. laevis* (Schönenberger *et al.,* 1986; Schönenberger and Escher, 1988); the normal developmental pattern of axon number decline in the oculomotor nerve was not observed in hypothyroid tadpoles. Unlike the laryngeal neuromuscular system, however, no sex difference in oculomotor nerve axon numbers has been reported.

Our evidence on the laryngeal nerve suggests that TH is also required to initiate the decline in axon number in both sexes. Without exposure to TH, axon numbers are maintained in both sexes and even increase by 100 days. Androgen secretion in males opposes the axon loss initiated by TH secretion and results in sexually differentiated laryngeal innervation. Thyroxine secretion is thus central to the developmental mechanisms by which sexual dimorphism in innervation arises but is not directly responsible for sexually differentiated innervation.

Androgen-Directed Sexual Differentiation: The Role of TH Secretion

The sex difference in the laryngeal innervation of stage 62 tadpoles, arising from androgen secretion in male tadpoles (Kelley and Dennison, 1990; Robertson *et al.,* 1994), is the earliest identified dimorphism in the neuromuscular system controlling mate calling in *Xenopus.* In the present study, hypothyroid tadpoles had no sex difference in laryngeal nerve axon numbers while, as expected (Kelley and Dennison, 1990), age-matched control (PM1 frogs) males had more axons than females. In the laryngeal nerve, androgen promotes the maintenance of axon numbers. This effect was severely attenuated in androgen-treated hypothyroid animals. We conclude that TH controls the onset of sexually differentiated laryngeal innervation by establishing androgen sensitivity.

How does TH induce androgen sensitivity in the laryngeal neuromuscular system? Thyroid hormone receptors (TR) are members of the steroid hormone receptor superfamily (Weinberger *et al.,* 1986; Sap *et al.,* 1986) and are believed to act as ligand gated transcription factors via interaction with TH response elements within transcriptional activation or repression domains of thyroxine-sensitive genes. In *Xenopus,* two classes of TR, α and β, have been identified (Yaoita *et al.,* 1990). Both the developing larynx and motor neurons express the α isoform of TR mRNA (Kawahara *et al.,* 1991; M. Cohen, unpublished). TH activation of this or another TR might then induce androgen receptor (AR) expression in the larynx and its motor neurons. However, this mechanism appears unlikely in view of the fact that AR mRNA expression precedes thyroxine secretion and is unaffected by PTU treatment (Cohen *et al.,* 1991; Cohen and Kelley, 1994). Another possibility is that androgen sensitivity is due to TH-induced stabilization of AR mRNA or protein. Alternatively, thyroxine control of androgen sensitivity might not involve the androgen receptor directly but instead reflect a requirement for an additional, TH-dependent transcription factor, without which androgen activated gene expression cannot proceed.

Gonadal Differentiation and Development

In contrast to the larynx (and to the majority of other tissues), gonadal differentiation is largely unaffected by TH blockade. The emancipation of gonadal development from thyroxine regulation seen in this study could reflect the absence of TR receptor expression. Most tissues of the developing tadpole express thyroxine receptors. However, close inspection of figures from a study in which *in situ* hybridization was used to localize TR mRNA in the *Xenopus* tadpole (Kawahara *et al.,* 1991) reveals little, if any, signal in developing gonads. Presently, several cell lines of *Xenopus* origin are used as TH insensitive models *in vitro* (Kanomori and Brown, 1993); tadpole gonads may represent a useful *in vivo* TH insensitive system for studies of TH and TR activity. In addition, the emancipation of gonadal development from control by thyroxine suggests a possible molecular mechanism for neoteny, a phenomenon seen in several urodele species whereby larval animals can become reproductively mature (Dent, 1968; Galton, 1992).

There are apparent limitations to the development of the gonads in hypothyroid animals. First, there were clear differences in gross morphology of the gonads between the hypothyroid and age-matched control animals. Additionally, failure of oocytes from PTU-treated tadpoles to advance beyond stage 1 likely reflects the TH dependence of certain aspects of maturation. The deposition of yolk in developing oocytes requires estrogen stimulation of vitellogenin secretion from the liver; metamorphic TH secretion induces the capacity of tadpole hepatocytes to respond to estrogen with vitellogenin synthesis (Kawahara *et al.,* 1987, 1989). Whether other cellular and molecular hallmarks of maturation are expressed in oocytes of hypothyroid tadpoles remains unknown. Presence of mature male gametes in testes of PTU-treated tadpoles is also of interest in view of the androgen dependence reported for some stages of spermatogenesis in other verte-

brates (Minucci *et al.,* 1992; Roberts and Zerkin, 1991). Thus, the gonads of hypothyroid *X. laevis* tadpoles could provide useful systems for studies of endocrine regulation of gameteogenesis.

ACKNOWLEDGMENTS

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