J Neurophysiol 94: 415–428, 2005.
First published March 9, 2005; doi:10.1152/jn.01279.2004.

Androgen-Induced Vocal Transformation in Adult Female African Clawed Frogs

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Submitted 14 December 2004; accepted in final form 24 February 2005

Potter, Kristen A., Tina Bose, and Ayako Yamaguchi. Androgen-induced vocal transformation in adult female African clawed frogs. *J Neurophysiol* 94: 415–428, 2005. First published March 9, 2005; doi:10.1152/jn.01279.2004. Sex-specific behaviors of some vertebrates are reversible by androgen administered in adulthood. Such behavioral transformations in adulthood provide opportunities to identify how neural systems reconfigure to produce sex-specific behavior. In this study, we focused on the vocalizations of the African clawed frog, *Xenopus laevis*. Male and female adult *Xenopus* produce sexually distinct vocalizations; males produce series of rapid clicks, whereas females produce slow trains of clicks. The differences in click rate can be reduced to differences in the firing rate of laryngeal motoneurons in vivo. This behavioral dimorphism is accompanied by various sex-specific characteristics throughout the vocal pathways, including functionally distinct laryngeal muscles and motoneurons in the sexes. In this study, we first determined whether and how testosterone (T) modifies the vocalizations of adult females and then examined changes underlying the behavioral modification at the laryngeal muscle and motoneuron levels. Our results show that, in response to T, the vocalizations of females were transformed within 13 wk. Vocal transformation was preceded by complete masculinization of muscle contractile properties and motoneuron soma size by the fourth week of T treatment, which suggests that the vocal pathways' peripheral components masculinize earlier than the behavior. Therefore the rate of transformation of vocal behavior must reflect a functional transformation of neurons in the central vocal pathways, which leads to the generation of male-like motor rhythms.

INTRODUCTION

Male and female vertebrates show sex-specific reproductive behavior. In most vertebrate species, the nervous systems of the sexes differentiate during development, mainly due to the organizational effects of steroid hormones (Phoenix et al. 1959). In most species, sex differences are irreversible; gonadectomy together with administration of androgen in adulthood fails to masculinize the behavior of females (Cooke et al. 1998). However, the sexual differentiation of behavior in some vertebrates remains flexible into adulthood, and female behavior can be masculinized by administration of exogenous androgen (Meyer 1983; Mills and Zakon 1991; Wade et al. 1993).

In adulthood, African clawed frogs (*Xenopus laevis*) generate sexually distinct vocalizations, which consist of series of clicks (Fig. 1). The rate at which clicks are produced is sex-specific; males produce clicks at rapid rates (~80 Hz; Wetzel and Kelley 1983), and females produce clicks at slower rates (~20 Hz; Kelley 1996). For example, advertisement calls, the most common adult male vocalization, are composed of alternating trains of fast and slow clicks, whereas release calls, the most common adult female vocalization, are made of slow, monotonous series of clicks (Fig. 1, *A* and *B*). Because *Xenopus* vocalizations do not rely on the respiratory system, the mechanisms of sound production are relatively simple. A click sound is produced by the larynx when a pair of laryngeal muscles contracts and pulls apart a pair of tightly apposed arytenoid discs; the fluid medium surrounding the discs implodes and generates a click sound (Kelley 1986; Tobias and Kelley 1987; Yager 1982). The laryngeal muscles themselves are controlled by the firing activity of laryngeal motoneurons in cranial nucleus IX–X (Yamaguchi and Kelley 2000). Thus sexually distinct click rates originate in the central vocal pathways.

The vocal pathways of male and female *Xenopus* are sexually distinct at various levels. For example, laryngeal muscle fibers are fast-twitch in males but slow-twitch in females, which indicates that the female larynx is physically incapable of producing male-typical rapid clicks (Tobias and Kelley 1987). The intrinsic and morphological properties of laryngeal motoneurons also are differentiated in the sexes; step depolarization evokes sex-specific firing patterns (Yamaguchi et al. 2003), and motoneuron somata are larger in males than in females (Yamaguchi et al. 2004).

A previous study reported that gonadectomy followed by administration of testosterone (T) in adult female *Xenopus* led to masculinized vocalization in a small proportion (~22%) of animals after 13 wk (Hannigan and Kelley 1986). However, contradicting Hannigan and Kelley (1986), a more recent study reports that T-treated, gonadectomized adult females continued to produce female-like vocalizations and failed to produce male-like vocalizations for as long as 15 mo (Watson and Kelley 1992). The discrepancy may be due to infrequent sampling of vocal behavior in both studies; in these studies, vocal recordings were made for four or fewer sessions, for 1.5 h/session, over 3–15 mo (a total of 6 h or less). Therefore it is possible that, in both studies, all the subjects produced some male-like vocalizations but that these vocalizations were not recorded in the latter study. If the vocalizations of adult female *Xenopus* indeed can be masculinized rapidly, such behavioral transformation provides an ideal opportunity to identify the manner in which neural and muscular systems reconfigure to produce sex-specific behavior.

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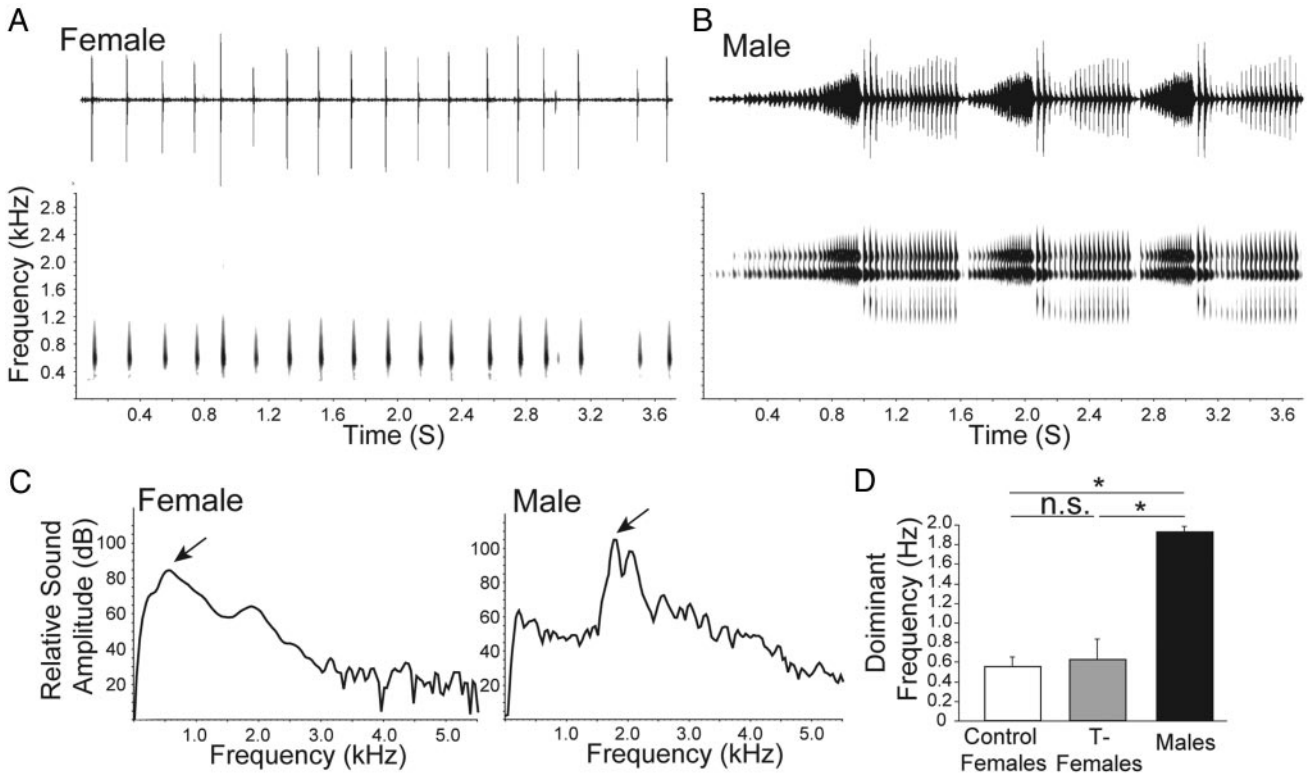

FIG. 1. Sexually distinct vocalizations of male and female *Xenopus laevis*. *A*: amplitude waveform (*top*) and sound spectrogram (*bottom*) of a release call, a female-typical vocalization. *B*: amplitude waveform (*top*) and sound spectrogram (*bottom*) of an advertisement call, a male-typical vocalization. Notice the difference in click rates and the frequency components of individual clicks in males and females. Sample rate = 11 kHz; discrete Fourier transform (DFT) size = 256, 3-dB bandwidth, 82 Hz for spectrograms. Sound was high-pass filtered at 200 Hz. *C*: frequency spectra of a male and a female click. Arrows indicate the dominant frequency (frequency at which sound energy is highest). DFT size = 256; frequency resolution = 43.1 Hz. *D*: mean and SD of dominant frequency of clicks from males, females, and females treated with testosterone (T) for 13 wk. Dominant frequency of T-treated and control female clicks are similar to each other, and distinct from that of male clicks. Ten clicks were sampled at random from 5 individuals in each group, and average dominant frequency for each individual was calculated.

In this study, we first determined whether and how vocalizations can be masculinized by T in adult female *Xenopus* by using automated sound recording systems that allowed comprehensive behavioral monitoring of the vocal transformation process. Surprisingly, rapid vocal transformation was observed. We next examined the rates at which the laryngeal muscles and the laryngeal motoneurons transformed in response to T. Both laryngeal muscle fibers and laryngeal motoneurons of male and female *Xenopus* express androgen receptors (Fischer et al. 1995; Kelley 1980; Kelley et al. 1975) and therefore may be masculinized at independent rates. Examination of vocal behavior, muscle physiology, and motoneuron size in parallel led us to conclude that the T-induced transformation of vocal behavior reflects the rate at which the central vocal pathways functionally transform to generate rapid motor rhythms; peripheral elements seem to be modified much more quickly.

METHODS

Animals

Thirty-seven sexually mature females [mean mass and snout-vent length before gonadectomy: 68.2 ± 1.8 (SE) g and 8.7 ± 1.0 cm, respectively] and 10 sexually mature males (49.0 ± 4.0 g; 7.5 ± 0.3 cm) were purchased from NASCO (Fort Atkinson, WI). The animals were kept in 15- and 20-gal glass aquaria, five to seven per tank, on a 12:12 light:dark cycle and at ~18°C, and males, control females, and T-treated females remained separate from each other except during recording sessions. All care and procedures adhered to National Institutes of Health standards for animal welfare.

Gonadectomy and T implants

Females were anesthetized with MS-222 (ethyl 3-amino benzoate methanesulfonic acid, Sigma Aldrich) and were ovariectomized by removing ovaries and fat bodies using a cauterizer through a small incision (~1 cm) in the abdomen. Immediately after ovary removal, T-filled (4-androsten-17β-ol-3-one, Sigma Aldrich T-1500) silastic tubes (2.16 OD × 1.02 ID; 0.5 mg/g body weight) were implanted into the ventral lymph sacs of 30 females. This treatment is known to elevate plasma levels of T from female-typical levels (1.1 ~ 2.3 ng/ml) (Kang et al. 1995) to very high levels (44 ng/ml) (Watson and Kelley 1992), about 70% higher than those of reproductively receptive males (26 ng/ml) (Kang et al. 1995) for >1 yr (Watson and Kelley 1992). The remaining seven ovariectomized females were implanted with empty silastic tubes (2 cm long, ends sealed with silicone elastomer) as controls.

Sound recordings

Sound recordings were made with a hydrophone (H2, Aquarian Audio Products, Shoreline, WA) suspended 2 cm below the surface

level in the center of a plastic 8-liter tank filled with 6.5 liters of water. Vocalizations were collected through a voice-activated recording system (Syrinx software, John Burt, www.syrinxpc.com). To facilitate vocal production in subject females, a sexually receptive stimulus male or female was introduced into the tank for each recording session. Stimulus males and females were brought into sexual receptivity by injecting them with human chorionic gonadotropin (0.6 ml/male, 1.0 ml/female; Sigma, St. Louis, MO) either 2–5 (males) or 8 h (females) before the beginning of a recording session. Stimulus females remained receptive for 8–24 h during oviposition, during which time they accepted clasping from males and T-treated females.

Recordings were made from 16 T-treated females and all control females. T-treated females were divided into two groups. In the first group ($n = 8$), recordings were made before surgery and 4, 8, 12, and 13 wk after T implantation (Table 1). Because vocalizations recorded 4 wk after T treatment in the first group of animals differed significantly from presurgery vocalizations, we recorded vocalizations from the second group of T-treated females ($n = 8$) during weeks 1, 2, and 3 to characterize the early process of vocal transformation (Table 1). Sound recordings of all seven control females were made before gonadectomy and 4, 8, and 12 wk after gonadectomy.

To determine whether T-treated females vocalize differently in the presence of males and females, recordings were made with stimulus animals of either sex. Males were used as stimuli for all recording sessions during presurgery and weeks 4, 8, and 12 for the first group of T-treated females and for all recording sessions during weeks 1, 2, and 3 for the second group (Table 1). Each stimulus male was paired with multiple experimental subjects. In addition, recordings were made in the presence of stimulus females from two T-treated females in the first group during week 8, from all animals in the first T-treated group during week 13, and from five animals in the second T-treated group during week 8 (Table 1). Control animals were paired with stimulus males for all recording sessions and additionally were recorded with stimulus females during weeks 8 and 12 (Table 1).

The total recording time for all animals was 2,396 h (113 recording sessions, average of 21.2 h per session). All recordings were made at 19–22°C, in dim light, between September and May.

Sound analysis

Vocalizations of T-treated/control females and stimulus males could be distinguished easily based on dominant frequency of clicks—i.e., the sound frequency with the highest sound energy within a single click, as measured from frequency spectra. Male clicks have a higher dominant frequency than those of both control females and T-treated females (Hannigan and Kelley 1986; Watson and Kelley 1992; Wetzel and Kelley 1983) (Fig. 1, *C* and *D*). During recording sessions with stimulus females, the vocalizations of T-treated/control females could not be distinguished from those of the stimulus animals based on dominant frequency. However, because our muscle physiology data (see RESULTS) showed that the larynges of T-treated females become capable of producing clicks faster than 20 Hz by the fourth week of T treatment, whereas the larynges of untreated females are not physically capable of producing such rapid clicks (see following text), in these sessions all vocalizations faster than normal release calls (>20 Hz) were ascribed to T-treated females, and any stereotyped release calls were ascribed to stimulus animals. Because this decision may have excluded some female-like release calls produced by T-treated females, we address this possibility when interpreting our data (see *Transformation of muscle properties*).

TABLE 1. *Sound recording schedule for testosterone-treated females and control females*

	Pre- and Postsurgery, wk							
	−1	1	2	3	4	8	12	13
T-females group 1 ($n = 8$)*	M				M	MF	M	F
T-females group 2 ($n = 8$)*		M	M	M		F		
Control females ($n = 7$)	M				M	MF	MF	

* The number of animals recorded with stimulus females in week 8 was 2 for group 1 and 5 for group 2. M, recordings made with stimulus males; F, recordings made with stimulus females.

Ten sound files were sampled randomly for analysis from each recording session of T-treated females and control females. Ten sound files also were sampled from each of 10 males for comparison. Almost all female files contained a minimum of 10 clicks (average number of clicks/file = 27.1). The interclick interval (ICI) for every pair of clicks (i.e., the interval between the amplitude peaks of 2 adjacent clicks) was measured with Raven software (Cornell University Bioacoustics Laboratory, Ithaca, NY).

To determine how the advertisement-like calls produced by T-treated females differ from the advertisement calls of males, calls recorded from five males and five T-treated females were analyzed. Ten ICIs before and 10 ICIs after a transition between fast and slow trills were sampled (Fig. 2C_3, *bottom*), and click rates were compared between subjects and males.

To evaluate how the overall click rate (i.e., the frequency distribution of click rates) of T-treated females changed over time, the following methods were used. First, instantaneous click rates (1/ICI in Hz) from each individual were plotted as normalized frequency histograms with a bin width of 1 Hz (Fig. 3). Next, each histogram was fitted with a bimodal Gaussian distribution using the Levenberg-Marquardt method to search for a model and the sum of squared errors method to identify the model that best fit the data. Finally, the mean values for both distributions ($\mu 1$ and $\mu 2$), which represent the two most common click rates produced by a subject, were compared with those of males and preop females.

To determine how T-treated females' maximum click rates changed over time, instantaneous click rates and sustained click rates (the average rate of 10 consecutive clicks) were measured from each individual. To do this, a pair of (or a group of 10) clicks produced by a subject in the shortest period of time were identified, and their click rates were calculated. Maximum rates of each subject were compared with those of males and preop females.

Because the vocalizations of males do not vary with the identity of the female with which they are paired [1-factor MANOVA for $\mu 1$, $\mu 2$, sustained, and instantaneous click rates obtained from 5 males paired with 3 females; Wilk's $\lambda = 0.036$; $F(16,22) = 2.71$; $P = 0.01$], individual males were used as units of analysis.

Contractile properties of laryngeal muscles

Animals were anesthetized using the method already described. The larynx was isolated in oxygenated saline and pinned to a petri dish coated with Sylgard (Dow Corning, Midland, MI). Laryngeal muscles and nerves were exposed by the careful removal of connective tissue and cartilage. The laryngeal nerve was stimulated with a suction electrode, with a train of pulses generated by Chart software (AD Instruments, Colorado Springs, CO), and delivered to the electrode via a MacLab/4e D/A converter (AD Instruments) running on a PC. A force transducer (model 1030, UFI, Morro Bay, CA; frequency response ≤ 500 Hz) attached to the tendon of the laryngeal muscle (just dorsal to its insertion into the arytenoid discs) was used to measure semi-isometric tension. The dorsal surface of the laryngeal muscles is entirely attached to the cricoid cartilage of the larynx, so the muscle length of the isolated larynx in vitro was the same as the resting state in vivo. The stimuli used were trains of square pulses of 1-ms duration repeated at frequencies ranging from 1 to 100 Hz in 10-Hz increments. Force outputs were amplified using BridgeAmp (AD Instruments) and digitized using a MacLab/4e A/D converter

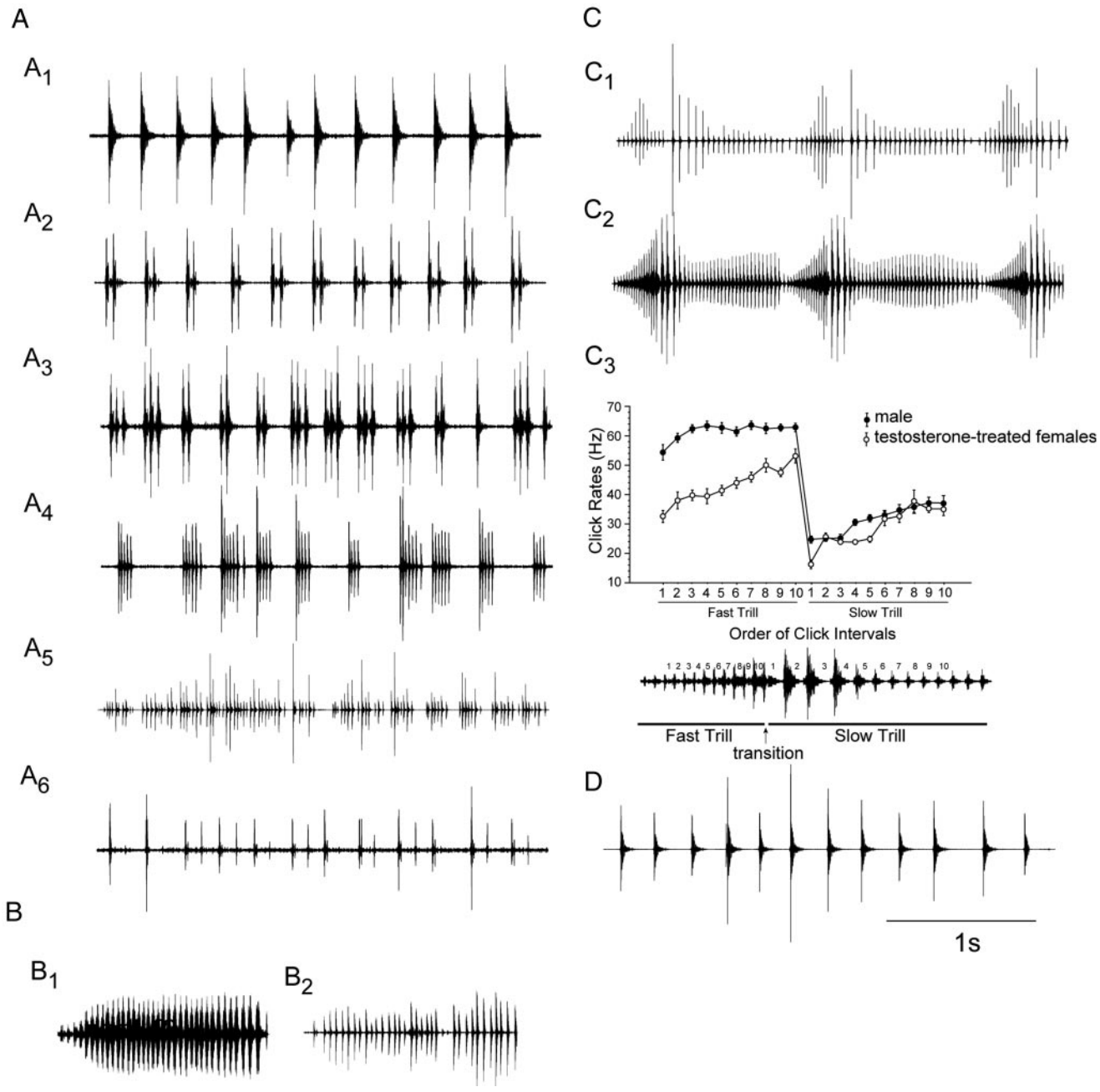

FIG. 2. Amplitude waveforms of example vocalizations recorded from T-treated females, control females, and males. *A*: vocalizations produced by T-treated females paired with stimulus males. A_1: typical female release call before T implantation. Vocalizations with (A_2) doublets, (A_3) triplets, and (A_4) bursts of clicks recorded in weeks 2, 3, and 8 after T treatment. A_5: rapid clicks with irregular intervals and amplitude modulation produced in week 12. A_6: release call produced in week 12. *B*: growl-like vocalizations produced by T-treated females paired with a stimulus male (B_1) and female (B_2). C_1: advertisement call-like vocalizations produced by a T-treated female in the 8th week of hormone treatment. C_2: male advertisement call. C_3: (*top*) click rates before and after the transition between fast and slow trills in male (●) and T-treated female (○) advertisement calls and (*bottom*) example amplitude waveform of advertisement call with click intervals and fast and slow trills labeled. *D*: release call of a control female 12 wk after gonadectomy.

with a sampling rate of 1 kHz. All experiments were carried out at 18 ± 0.5°C.

In addition to the altered contractile properties of muscle, the size of the cartilaginous cricoid box (laryngeal box) also increased slightly in response to T. However, these changes are considered to merely modify the frequency spectrum of clicks (i.e., the frequency components of each individual click) and do not affect click rates (Yager 1992). Therefore morphological changes in cartilage are not reported here.

Muscle physiology data analyses

The temporal profile of a single twitch tension elicited from a laryngeal muscle was characterized by two parameters: slope to peak twitch tension and half-relaxation time. Slope was measured by fitting a straight line from the onset of twitch tension to the peak, and half-relaxation time was defined as the time between the peak of twitch tension and its return to 50% of peak value.

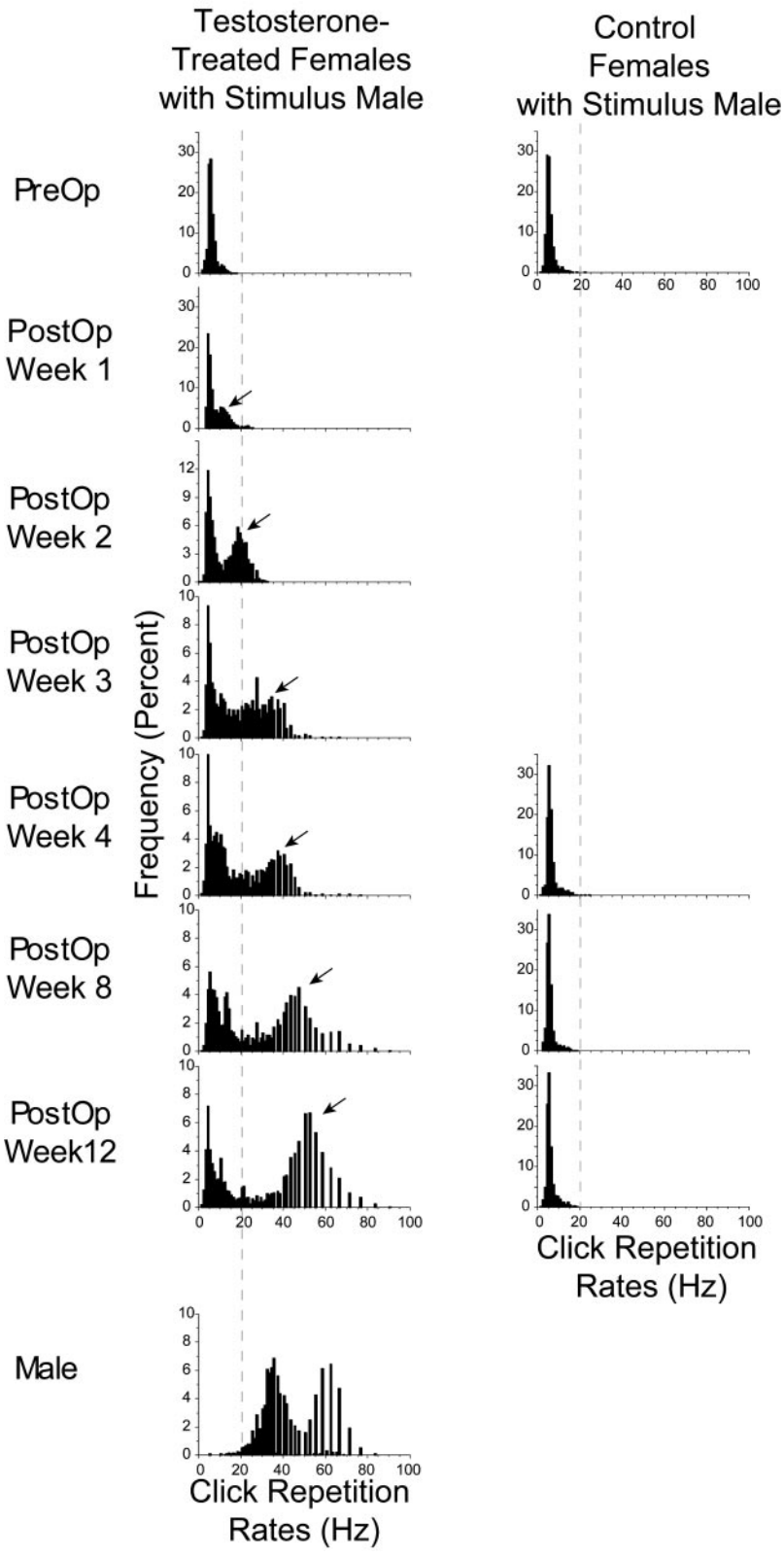

FIG. 3. Frequency histograms of instantaneous click rates of T-treated females (*top 7 rows* of *left column*), males (*bottom row* of *left column*), and control females (*right column*). T-treated females and control females were recorded with stimulus males. Bin width for all histograms is 1 Hz, and all histograms are normalized average across 8 individuals in both groups. Arrows in the histograms of the *left column* indicate appearance of the 2nd peak.

Repetitive stimulation of laryngeal muscles at frequencies higher than the fusion frequency produces a sustained contractile force (tetany). Fusion frequency is lower in females (~20 Hz) than in males (~70 Hz) (Tobias and Kelley 1987). Development of significant fused (tetanic) tension is considered a hindrance to the production of click trains in the *Xenopus* larynx, because such tension retracts and holds apart from each other the arytenoid discs, the movement of which is essential for producing clicks (Yager 1992). To assess the capability of laryngeal muscles to produce clicks at various rates, we measured the ratio of fused tension relative to the total tension (i.e., the sum of

transient and fused tensions). A low fused tension ratio translates to a faithful production of clicks at the stimulus frequency, whereas a high ratio indicates failure to produce repetitive clicks. All measurements were made using Clampfit (Axon Instruments) and Igor (Wavemetrix).

Retrograde labeling of laryngeal motoneurons

For morphometric analyses of laryngeal motoneurons, a subset of the T-treated females used in the behavioral experiment were used along with the additional, unrecorded females: females treated with T for 1 ($n = 4$), 2 ($n = 5$), 4 ($n = 5$), 8 ($n = 5$), and 15 wk ($n = 6$) and control gonadectomized females treated with empty silastic capsules for 17 wk ($n = 3$). In addition, adult males ($n = 5$) and intact adult females ($n = 5$) were used for comparison.

Each frog was anesthetized as previously described and perfused transcardially with ice-cold saline. The brain stem was isolated, and the laryngeal motoneurons were retrogradely labeled with 5% neurobiotin (Vector Laboratories, Burlingame, CA) via the fourth rootlet of nerve IX–X in oxygenated saline (4°C) for 5–12 h. The fourth rootlet contains the axons both of the laryngeal motoneurons and of general visceral efferent neurons, but the two populations of neurons can be distinguished based on location (general visceral efferent somata are more rostro-medial than those of motoneurons; Simpson et al. 1986). The brain was fixed overnight in 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), embedded in gelatin (Fisher, Fair Lawn, NJ), and sectioned coronally at 80 μm thickness with a Vibratome (series 1000, Technical Products International, St. Louis, MO). Labeled motoneurons were visualized using 0.5% Fluorescein Avidin D (Vector Laboratories). The tissue was mounted on glass slides and coverslipped using 50% glycerol in phosphate buffer. The subsequent image acquisition and analysis were performed blind (identification of the animal was concealed from the experimenter) to minimize bias.

Morphometric analyses of laryngeal motoneurons

An Olympus laser confocal microscope controlled by Fluoview software was used to acquire 8-bit images of laryngeal motoneuron somata. Argon laser intensity was set at 20%, and a confocal aperture of 2 was used to excite fluorescein in the tissue at a wavelength of 488 nm. Z-stacks through the somata were acquired using a ×40 oil objective at increments of 1 μm.

Laryngeal motoneuron soma volume was estimated using the following semi-automated procedure. First, a projected image of the soma was constructed from Z-stacks of confocal optical sections using the Sync Measure 3D plug-in for ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/). The outer perimeter of the soma in the *XY*-plane was determined from this projected image and outlined manually. For each optical section in the Z-stack, this perimeter outline was superimposed on the section, and pixels inside the perimeter were thresholded. Thresholding within this maximal soma perimeter ensured that the pixels counted were part of the soma and not part of nearby dendrites or axons. Each thresholded pixel was assigned a volume of $2.4 \times 2.4 \times 1$ μm^3, and all were summed to arrive at an estimate of total soma volume.

The following method was used to determine the boundary between soma and dendrites. Laryngeal motoneurons can be divided into three distinct shapes: ovoid, triangular, and oblong (Simpson et al. 1986). The boundary between soma and dendrites could be identified easily in ovoid and triangular neurons, but in oblong neurons, the boundary was difficult to establish visually. Therefore we used the average diameter of dendrites at the soma boundary obtained from triangular and ovoidal neurons (4.96 ± 0.96 m, $n = 60$) to define the soma-dendrite boundary in oblong neurons. Six to 30 neurons from each individual were used to measure volume [mean, 14.9 ± 5.8 (SD) neurons/animal].

Statistical analyses

The dominant frequency of male, female, and T-treated female clicks were compared using a Mann-Whitney *U* test with overall significance levels set at 0.05. The click rates of fast and slow trills produced by males and T-treated females also were compared using a Mann-Whitney *U* test.

For all data obtained from preop and T-treated females, we first determined whether a measured parameter changed in response to T using a Kruskal-Wallis test (μ1, μ2, maximum instantaneous and sustained click rates, slope and half-relaxation time of a single muscle twitch, soma volume). For all behavioral parameters, the mean value calculated for each animal on a given day was used in statistical analyses. For the physiological and morphological parameters, the mean value calculated from each individual was used in statistical analyses. The factor tested in the Kruskal-Wallis tests was time after T treatment, which consisted of seven levels (preoperative and 1, 2, 3, 4, 8, and 12 wk after treatment). These tests were followed by six posthoc multiple comparisons (each postoperative week compared with the preoperative condition), with overall significance levels for all comparisons set at 0.05 to determine when a parameter differentiated significantly from the preoperative condition (the timing of defeminization). Mann-Whitney *U* tests also were used to determine whether measured parameters differed between males and T-treated females (the timing of masculinization). To evaluate the T-induced change in the fused tension ratio of laryngeal muscles, two-factor repeated measure ANOVA was performed. In this analysis, T treatment was one factor with eight levels (control females, intact females, females treated with T for 1, 2, 4, 8, and 13 wk, and males), and repeated observation of the laryngeal response to four different stimulation frequencies (10, 30, 50, and 70 Hz) was the second, within-subjects factor. The ANOVA was followed by Scheffe's posthoc comparisons to examine when the laryngeal muscles of T-treated females became defeminized and masculinized.

To determine whether T-treated females vocalized differently in the presence of stimulus males and females, the Wilcoxon signed rank test was used to compare maximum instantaneous click rates, maximum sustained click rates, μ1, and μ2. All statistical analyses were carried out using StatView.

RESULTS

Change in type of call produced

All T-treated and control females vocalized during every recording session, with the exception of four of seven T females paired with stimulus females in week 8 and one of eight T females paired with a stimulus male in week 12. The number of control females that vocalized during a recording session with a stimulus female was not clear, because vocalizations of subject and stimulus animals were indistinguishable (see METHODS).

T induced rapid changes in the acoustic morphology of female vocalizations. Before surgery, all females produced normal release calls (Fig. $2A_1$). The subjects began to produce calls that included doublets repeated at male-like click rates within 2 wk (Fig. $2A_2$), triplets within 3 wk (Fig. $2A_3$), and rapid bursts of clicks (Fig. $2A_4$) interspersed with silent intervals of duration similar to those in normal release calls within 8 wk of T treatment. These calls were produced predominantly during recording sessions with stimulus males. By the 12th wk,

subject females in the presence of stimulus males produced rapid clicks with random interclick intervals and amplitude modulation (Fig. $2A_5$). In addition to these abnormal calls, subject females continued to produce calls that resembled normal release calls in the presence of stimulus males (e.g., Fig. $2A_6$). Within 3 wk of T treatment, brief bursts of clicks resembling the growl of a male (an aggressive, male-typical vocalization) were recorded from subject females (Fig. 2*B*). These vocalizations were produced during recording sessions with either stimulus males (Fig. $2B_1$) or females (Fig. $2B_2$). Furthermore, calls that resembled male advertisement calls (Fig. $2C_2$), containing amplitude modulated clicks repeated at alternating fast and slow rates, were recorded from all T-treated females (Fig. $2C_1$). These calls first appeared within 5 wk of T treatment, and their acoustic morphology stabilized after 8 wk of treatment (i.e., calls recorded in the 13th wk of T treatment did not differ from those recorded in the 8th wk). Although these calls were similar to male advertisement calls, their constituent clicks were produced at rates significantly lower than those of males; average click rates during the fast and slow trills of males were 61.53 and 31.52 Hz and those of females were 40.97 and 27.52 Hz ($U = 3258.0$ and 11819.5, $P < 0.0001$ and 0.0001 for fast and slow trills, respectively; Fig. $2C_3$). Interestingly, these calls never were recorded from T-treated females paired with stimulus males. This suggests that T-treated females limit the use of advertisement call-like vocalizations to social contexts involving sexually receptive females (see *Social context and vocalization*).

In contrast, control females showed no change in vocalization throughout the experiment (Fig. 2*D*). Calls of control females recorded with stimulus males all were stereotyped release calls. Calls recorded during recording sessions with stimulus females also all were release calls with click rates slower than 20 Hz, although in these cases, the caller could not be identified. Thus gonadectomy alone seems not to modify the vocalization of adult females.

Change in overall click rates

The transitional vocalizations of T-treated females described above were not well stereotyped. To characterize quantitatively the *overall* changes in vocalizations, we focused on the click rates of T-treated and control females and examined how click rates changed in response to T by plotting frequency histograms (Fig. 3). All clicks produced by the subjects before surgery (preoperative) were <20 Hz, with mean frequency at 6 Hz. After implantation, in addition to these slow clicks, T-treated females began to produce progressively faster clicks during recording sessions with stimulus males, as indicated by the rightward shift of the histograms' second peak (Fig. 3, *left column*, indicated by arrows). Throughout the experiment, control females showed no change in the frequency distribution of their click rates (Fig. 3, *right column*).

To characterize quantitatively the overall change in click rates, click frequency histograms were modeled with bimodal Gaussian distributions, and the two model means, $\mu 1$ and $\mu 2$, were compared (Fig. 4*A*). All of the histograms, including those of control and preoperative T-treated females, were fitted better with bimodal than unimodal Gaussian distributions, as

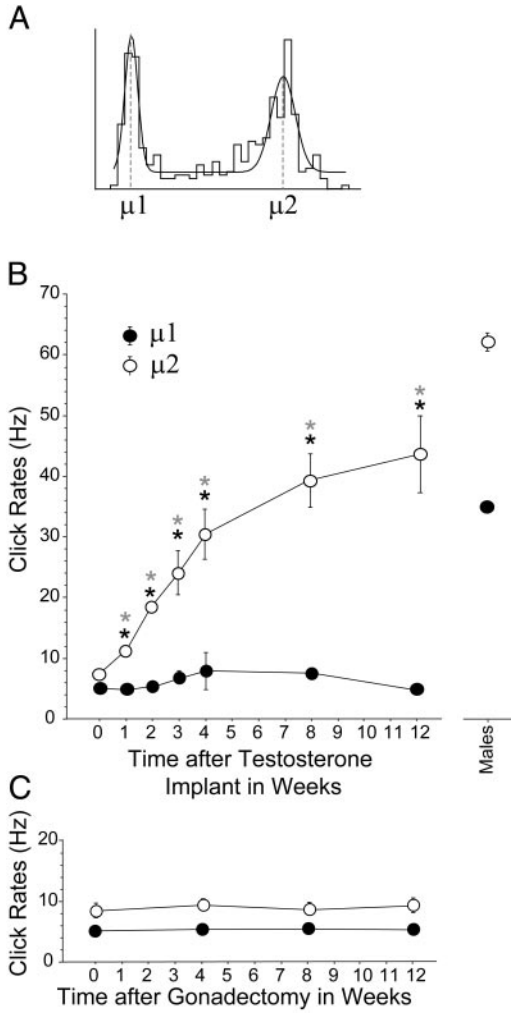

FIG. 4. Two most common click rates, $\mu 1$ and $\mu 2$, estimated from bimodal Gaussian distribution fitted to frequency histograms. *A*: example Gaussian distribution fitted to a frequency histogram. *B*: average and SE of $\mu 1$ and $\mu 2$ recorded from T-treated females and intact males. In T-treated females recorded with stimulus males, $\mu 2$ increased progressively over time, whereas $\mu 1$ remained constant. The $\mu 2$ of T-treated females became significantly higher than the presurgical value (indicated as 0 in *x*-axis) within 1 wk of T treatment but never equaled that of males. Black and gray asterisks indicate significant difference from presurgical females and from males, respectively. Black and gray bars indicate resemblance to presurgical females and males, respectively. *C*: average and SE of $\mu 1$ and $\mu 2$ recorded from control females. Both $\mu 1$ and $\mu 2$ do not change over time in control females.

indicated by the consistently smaller sum of squared errors in all cases (Wilcoxon signed rank test, $Z = -6.4$, $P < 0.0001$).

Before T treatment, $\mu 1$ and $\mu 2$ of subject females were 5.1 ± 0.1 and 7.3 ± 0.5 (SE) Hz. After T treatment, $\mu 1$ remained constant throughout the experiment in recording sessions with stimulus males (Kruskal-Wallis test, $H = 12.14$; $P = 0.06$), whereas $\mu 2$ increased progressively over time ($H = 35.99$; $P < 0.0001$; Fig. 4*B*). In as little as 1 wk after T treatment, $\mu 2$ was significantly higher than the presurgical value, and by the 12th wk, it reached 43.6 ± 6.3 Hz (Fig. 4*B*). However, never during our experimental period did $\mu 2$ of T-treated female vocalizations come to equal that of male vocalizations (62.1 ± 1.5 Hz; Fig. 4*B*). Thus, whereas T induces females to produce progressively faster clicks, the

most frequently produced clicks remained slower than those of males.

Control females, in contrast, showed constant $\mu 1$ and $\mu 2$ values for 12 wk (Kruskal-Wallis test, $H = 3.61$ and 0.91; $P = 0.31$ and 0.82 for $\mu 1$ and $\mu 2$, respectively; Fig. 4*C*), which indicates that gonadectomy alone does not influence the click rates of adult females.

Maximum click rates

We next asked how the maximum click rates produced by T-treated females increased over time. Both maximum instantaneous (2 clicks) and sustained (10 clicks) click rates increased progressively after T treatment (Kruskal-Wallis test, $H = 45.99$ and 46.79; $P < 0.0001$ and 0.001 for instantaneous and sustained click rates, respectively; Fig. 5, *A* and *B*), whereas in control females, maximum click rates did not change over time ($H = 0.83$ and 0.74; $P = 0.84$ and 0.86 for instantaneous and sustained click rates, respectively; Fig. 5, *A* and *B*). Maximum instantaneous click rates were significantly higher than preoperative rates within 3 wk of T treatment and became indistinguishable from those of males by the eighth week (Fig. 5*A*). Maximum sustained click rates became significantly higher than those of preoperative females within 1 wk of T treatment, but never during our experiment did they match male rates (Fig. 5*B*). The results were the same when repetition rates for 5 consecutive clicks (instead of 10) were compared (data not shown). Our results indicate that T-treated females attained the capacity to produce click doublets at rates equal to those of males within 8 wk. However, they never were able to sustain male-like rates for a train of clicks.

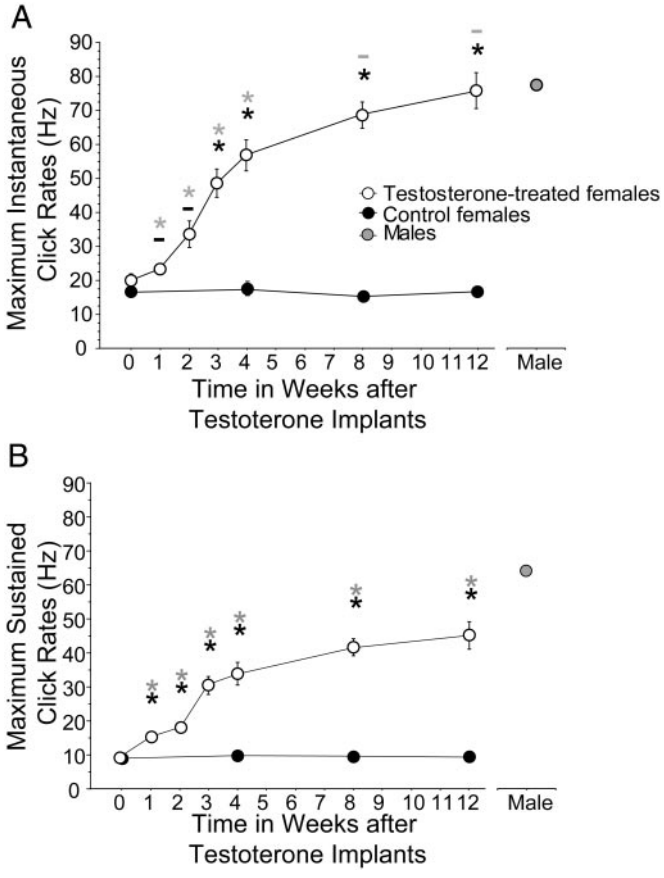

FIG. 5. Maximum instantaneous and sustained click rates. *A*: maximum instantaneous (2 clicks) click rates increased progressively after T treatment. Rates became significantly higher than those of preoperative females within 3 wk and became similar to those of males within 8 wk. *B*: maximum sustained (10 clicks) click rates also increased progressively over time after T treatment. Rates became significantly distinct from those of preoperative females within 1 wk but never became similar to those of males by the end of the experiments. All clicks analyzed were recorded in the presence of stimulus males. Black and gray asterisks and bars indicate statistical significance/nonsignificance as in Fig. 4.

Social context and vocalization

To determine whether T-treated females produce clicks at different rates in the presence of stimulus males and females, clicks recorded in two social settings were compared using T-treated females during weeks 12 and 13. Maximum click rates, both instantaneous and sustained, did not differ significantly in the two social contexts (Wilcoxon signed rank test, $Z = -0.84$ and -1.18; $P = 0.40$ and 0.24 for instantaneous and sustained click rates, respectively). Thus T-treated females do not necessarily employ their fastest clicks discriminately in the presence of either sex.

However, social context did seem to influence the *types* of calls produced by T-treated females; calls resembling male advertisement calls, with alternating fast and slow trills (Fig. $2C_1$), were produced only in the presence of stimulus females, whereas calls resembling release calls with bursts of two to five clicks (Fig. 2, A_2–A_4) were recorded predominantly in the presence of stimulus males. The difference in call type between the two contexts is reflected in the click frequency histogram (Fig. 6*A*). The lower of the most common click rates ($\mu 1$) of T-treated females paired with stimulus females (26.8 ± 0.9 Hz) was significantly higher than that of the same females paired with stimulus males (5.0 ± 0.3 Hz; Wilcoxon signed rank test, $Z = -2.366$, $P = 0.018$; Fig. 6*B*); the higher of the most common click rates ($\mu 2$) was the same in both social contexts ($Z = -0.507$, $P = 0.61$; Fig. 6*B*). Although the higher $\mu 1$ obtained from T-treated females paired with stimulus females may result from our failure to sample female-like slow clicks (see METHODS), the addition of such clicks to the histogram should not convert it into the histogram of T-treated females paired with stimulus males. Instead, the addition of female-typical slow clicks is expected to produce a third histogram peak centered around 6 Hz. Taken together, these results indicate that T-treated females use different types of vocalizations in the presence of sexually receptive females and males.

Transformation of muscle properties

T-treated females were not able to sustain male-like rapid click rates. This may be due either to incomplete masculinization of the contractile properties of laryngeal muscles or to the inability of central vocal pathways to generate sufficiently rapid motor rhythms. To determine which was the case, we examined the contractile properties of laryngeal muscles in T-treated females. In subject females, the single twitch tension of laryngeal muscles showed a rapid change in temporal profile (Fig. 7*A*). The onset of twitch tension, measured as the slope to peak tension, became significantly faster (Kruskal-Wallis test,

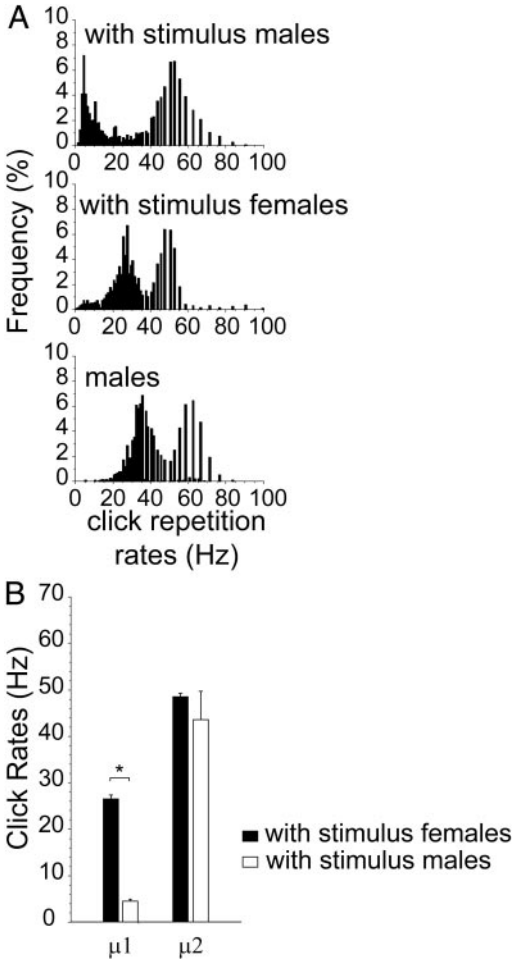

FIG. 6. Social context of vocalizations. *A*: frequency histogram of instantaneous click rates of T-treated females recorded with stimulus males (*top*), stimulus females (*middle*), and intact males (*bottom*). *B*: Average and SE of μ1 and μ2 recorded from T-treated females paired with stimulus males or females during the 12th–13th wk after T treatment. μ1 is higher in the presence of females than in the presence of males, whereas μ2 is the same in both social contexts.

$H = 9.58$, $P = 0.048$; Fig. 7, *A* and *B*), and the half-relaxation time of twitch tension decreased significantly over time ($H = 17.33$, $P = 0.001$; Fig. 7, *A* and *C*). By the fourth week, the tension onset slope of subject female laryngeal muscles became indistinguishable from those of male laryngeal muscles (Fig. 7*B*). By the fourth week, half-relaxation time also became significantly different from those of intact females and was not distinguishable from those of males (Fig. 7*C*). Thus T completely masculinized the temporal profile of twitch tension within 4 wk. In control females, in contrast, the slope of twitch tension decreased (Mann-Whitney *U* test, $U = 4.0$, $P = 0.03$), and the half-relaxation time increased significantly ($U = 1.0$, $P = 0.07$) within 17 wk of gonadectomy (Fig. 7, *B* and *C*), which suggests that in females a lack of ovarian hormones modifies the laryngeal muscles by slowing the onset and offset times of contraction.

When the laryngeal muscles of intact females were stimulated with repeated pulses of increasing frequency, fused tension (tetanus) developed at frequencies >20 Hz at 18 ± 0.5°C (e.g., Fig. 8*A*, control female 30, 50, and 70 Hz). In the majority of males, in contrast, fused tension was not seen until frequencies exceeded 70 Hz [average fusion frequency, 90 + 6.3 (SE) Hz; $n = 5$ males]. In females treated with T, the fusion frequency (the frequency at which fused tension was reached) of laryngeal muscles increased progressively after the treatment (Fig. 8*A*). These changes were characterized quantitatively by measuring the ratio of fused tension to total tension (the sum of transient and fused tensions). The results show that the fused tension ratio changed after T treatment [2-factor repeated measure ANOVA, $F(7,34) = 26.6$, $P < 0.0001$ for T treatment factor]. By the fourth week of T treatment, the ratios of subject females decreased significantly in comparison to those of intact females and were similar to those of males (Fig. 8*B*). In contrast, control females showed fused tension ratios similar to those of intact females 17 wk after gonadectomy. Taken together, these results indicate that T masculinizes laryngeal muscles relatively quickly, so that they become capable of producing male-typical vocalizations *if* driven by male-like neuronal signals.

Increase in motoneuron somata volume

Finally, we examined whether motoneuron size increased over time in T-treated females. In adult *Xenopus*, the size of laryngeal motoneuron somata is sexually distinct, being larger in males than in females (Yamaguchi et al. 2004). T treatment significantly increased the size of motoneuron somata in adult females ($H = 24.3$, $P = 0.0002$). In as little as 1 wk after T treatment, somata volumes became indistinguishable from those of males (Fig. 9). We conclude that, at least by one morphological measure, motoneurons become masculinized relatively quickly in response to T.

DISCUSSION

The goal of this study was to characterize the process of T-induced vocal transformation in adult female *Xenopus* and to determine the relative rates at which laryngeal muscles and the central vocal pathways transform. To this end, we examined in parallel the effects of T at the levels of vocal behavior, laryngeal muscles, and laryngeal motoneurons. Some of the parameters measured in this experiment first *defeminized* (differentiated from that of preoperational females) and then *masculinized* (became indistinguishable from those of adult males), whereas other parameters masculinized without first undergoing defeminization.

T-induced vocal transformation

Our behavioral results show that, although they never masculinized entirely, the vocalizations of adult female *Xenopus* defeminized within 1 wk of T treatment and underwent a drastic transformation during the following weeks. All females began to produce rapid clicks in the form of abnormal release calls or male-like growling within 2–3 wk of T treatment and produced a call resembling male advertisement calls by the 13th wk. Although these vocal transformations have been described elsewhere (Hannigan and Kelley 1986; Watson and Kelley 1992), our results show that these changes take place more rapidly and to a greater extent than previously reported. We found a significant increase in click rates in as little as 1 wk

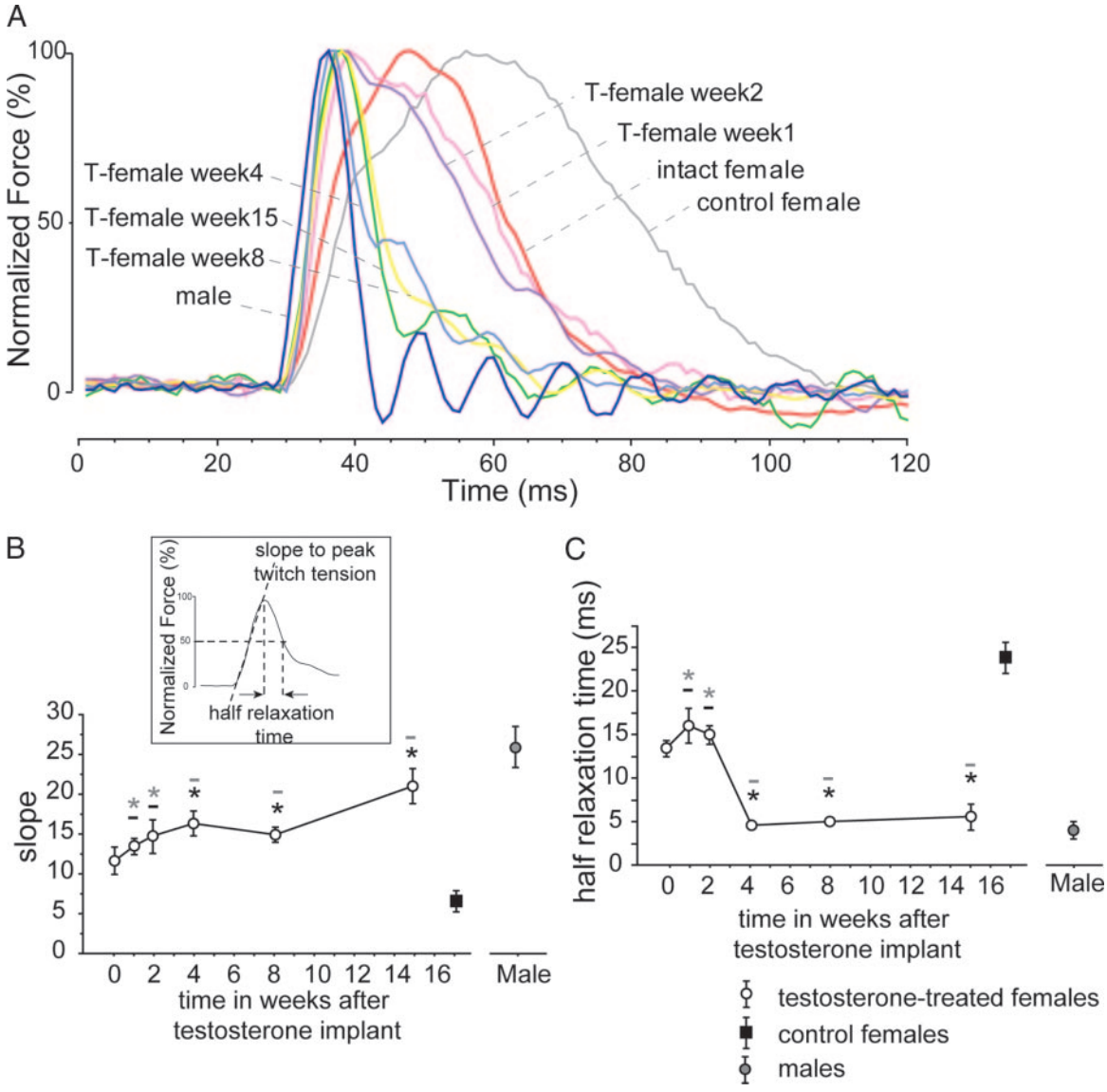

FIG. 7. Single twitch tension profile of laryngeal muscles. *A*: average tension profiles of a single twitch recorded from the laryngeal muscles of males, control females, intact females, and T-treated females. *B*: slope of twitch tension onset to tension peak decreased after T treatment in females. By the 4th week of treatment, the slope became similar to that of males. *Inset*: diagram explaining how slope to peak and half-relaxation time were measured. *C*: half-relaxation time of twitch tension also decreased after T treatment in females. It became significantly shorter than that of control females within 4 wk and became indistinguishable from that of males. Black and gray asterisks and bars indicate statistical significance/nonsignificance as indicated in Fig. 4, except that the comparisons indicated in black were made to intact females not to presurgical females.

after T treatment, 3 wk earlier than previously reported (Hannigan and Kelley 1986), and advertisement call-like vocalizations appeared as early as the fifth week of T treatment, 8 wk earlier than previously reported (Hannigan and Kelley 1986). Furthermore, by the end of our experiment, 100% of the T-treated females had produced advertisement call-like vocalizations (cf. 22% in Hannigan and Kelley 1986; 25% in Watson and Kelley 1992). The discrepancy between the results of this study and those of others cannot be due to the sexual maturity of the animals used here, because they were of an age and body size similar to those in the other studies (68.2 ± 1.8 g in this study; average of 51.6 and 54.7 g in Hannigan and Kelley 1986 and Watson and Kelley 1992, respectively). Rather, the discrepancy is likely to be due to the different sampling methods used in our experiments. In previous studies, T-treated females were recorded for four or fewer sessions, for 1.5 h/session, over 4–18 mo (a total of ~6 h; Hannigan and Kelley 1986; Watson and Kelley 1992). In this study, females were recorded for eight sessions, for an average of 21.2 h/session (a total of ~170 h), over a 13-wk period. We believe that this intensive sampling of behavior enabled us to record even infrequently produced vocalizations and revealed novel information about the dynamics of vocal transformation in female *Xenopus*. Further possible explanations for the discrepancy include the recording conditions used in the studies and the method of T application. Watson and Kelley (1992) may not have induced male-like vocalizations efficiently from their subjects because they used sexually nonreceptive females as stimulus animals during recording sessions. The plasma levels of T in the female subjects examined by Hannigan and Kelley

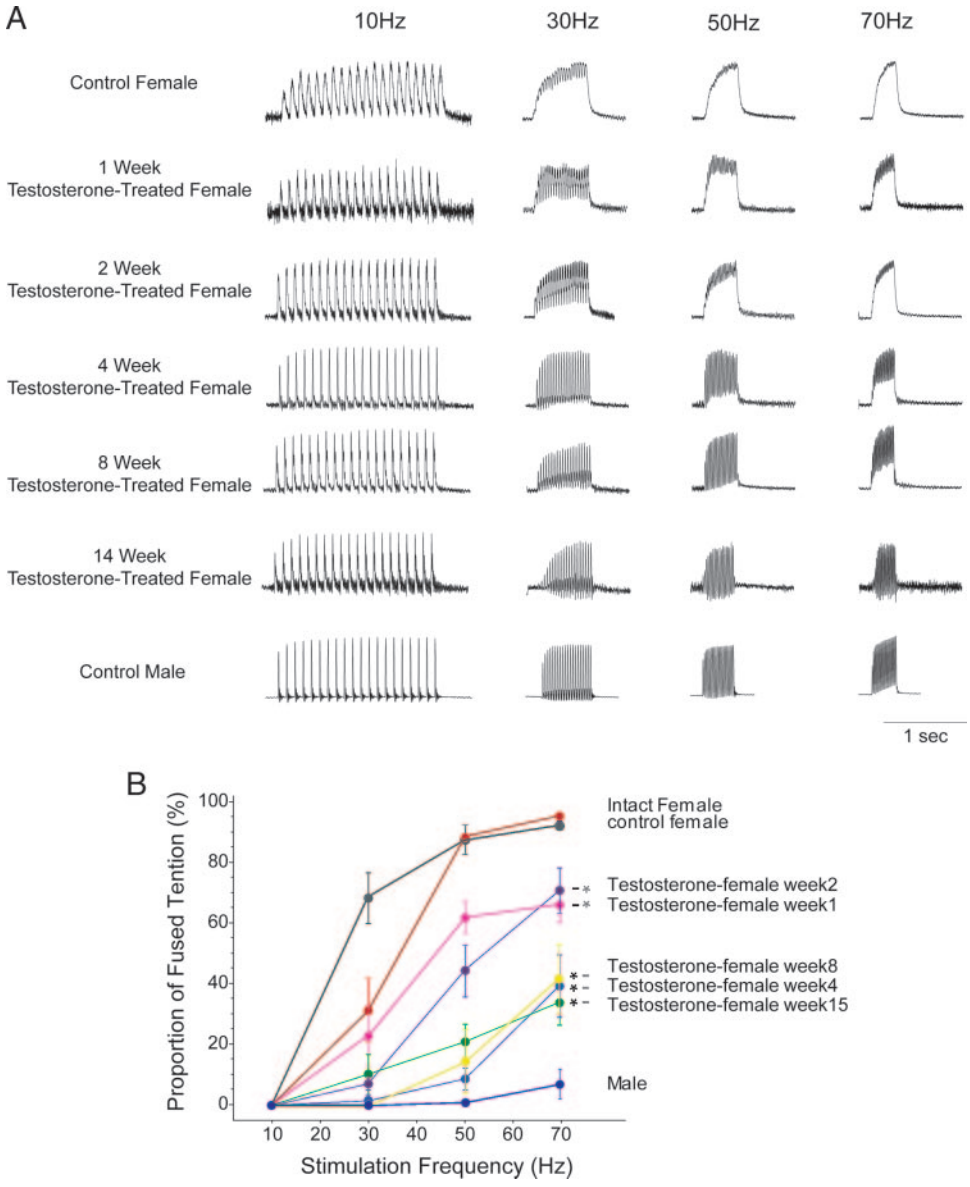

FIG. 8. Fused tension of laryngeal muscles in response to repeated stimulation. *A*: example traces of muscle tension of a control female, T-treated female, and male in response to repeated stimulation at 10, 30, 50, and 70 Hz. *B*: ratio of fused tension relative to total tension as a function of stimulus frequency. By the 4th wk of testosterone treatment, the fused tension ratio became significantly different from that of control or intact females and became similar to that of males. Black and gray asterisks and bars indicate statistical significance as described in Fig. 4.

(1986) may differ significantly from those of the subjects in both this study and the study by Watson and Kelley (1992), because in the experiments of Hannigan and Kelley (1986), T crystals pressed into pellets, not T-filled silastic capsules, were administered.

Although the vocalizations of T-treated females came to resemble those of males, they never masculinized entirely. The maximum sustained click rates, fast and slow trills of advertisement-like calls, and the higher of the most commonly produced click rates (μ2) all were slower than those of males (e.g., Figs. 2C_3, 4*B*, and 5*B*). This incomplete masculinization is unlikely to be due to insufficient plasma levels of T, because silastic implants have been shown to elevate T levels beyond those of sexually active males (Watson and Kelley 1992). Thus at least within the length of our study, exposure to T in adulthood is not entirely sufficient to masculinize the vocal behavior of female *Xenopus*. Differential exposure to androgen early in development (Phoenix et al. 1959) and/or expression of sex chromosome-linked genes (reviewed by Arnold et al. 2003) may account for the remaining differences in vocalization between adult males and T-treated females. Alternatively, pharmacological levels of T in our subjects may have interfered with the masculinization process through as yet unidentified mechanisms.

Transformation of laryngeal muscles

T rapidly masculinized the laryngeal muscles of gonadectomized females. Muscle contractile properties became masculinized within 4 wk of T treatment and changed little thereafter. These results are consistent with an earlier observation that T increases the maximum twitch rate of adult female laryngeal muscles within 2–4 wk (Tobias and Kelley 1987). The shortening of twitch tension duration allows the production of faster clicks by increasing fusion frequency and decreasing the fused tension ratio in response to repeated stimulation (Fig. 8).

The functional masculinization of laryngeal muscles is likely to be the result of the physiological transformation of existing

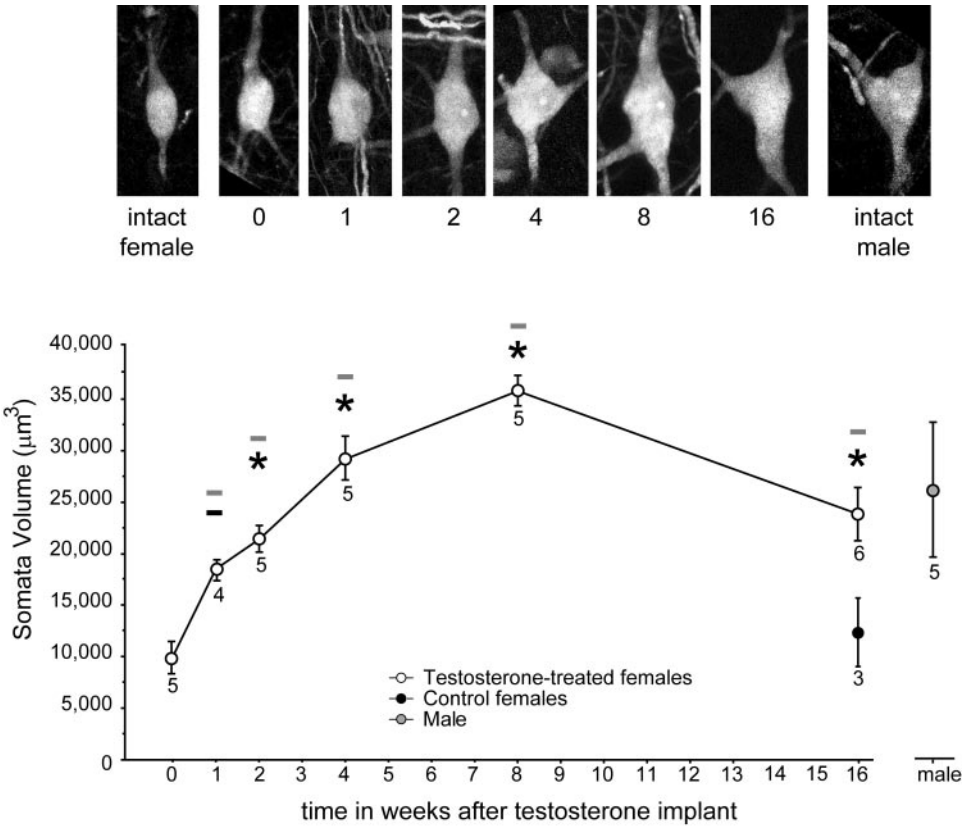

FIG. 9. Size of motoneuron somata. In response to T treatment, the volume of laryngeal motoneurons increased in females. *Top*: example confocal images of laryngeal motoneurons of T-treated females. *Bottom*: average and SE of laryngeal motoneuron soma volume. Numbers below each data point indicate number of animals used. Volume changed significantly after T treatment. Motoneurons of T-treated females became indistinguishable from those of males within 1 wk of hormone treatment. Black and gray asterisks and bars indicate statistical significance as in Fig. 4.

muscle fibers (Sassoon et al. 1987) rather than the result of newly added male-like fibers, because T does not increase myogenesis in adult females (Sassoon et al. 1986), and the number of laryngeal muscle fibers is known to remain constant after T treatment (Watson et al. 1993). At the molecular level, a drastic decrease in half-relaxation time suggests that the time required for cytosolic Ca^{2+} to be resequestered by the sarcoplasmic reticulum becomes much shorter in T-treated laryngeal muscle fibers. T may achieve these results by increasing Ca-ATPase activity and/or by increasing the expression of a sarcoendoplasmic reticulum Ca^{2+}-ATPase isogene, SERCA1, that is specific to fast-twitch fibers (Brandl et al. 1986).

Laryngeal motoneuron soma size

T also induced rapid growth of female laryngeal motoneurons. The size of female motoneuron somata caught up with the size of male motoneuron somata by the first week. These results differ from a previous report that the somata of Golgi-stained neurons in the nucleus IX–X are of similar sizes in males and females (Kelley et al. 1988). The discrepancy may be due to different measuring methods and to the identity of the neurons measured. This study selectively labeled and measured the volume of laryngeal motoneurons using confocal microscopy, whereas the previous study measured the cross-sectional area of the somata of neurons within nucleus IX–X, which likely resulted in the inclusion of both moto- and interneurons (Kelley et al. 1988).

Previously, we have shown that the functional properties of motoneurons are also sexually distinct (Yamaguchi et al. 2003). The differences include passive membrane properties (a smaller input resistance and a larger membrane capacitance in males than in females), firing patterns (in response to step depolarization, most male neurons fire phasically, while female neurons fire tonically), and sex-specific expression of two types of ionic currents (hyperpolarization-activated cationic currents and low-threshold potassium currents are more common in male than in female motoneurons; Yamaguchi et al. 2003). The functional specialization of male laryngeal motoneurons is thought to enable the precise transformation of a rapid series of synaptic inputs received from premotor neurons into a rapid train of spikes, which in turn underlies a rapid series of clicks (Yamaguchi et al. 2003). The rapid enlargement of laryngeal motoneurons in T-treated females shown in this study indicates that passive membrane properties associated with soma size are masculinized within 1 wk of T treatment. In addition, a preliminary study from our laboratory shows that the functional properties (firing properties and the expression of ionic currents) of motoneurons also are masculinized by at least the 13th wk of T treatment (Yamaguchi et al. 2004). While the exact timing of physiological masculinization within 13 wk of T treatment is yet to be examined, laryngeal motoneurons, like laryngeal muscles, become functionally masculinized in response to T. The association between the morphological and physiological masculinization of laryngeal motoneurons and the production of rapid clicks in T-treated females suggests that these male-typical neuronal properties make possible the precise transduction of newly generated rapid synaptic inputs from premotor neurons into a train of action potentials driving masculinized muscle fibers.

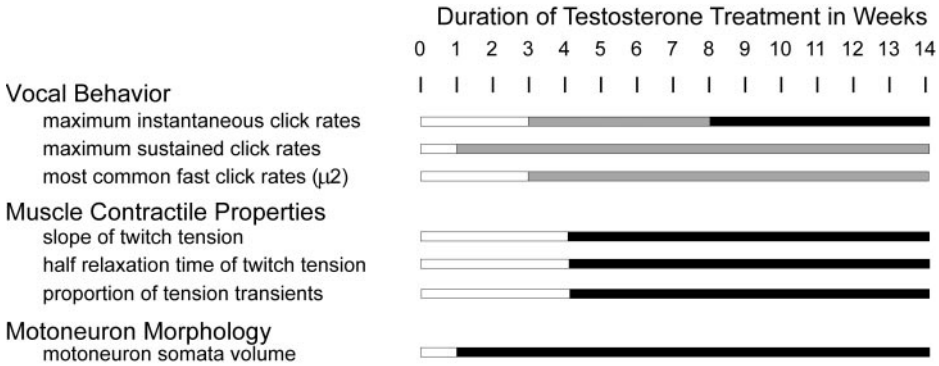

FIG. 10. Timing of defeminization and masculinization in response to T. Transitions from white to gray indicate defeminization and from gray or white to black indicate masculinization.

Functional transformation of the central vocal pathways

Female vocalizations defeminized within 1 wk of T treatment but never completely masculinized (Fig. 10). In contrast, the laryngeal muscles were masculinized by the fourth week of T treatment, which indicates that they would have been capable of producing male-typical calls if adequately driven. Together, the results indicate that, after the fourth week of treatment, peripheral muscles do not limit click production rates in T-treated females. Instead, the limiting factor seems to be the functional transformation of central vocal pathways.

What transformations might take place in the central vocal pathways? Although vocalizations were not completely masculinized, the behavioral results indicate that, in response to T treatment, the central vocal pathways began to produce novel motor rhythms that underlay both rapid bursts of clicks as well as alternating fast and slow rhythmic trills that resembled male advertisement calls. Although T-induced reconfiguration of the underlying neuronal network is likely to be complex, androgen receptor expression in the laryngeal motor nucleus and in the dorsal tegmental area of the medulla (a presumed premotor nucleus) of adult female *Xenopus* (Kelley 1986) makes these nuclei likely candidate loci for major modification. In this study, we identified one such change at the level of motoneuron morphology. The functional transformation of active properties of laryngeal motoneurons also is likely to be important for generating male-typical vocalizations. Because the size of somata often correlates with dendritic arborization (Fiala and Harris 1999), we predict that these masculinized laryngeal motoneurons have more extensive dendritic arbors and form new synapses with premotor neurons. Our next goal will be to identify functional changes in the laryngeal motor nucleus and the dorsal tegmental area of the medulla that underlie the vocal masculinization process.

ACKNOWLEDGMENTS

We thank D. Katz for advice on statistical analyses, J. Burt for allowing us to use Syrinx software, S. Kandarian for helpful discussion on muscle physiology, and M. Wachowiak, V. Dionne, and two anonymous reviewers for comments on earlier versions of the manuscript.

GRANTS

This work was supported by startup money provided by Boston University and by a Clare Boothe Luce Professorship awarded to A. Yamaguchi.

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