Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate, and frequency spectra

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Summary. In *Xenopus laevis*, adult males but not females produce courtship songs comprised of rapid trills. Two experiments were conducted to determine whether male-typical singing could be induced in females. At 6 different juvenile stages, male and female frogs were gonadectomized and implanted with testes, grown to sexual maturity, and tested for vocal behavior. All frogs with functional testicular implants sang; females sang as much as males. The frequency spectra of the clicks within trills were fully masculinized in females implanted at PM0, PM1, and PM2. There were deficiencies in song quality in females implanted late in juvenile life. Females receiving testis implants at PM3, PM4, and PM5 did not produce clicks with masculine spectral qualities. In a concurrent experiment, adult males and females were gonadectomized and implanted with testes or silicone tubes containing testosterone propionate. When tested for vocal behavior 10 to 15 months after implantation, 8/10 androgen-treated males, 3/12 androgen-treated females, 5/5 testes-implanted males, and 2/4 testes-implanted females sang. The females that did sing spent much less time singing than males. The click rates of females were uniformly slower than males and no female produced clicks with a masculine frequency spectrum. Thus, testicular secretions can induce male-typical singing in females until late in juvenile development. However, females exhibit a progressive decline in vocal potential with increasing age, culminating in an almost complete loss of singing ability by adulthood.

Key words: Sexual differentiation - Song - Larynx - Critical period - Organizational-activational

Introduction

In African clawed frogs (*Xenopus laevis*), adult males produce a repeated series of amplitude-modulated, alternating rapid (~60 Hz) and slow (~30 Hz) trills which serves to attract gravid females (Picket 1983). Adult females do not sing but do produce a slow (~6 Hz) trill, ticking, which initiates release from a clasping male (Weintraub et al. 1985; Hannigan and Kelley 1986). The trills of courtship song and ticking are comprised of brief, noisy clicks produced by the vocal organ, the larynx. Clicks are generated by contraction of intrinsic laryngeal muscles and resultant movements of a pair of specialized laryngeal cartilages, the arytenoid disks (Ridewood 1898; Tobias and Kelley 1987; Yager 1992a, b). Courtship song in adult male *Xenopus laevis* is induced by androgens secreted from the testes; adult males do not sing following castration unless exogenous androgen is supplied (Wetzel and Kelley 1983). Adult females do not produce courtship song; adult females treated with exogenous androgen for 4 months occasionally emit song-like vocalizations which lack the fast trill rates and high frequency spectral components characteristic of male song (Hannigan and Kelley 1986).

The role played by the sexually dimorphic larynx in the production of male courtship song has been explored in detail. The fast trills of courtship song require rapid laryngeal muscle contraction and relaxation, which rely on a muscle composed entirely of fast twitch fibers (Sasnoon et al. 1987; Tobias et al. 1991a). Amplitude modulation is correlated with the progressive recruitment of muscle fibers during a trill and the presence of weak facilitating neuromuscular synapses (Tobias and Kelley 1987). Female laryngeal muscle is not capable of the fast twitch rates produced by male muscle and does not exhibit progressive fiber recruitment. The component sound frequencies of individual clicks also differ between males and females (Hannigan and Kelley 1986). Since there are no obvious sex differences in the vocal tract other than within the larynx, and the glottis is closed during vocalization, we believe sex differences in sound
frequencies are largely due to sex differences in larvalgial cartilages. The complexity in shape of the larvalgial cartilage skeleton is greater in males than in females (Sassoon and Kelley 1986); hyaline cartilage is more abundant in males than in females and elastic cartilage is restricted to males (Fischer and Kelley, unpublished observations; see also Yager 1992a). For these reasons, the female larynx is incapable of producing male-typical songs.

The male larynx acquires the ability to produce courtship song during post-metamorphic (PM) juvenile development. Rapid myogenesis during the first 6 months of PM life produces adult male-typical larvalgial muscle fiber number (Marin et al. 1990). These muscle fibers convert to all fast twitch between 6 and 9 months PM, and fiber recruitment is masculinized between one and two years PM (Tobias et al. 1991). The development of the larvalgial directed by androgen and appears permanent (Tobias et al. 1991a). Male-like development of the larynx can be produced in females by administration of exogenous androgen. Larvalgial muscle fiber twitch rate and fiber recruitment can be masculinized in adult females with prolonged (>9 month) androgen treatment (Tobias et al. 1991b).

The finding that larvalgial muscle physiology can be masculinized in females at any age by prolonged androgen exposure raises the possibility that adult females given short-term androgen treatment in a previous experiment (Hannigan and Kelley 1986) did not sing because of incomplete masculinization of the larynx. If so, then prolonged androgen treatment should be sufficient to induce singing in females. Alternatively, there may be a requirement for androgen exposure during juvenile development. Here we address these possibilities by examining the vocal behavior and larvalgial physiology of testis-implanted developing and adult frogs and of gonadectomized adult females exposed to prolonged testosterone treatment.

Methods

General procedures. Juvenile and adult African clawed frogs (Xenopus laevis) were obtained from Nasco (Fort Atkinson, WI). Post-metamorphic development of males extends over a two year period which we have divided into 7 stages based on the attainment of certain milestones of larvalgial masculinization (Tobias et al. 1991a); stage PM0 is the end of metamorphosis and stage PM6 is adulthood. Juvenile frogs (PM0–PM3) were staged by body weight as described in Tobias et al. (1991a); male body weights were within the semi-interquartile range for the stage, females were age-matched (taken from the same batch as staged males). Frogs were kept in 35 × 44 cm polycarbonate tanks (Nalgene) in 5 l of standing 0.4% salt water, treated with Novacare water conditioner (Novacare, Hayward, CA), on a photoperiod of 12L:12D (L = 0500–1700). Frogs were housed under constant temperature (19 °C), and their water was changed twice weekly after feeding. All frogs were individually toe-clipped for identification and housed in same sex groups of 2–8 per tank, depending on size. Stimulus females were un.injected, intact, sexually mature and ~30% larger than each experimental subject at the time of testing.

Testicular implants. Because it is difficult provide physiological levels of exogenous androgen to post-metamorphic frogs over the prolonged developmental period in which androgen normally acts, we used testicular implantation, a procedure known to be effective in masculinizing muscle fiber number, twitch tension and EMG potentialization (Marin et al. 1990; Tobias et al. 1991b). Frogs were anesthetized by immersion in buffered 0.115% MS–222 (2-amino-2-methyl-1-propanol, Sigma), gonadectomized (as described previously; Kelley et al. 1978), and given testicular implants at stages PM0, PM1, PM2, PM3, PM4, PM5 and PM6. Testicular implants were a single testis from a sibling as described previously (Marin et al. 1990) or 2–6 testes from PM2 males. Because implanted testes from older, larger males were not viable when implanted (such a result has also been encountered in rats: Pfeiffer 1936), multiple PM2 testes were used in older frogs. Of the frogs with viable testes at the end of the experiment, 2 PM3 females, 4 PM4 females, 1 PM4 male, 3 PM5 females, 1 PM5 male, 3 PM6 females, 1 PM6 male, and 4 PM6 males had multiple testes. Control females were laparatomized at stage PM2 and then maintained under the same conditions as testis-implanted frogs. Control males were 5 unoperated PM0 males raised under identical conditions over the same period of time. All testis-implanted frogs were maintained for at least 10 months, an interval long enough to allow the PM0 frogs to reach sexual maturity.

Sixty-eight of 101 testis-implanted females and 47 of 62 testis-implanted males survived for 10 months or more. At the time of behavior testing, 36 females and 25 males had nuptial pads, indicating the implanted testes had survived and was producing circulating androgen. At sacrifice, it was found that all frogs with nuptial pads had at least one viable testis and frogs without nuptial pads lacked testicular tissue. One PM4 implanted female and all 12 PM5 implanted males, even though the nang and was found to have testicular tissue when killed. Therefore, the behavioral data presented in Figs. 1–3 and the “sacrifice” data in Table 1 reflect only those frogs with testicular tissue at sacrifice.

Testicular implants. Hormone was administered in silicone tubing to adults. Crystalline testosterone propionate (T; Sigma) was dissolved in a glass beaker heated in a salt water bath. Molten steroid was drawn up into 10–15 cm lengths of Silastic® (Dow Corning, Midland, MI) tubing (2.41 mm O.D. × 1.57 mm I.D.) using a 10 ml syringe. The TP-filled tubes were cut to length and sealed with Silastic 891 type A adhesive. A total TP dose of 0.5 mg/kg body weight was administered to each frog. Blank implants were filled with Silastic adhesive. Frogs were gonadectomized under anesthesia and a TP-filled or blank tube was placed into the dorsal lymph sac.

Tube-implanted frogs were maintained for 35 months. The darkening of forearm nuptial pads, which can be induced by androgen in both sexes, was used to assess androgen release. Androgen implants were replaced if a frog was found to have pale nuptial pads during cage cleaning. There was significant mortality in the gonadectomized male and sham-operated female groups between 22 and 33 months after implantation.

Behavior testing. The recording tanks for all behavior tests were 10 inch glass aquariums, lined with open cell foam and polyethylene garbage bags, filled with 35 l of standing tap water, and fitted with Plexiglas covers. Hydrophones (model HSX, Wilcoxen research, Rockville, MD) were suspended from the Plexiglas cover at a height of 7 cm from the floor of the tank and vocalizations were recorded on 3M 807 magnetic tape using Akai GX-255 tape recorders. A randomly chosen experimental subject was placed alone in the recording tank between 1000 and 1100 h. Six hours later, a stimulus female was introduced to the tank, the recording room was darkened, and the 90 min recording session was begun (see Wetzel and Kelley 1983 for details). Tube-implanted adults were tested at 18–22 months and 34–35 months after Silastic implantation. All testis-implanted frogs were recorded 10–15 months after implantation, whether they had darkened nuptial pads or not. Two animals of the same age, one male and one female implanted during the same week, were tested in each session. Each testis-implanted frog was injected between 1000 and 1100 h with 2.5 IU/kg BW human chorionic gonadotropin (HCG,
Table 1. Average body weights, larynx weights, and serum androgen for implanted frogs. All values are means ± S.E.Ms

<table>
<thead>
<tr>
<th>Group</th>
<th>Males implanted at sacrifice</th>
<th>Females implanted at sacrifice</th>
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<tbody>
<tr>
<td></td>
<td>N body weight (range-g)</td>
<td>N body weight (g)</td>
</tr>
<tr>
<td>PM0</td>
<td>5 1.2 ± 0.1</td>
<td>12 7.6 ± 0.1</td>
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<tr>
<td>PM1</td>
<td>6 4.3 ± 0.3</td>
<td>2 23.7 ± 2.9</td>
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<tr>
<td>PM2</td>
<td>8 6.5 ± 0.2</td>
<td>4 28.8 ± 6.4</td>
</tr>
<tr>
<td>PM3</td>
<td>6 8.6 ± 0.3</td>
<td>6 17.9 ± 3.1</td>
</tr>
<tr>
<td>PM4</td>
<td>12 10.4 ± 0.2</td>
<td>6 36.1 ± 4</td>
</tr>
<tr>
<td>PM5</td>
<td>10 11.6 ± 1.1</td>
<td>3 27.3 ± 1.3</td>
</tr>
<tr>
<td>PM6</td>
<td>15 42.9 ± 3.9</td>
<td>5 30.8</td>
</tr>
<tr>
<td></td>
<td>11 1.2 ± 0.1</td>
<td>15 19.2 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>9 4.3 ± 0.3</td>
<td>4 12.7 ± 2</td>
</tr>
<tr>
<td></td>
<td>12 2.4 ± 0.4</td>
<td>9 25 ± 7.4</td>
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<tr>
<td></td>
<td>8 8.8 ± 0.2</td>
<td>6 28 ± 3</td>
</tr>
<tr>
<td></td>
<td>13.6 ± 0.5</td>
<td>6 28 ± 3</td>
</tr>
<tr>
<td></td>
<td>15 22.1 ± 3</td>
<td>5 33.4 ± 3</td>
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<tr>
<td></td>
<td>17 57.4 ± 3.5</td>
<td>4 69.9 ± 10.2</td>
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<tr>
<td>B. Silastic pellet-implanted:</td>
<td></td>
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<td></td>
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<tr>
<td>Shm</td>
<td>9 48.3 ± 1.7</td>
<td>8 61.7 ± 2.9</td>
</tr>
<tr>
<td>Gnx</td>
<td>10 48.0 ± 4.1</td>
<td>5 48.6 ± 2</td>
</tr>
<tr>
<td>TP</td>
<td>11 43.3 ± 3.2</td>
<td>10 45.7 ± 1.9</td>
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<tr>
<td></td>
<td>12 61.4 ± 2.5</td>
<td>11 54.9 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>16 64.8 ± 3.7</td>
<td>12 61.4 ± 2.5</td>
</tr>
</tbody>
</table>

Sigma) and placed alone in a recording tank. Because in a previous experiment, testosterone treatment combined with human chorionic gonadotropin (HCG) injection resulted in abnormally low numbers of larval growth in females (Hannigan and Kelley 1986), tube-implanted adults were not injected with HCG in this study. Tube-implanted frogs were treated identically otherwise. Six hours after the experiment, the sample was introduced to each recording tank and the recording session was begun.

Data analysis. Tape recordings of behavior tests were monitored and the types and durations of all calls were noted. The male song consists of repetitions of alternating fast and slow trills of clicks, with a period of approximately one second. The number of frogs singing was compared between the sexes within an implant group using the Fisher exact probability test (Siegel 1956). Data on time spent calling, interclick intervals, and frequency maxima are described as means and compared using Kruskal-Wallis one-way analysis of variance followed by Mann-Whitney U tests (Siegel 1956), where appropriate. Probability for all tests was one-tailed.

The temporal and spectral characteristics of songs were analyzed. A single song was defined as one fast trill followed by one slow trill. Three 4-s song samples from each frog were analyzed using the MacSpeech Lab II speech analysis software (GW Instruments, Somerville, MA) on an Apple Macintosh II computer. The click rate was measured by determining the time between successive clicks, the interclick interval (ICI). The mean ICI for each fast or slow trill was calculated by measuring the duration of 8–21 trills. A fast Fourier transform (FFT) was prepared for 4 individual clicks per recording (12 clicks per frog). Clicks from the slow trill were chosen for FFT analysis, because in many recordings the fast trill clicks overlapped with echoes from preceding clicks. There were no temporal or spectral differences in clicks from the fast and slow portions of the trill in tapes where both were analyzed. The characteristic frequency of each peak from the FFT with an amplitude ≥ 25% of the largest peak was recorded. Just the sound energy between 0.5 and 4 kHz was analyzed, because previous experiments had shown that the resonant frequency of the tanks used was 4.2 kHz (Wetzel and Kelley 1983).

Sacrifice and post-mortem analysis. Frogs were killed by anesthetic overdose. A blood sample was collected, and the larynx was removed, weighed, and tested for muscle tension and fiber recruitment as described in Tobias and Kelley 1987 (see below). Gonadal tissue from every frog was removed and weighed. The body weights at the start of the experiment and body weights and larynx weights at sacrifice of the frogs used in this study are given in Table 1. There was significant mortality in the testis-implanted adult groups (unrelated to implantation) between behavior testing and sacrifice, so the larynx and RIA data for these treatments are from only one male and two females. Frogs implanted at or before PM3 had body weights significantly less than those implanted after PM3 (overall mean ± S.E.M weights: PM0-PM3 implanted = 21.6 ± 9.6, PM4-PM5 implanted = 31.6 ± 10.6, P < 0.005). The slower growth rate of the earlier stages was probably due to the conditions in this laboratory rather than to the surgical procedures; body weights of the control males were not significantly different from the PM0 testis implanted frogs (PM0 implanted = 15.7 ± 4.6, PM0-males = 19.2 ± 4.6, P > 0.1).

Laryngeal physiology. Because both singing and ticking can be produced in vitro by the larynx in response to appropriate nerve stimulation, it is possible to isolate the contribution to call production made by the vocal organ (Tobias and Kelley 1987). Following sacrifice, the larynx was isolated from each adult and each frog with a parotid gland and/or a visible testis implant. The laryngeal nerve was stimulated (250 ms, 71 Hz) while recording electrical activity and tension produced by the laryngeal muscle (Tobias and Kelley 1987; Tobias et al. 1991a). The production of discrete tension transients with complete relaxation to baseline tension during the interstimulus interval represents 100% transient tension (e.g., Fig. 6A). Electromyogram potentiation was summarized as "potentiation index", a score obtained by dividing the height of the largest EMG spike in the train by the height of the first.

Androgen assay. In intact adult males, blood levels of androsterone decline to baseline levels within 12 h after HCG injection (Lambdin and Kelley 1986), so sacrifice followed HCG injection by at least two days. At sacrifice, the ventricle was slit and 0.5-2 ml of blood were collected into 1-2 centrifuge tubes. Samples were spun at 2500 rpm for 5 min. The serum fraction was collected and stored at −20 °C until assayed. A single antibody radioimmunoassay for 1-testosterone (Amersham TRK 600; inter- and intra-assay C.V. < 5%, sensitivity of 5 pg/tube) was used to measure total serum androgen. The antibody has ~50% cross-reactivity with dihydrotestosterone and minimal cross-reactivity with progestins, estrogens, or other androgens.

The effects of the various implants on circulating androsterone are summarized in Table 1. The serum androgens in the TP-implanted frogs was approximately 10 times higher than the level seen in the sham-operated males. Castration reduced androsterone levels by an
order of magnitude to levels in the intact female range. Among the
testis-implanted frogs, there were no sex differences in circulating
androgen; the mean serum androgen concentrations were not signifi-
cantly different from the sham-operated adult males in the TP
implant experiment. There was no significant correlation between
the number of testes implanted and androgen level so the behavioral
data were analyzed without regard for testis implant number.

Results

Testis-implanted juvenile frogs. All juvenile females with
viable testicular implants sang when they reached adulthood.
Of the frogs with viable testis implants the only individual that did not sing was a male, implanted at
PM3. In contrast, all 25 juvenile-implanted frogs that
lacked testicular tissue at sacrifice and all five control
females were silent in behavior tests (data not shown).
The songs produced by all testis-implanted females were
alternating fast and slow trains of clicks, with amplitude
modulation apparent in the fast portion, and often the
slow portion as well (examples are shown in Figs. 1 and 2). Testis-implanted juvenile females sang as much as
their identically-treated male siblings and as much as
intact, adult males (Fig. 3A). There was no significant
effect of implantation age on total duration of singing
(df = 9, H = 13.4, P > .03). Furthermore, all females
receiving testicular implants produced clicks at rates equiva-
tent to males: there were no significant sex differences in
interclick interval in the fast trill portion of the songs
(Fig. 3B).

The frequency spectra of songs by adult males are
highly stereotyped (Wetzel and Kelley 1983). In the
present study, adult males produced clicks with the
greatest energy in the 2 kHz range (1999 ± 53 Hz, N = 6),
with 4 lower amplitude frequency maxima at
1259 ± 36 Hz, 2542 ± 90 Hz, 2946 ± 65 Hz and
3398 ± 54 Hz (Figs. 1A and 2A). The range of variability
(S.E.M.) for clicks within individuals was 33–199 Hz
around the 2000 Hz peak. The low within and between
individual variability in the frequency maxima of clicks
allowed us to categorize the frequency spectra of implanted
clicks as either masculinized or non-masculinized. The
frequency spectra of clicks were fully masculinized in
females implanted at PM0, PM1, and PM2 (Fig. 1B, C,
D). Most females implanted at or after PM3 produced
aberrant clicks. Although their clicks contained some
maxima at the same frequencies as males, either the frequency with the greatest amplitude was outside the 17.5-22 kHz range, or their clicks contained two maxima between 1.75 and 2.25 or both (Fig. 2B, C, D). The proportion producing masculinized clicks was significantly less than males in the PM3, PM4, and PM5 groups (Fig. 3C).

Androgen- and testis-implanted adult frogs. While androgen implantation activated singing in castrated adult males, there was no significant activation of singing in implanted adult females regardless of the source of the androgen. The results of vocal behavior tests on all adult frogs are summarized in Fig. 4. Only the data for the 1.5 year tests of the TP-implanted adults are shown, as this implant duration was similar to the testicular implant duration, and the mortality in the gonadectomized male and sham operated female groups occurred after the 1.5 year tests. (The singing behavior of the TP-implanted adults after three years was similar: no addi-
A

Sham Male

C

TP Female

B

TP Male

D

Testis-Implanted Female

E

Fig. 5A–D. Adult female frogs that did sing produced songs that were not fully masculinized. All females produced the male-typical temporal pattern; oscillograms of sample songs are shown in the top traces. Fast Fourier transforms (bottom traces) show the degraded spectral qualities of song produced by females. Note the multiple large peaks, and/or lack of a peak at 2 kHz. Sample data are from (A) a normal adult male, (B) an adult male implanted with TP, (C) an adult female implanted with TP, and (D) an adult female implanted with a testis.

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tional females sang, and the number of males singing was not significantly different). As expected, castration silenced singing in males; TP implantation restored singing. Androgen implantation had no significant masculinizing effect on vocal behavior of females; 9 of 12 were completely silent \( (P > 0.05 \text{ vs. sham females}) \), and the 3 that called spent significantly less time singing than TP-implanted males. Testicular implantation was slightly more effective in inducing adult females to sing than was androgen implantation: two of 4 testes-implanted females sang \( (P > 0.05 \text{ vs. TP or sham females}) \), one for 229 s and one for 38 s \( (P = 0.1 \text{ vs. TP females}) \). In contrast, both types of implants induced singing in castrated males. Eight of 10 TP-implanted males sang, and their average duration was not different from that of intact males \( (P > 0.5) \). Testicular implantation restored singing in all 5 adult male frogs and their total duration singing per test was not significantly different from HCG-injected, unoperated adult males \( (P > 0.1; N = 6, \text{ data not shown}) \). Only testis-implanted frogs received HCG injections, and their longer singing than the un.injected, tube-implanted males \( (P = 0.015 \text{ vs. sham males}, P > 0.047 \text{ vs. TP males}) \) is consistent with previous studies in which HCG increased the total singing duration (Wetzel and Kelley 1983). To facilitate comparisons, the behavioral effects of testicular implants in adults are included in Fig. 4.

All songs by adult females, whether androgen-implanted or testes-implanted, had the alternating fast and slow pattern of clicks characteristic of the male song (Fig. 5C, D), but were not masculine in other respects. Androgen- and testis-implanted adult females that sang produced songs that were similar. The intercall interval in the fast portion was 18.5 ± 0.05 ms (for all adult females combined), and was significantly slower than comparably treated males (Fig. 4C). While TP-implanted males produced normal clicks (Fig. 5B), the 5 adult females that sang produced clicks that contained only 3 frequency maxima, at 1429 ± 64 Hz, 2285 ± 51 Hz, and 3027 ± 100 Hz (see Fig. 5C and D).

In vitro stimulation indicated that both androgen and testicular implantation resulted in fully masculinized laryngeal muscles. Laryngeal muscles from all juvenile testis-implanted frogs, all androgen-treated adults, and the two testis-implanted adults tested produced masculine 100% transient tension in response to 71 Hz stimu-
lus trains. Furthermore, their EMG potentiation was not significantly different from either TP males or sham males (P > 0.4 compared to both groups of males). Examples of tension traces and EMGs from androgen- and testis-implanted adult females are shown in Fig. 6 (data from frogs implanted as juveniles not shown). Thus, when driven by electrical stimulation of the laryngeal nerves, larynges from androgen-treated adult females were capable of contracting as fast as those of adult males, and showed masculinized fiber recruitment, so the inability of androgen-treated adult females to sing was not due to laryngeal muscle constraints on vocal production.

Male-like tension and fiber recruitment properties of laryngeal muscle were maintained in castrated males for almost 3 years (also shown in Fig. 6). While some decrement was apparent, especially in percent transient tension, both measures were still significantly greater than intact females.

Discussion

These results represent the first time singing has been masculinized in female Xenopus laevis. All females maintaining juvenile-implanted testicular tissue into adulthood sang, they sang as much as males, and the overall temporal patterns of their songs were masculine. All the features of songs that we measured were masculinized in females implanted at or before PM2, and the deficiencies in the quality of songs produced by females implanted later in juvenile development (PM3–PM5) were relatively slight.

In contrast, androgen implantation failed to induce singing in a significant number of adult females. Three of 12 females sang, each for less than 30 s. The longer duration androgen treatment employed in the current experiment was no more effective in inducing singing than short-term androgen exposure in a previous experiment (Hannigan and Kelley 1986). The only difference between the songs of females in the present experiment and those of females implanted with androgen for only 4 months was that the overall temporal pattern of songs and interclick intervals were more masculinized.

While the number of adult females that maintained viable testes was small, nonetheless the available data suggest that adult testis implantation is less effective than juvenile implantation in promoting song production. All females that maintained testicular implants from juvenile stages sang, while only two of four females testis-implanted as adults sang. Thus, while prolonged androgen implantation and testicular implantation were more effective than shorter androgen treatment, neither adult treatment was as effective as juvenile testicular implantation. We conclude that singing in X. laevis requires hormonal influences on the vocal system during juvenile development.

Providing females with testicular hormones for only part of post-metamorphic life has allowed us to dissect components of the masculinization process. Prior to PM2, all aspects of male song can be masculinized in testis-implanted females. After PM2, the potential for masculinization diminishes. The majority of females implanted at PM3, PM4, and PM5 produced clicks with unmuscularized frequency spectra. The distortion of song seen in the majority of females implanted later in juvenile development was also evident in those females, implanted in adulthood, that produced male-like calls. Click frequency spectra were not masculinized, and in addition, click rates were consistently slower than males.

The physiological and cellular bases for some song features are known, so the specific contributions of laryngeal motor neurons, muscles, and cartilage can be related to the ability to produce specific song components. Songs of all androgen- or testis-implanted females were amplitude-modulated and fiber recruitment was masculinized. In males, the amplitude modulation of the songs is thought to be due to increasing muscle fiber recruitment over the course of a trill and requires a masculinized neuromuscular synapse (Tobias and Kelley 1988). In the present study, male-like fiber recruitment was induced by testicular implants, even in adult females, suggesting that the neuromuscular synapse retains sensitivity to testicular secretions (androgens) into adulthood. However, the duration of exposure to the testicular implant was long (10 to 15 months); sex differences in sensitivity might be revealed at a shorter, post-transplant interval.

Production of rapid trills requires a laryngeal muscle capable of producing 100% transient tension at 70 Hz and composed of entirely fast twitch muscle fibers (Tobias and Kelley 1987; Tobias et al. 1991b). All androgen and testis-implanted females' laryngeal muscles achieved 100% transient tension at 71 Hz. Thus, the slow click rates of the few adult females that did sing and the silence of the remainder cannot be due to insufficient masculinization of the twitch characteristics of laryngeal muscle. The most likely explanation is insufficient masculinization of central nervous system components that drive song production. The central vocal circuit consists of laryngeal motor neurons, reticular interneurons, and a tegmental pattern generator (Schmidt 1976) which receives sensory and preoptic input (Wetzal et al. 1985). All nuclei in the central vocal circuit contain androgen-sensitive neurons; many of these neurons differ in number and morphology in adult males and females (reviewed in Kelley 1988). Masculinization of the central vocal circuit may exhibit a developmental critical period whose sensitivity diminishes at PM6.

Finally, the frequency spectrum of clicks was not masculinized in all adult females nor in most females implanted late in juvenile development. The acoustic characteristics of the individual clicks probably depend on the complexity of the laryngeal cartilage morphology (Yager 1992a; Hannigan and Kelley 1986; Sassoon and Kelley 1996). The features of songs produced by females in the present experiment suggest that masculinization of the cartilaginous structure of the larynx has an early critical period.

The results of the present study bear on an influential theory in behavioral endocrinology, originally formulated to explain the developmentally-specific effects of gonadal secretions on mammalian reproductive behav-
ior (Phoenix et al. 1959). This hypothesis postulates that gonadal steroids act early in development to irreversibly organize the capacity to exhibit sex-specific behaviors in adulthood. Some features of the masculinization of song in Xenopus laevis resemble an organizational process. Masculinization X. laevis occurs most readily during a sensitive period in development. Testicular hormones are largely ineffective at inducing singing in adult females. Once the larynx is masculinized, further androgen exposure is not necessary to maintain its masculine properties. For example, castration of adult males for 3 years did not feminize laryngeal tension properties or fiber recruitment essential for singing. In comparison to birds and mammals, however, the timing of the sensitive period for masculinization in Xenopus laevis is extended. Females given testicular implants as late as PMS, the late juvenile/preadult stage, sang as much as males, and their click rates were as fast as males. Late organizational effects of hormones have been reported in the pig in which the critical period for estradiol desensitization to receptor extends to the prepubertal period (Adkins-Regan et al. 1989). The only other reported organizational effect of hormones on vocal behavior is the finding that early estrogen treatment masculinizes zebra finch song control regions (Gurney 1981; Gurney and Konishi 1980) and, to some extent, singing (Adkins-Regan and Asenzi 1987; Gurney 1982). The endogenous hormonal basis for sexual differentiation of song in intact zebra finches remains elusive, however, since it is unclear how or even whether the gonads are involved (Adkins-Regan and Asenzi 1990; Arnold and Schlinger 1991). In contrast, sexual differentiation of courtship song in X. laevis seems to follow the mammalian model: the female is the neutral sex, early gonadectomy results in a feminine larynx and lack of singing, and testicular hormones fully masculinize the larynx and behavior (Marin et al. 1990; Tobias et al. 1991b; and this report).

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References


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