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Zornik E, Yamaguchi A. Vocal pathway degradation in gonadectomized *Xenopus laevis* adults. *J Neurophysiol* 105: 601–614, 2011. First published December 8, 2010; doi:10.1152/jn.00883.2010. Reproductive behaviors of many vertebrate species are activated in adult males by elevated androgen levels and abolished by castration. Neural and muscular components controlling these behaviors contain numerous hormone-sensitive sites including motor initiation centers (such as the basal ganglia), central pattern generators (CPGs), and muscles; therefore it is difficult to confirm the role of each hormone-activated target using behavioral assays alone. Our goal was to address this issue by determining the site of androgen-induced vocal activation using male *Xenopus laevis*, a species in which androgen dependence of vocal activation has been previously determined. We compared in vivo calling patterns and functionality of two in vitro preparations—the isolated larynx and an isolated brain from which fictive courtship vocalizations can be evoked—in castrated and control males. The isolated larynx allowed us to test whether castrated males were capable of transducing male-typical nerve signals into vocalizations and the fictively vocalizing brain preparation allowed us to directly examine vocal CPG function separate from the issue of vocal initiation. The results indicate that all three components—vocal initiation, CPG, and larynx—require intact gonads. Vocal production decreased dramatically in castrates and laryngeal contractile properties of castrated males were demasculinized, whereas no changes were observed in control animals. In addition, fictive calls of castrates were degraded compared with those of controls. To our knowledge, this finding represents the first demonstration of gonad-dependent maintenance of a CPG for courtship behavior in adulthood. Because previous studies showed that androgen-replacement can prevent castration-induced vocal impairments, we conclude that degradation of vocal initiation centers, larynx, and CPG function are most likely due to steroid hormone deprivation.

INTRODUCTION

In their investigation of mammalian reproductive behaviors, Phoenix et al. (1959) first proposed that gonadal hormones play two roles—organizational and activational—in controlling reproductive behaviors. During development, hormones "organize" behavioral pathways, inducing long-lasting changes. In adulthood, hormones "activate" these networks via transient neuronal and muscular changes that permit behavioral expression. With some exceptions and caveats, the organizational–activational hypothesis is supported in many mammalian and nonmammalian species (see reviews by Arnold 2009; Arnold and Breedlove 1985; Schulz et al. 2009; Zornik and Yamaguchi 2008). A complicating factor, however, is that steroid hormones influence many neural and muscular structures (Arnold et al. 1976; Jordan et al. 1997; Kelley 1980; Lieberburg and Nottebohm 1979; Pfaff 1968; Segil et al. 1987). Therefore castration may eliminate behavior by preventing hormone-dependent activation of motor initiation centers, by demasculinizing muscles that transduce nerve signals into behavior, and/or by disabling central pattern generators (CPGs). Behavioral assays merely reveal the absence of behavior, but cannot distinguish among these scenarios. Thus understanding the mechanisms of hormonal activation requires assays at multiple levels.

Courtship vocalizations of African clawed frogs (*Xenopus laevis*) are an excellent model for studying hormonal organization and activation because of the wealth of available knowledge. Androgens and their aromatized products, estrogens, target various sites within the vocal pathway. Midbrain auditory and hindbrain CPG nuclei concentrate androgens (Kelley 1980), as do laryngeal muscles (Segil et al. 1987); forebrain nuclei—central amygdala and preoptic area—concentrate estradiol but not androgens (Kelley 1981; Morrell et al. 1975). Androgens organize some features of male vocal pathways. For instance, sexually dimorphic motoneuron and muscle fiber numbers are permanently organized in juveniles by androgen (Kay et al. 1999; Watson et al. 1993). Androgens also activate male vocal behaviors. Castration eliminates male calling and androgen treatment rescues the vocal loss (Watson and Kelley 1992; Wetzel and Kelley 1983), indicating that androgens, not other testicular secretions, are required for vocal activation. Thus calling in adult males is androgen dependent, as in other vocal systems (Floody et al. 1979; Harding and McGinnis 2004; Harding et al. 1988; Zimmerman 1996). Still unclear, however, is what vocal pathway components are activated by androgens.

Vocal behaviors are unchanged in androgen-treated castrated males (Watson and Kelley 1992), indicating that castration-induced effects on vocal pathways are most likely androgen dependent. We combined in vivo and in vitro experiments in adult males to determine which vocal pathway components are affected by castration. We used the in vitro larynx preparation (Tobias et al. 1987) to assess whether long-term castration demasculinizes laryngeal function. We also used an in vitro brain preparation that produces fictive vocalizations (Rhodes et al. 2007). Fictive calling can be elicited after removing the midbrain and forebrain (Yu and Yamaguchi 2010), which contain sensory and motor initiation centers—torus semicircularis, central amygdala, preoptic area—involved in the *Xenopus* vocal pathway (Kelley 1980; Morrell et al. 1975; Wetzel et al. 1985). Thus we can directly activate the vocal CPG, bypassing sensory and vocal initiation centers. This allowed us to test, for the first time, whether the gonads are required for the maintenance of CPG function in adulthood.

METHODS

Animals and experimental overview

We purchased 41 adult *Xenopus laevis* (39 males, 2 adult females) from Nasco (Fort Atkinson, WI). Animal care protocols conformed to National Institutes of Health guidelines. To assess the effects of

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castration on male vocal capacity, we first recorded vocalizations of each animal. Animals were then randomly selected to undergo castration or sham surgery. Animals were then retested for vocal production (and in vitro testing) between 5 and 18 mo after surgery so that any time-dependent changes could be identified.

Gonadectomy and control surgeries

Twenty-six males were castrated and 13 males served as sham-operated controls. For gonadectomies, we performed caudal laparotomies (~1 cm along the anterior–posterior axis, ~0.5 cm lateral of the midline), extracted the abdominal fat bodies and testes with forceps, and removed both using a cauterizer (Aaron Medical Industries, St. Petersburg, FL). For sham-operated controls, only the fat bodies were removed. To close the incision, individual stitches were made separately in the muscle and skin using absorbable sutures (6–0 diameter, P-1 needle; Ethicon, Somerville, NJ). Each animal was inspected at the end of the experiment to ensure that gonadectomies were complete and no signs of testicular recrudescence were present. Using skin patterns to identify individuals, 30 of 39 animals (20 castrates; 10 controls; randomly selected) were used to determine whether morphological characteristics of experimental and control animals changed systematically before and after surgery.

Vocal recordings

Vocalizations were monitored with a hydrophone (H2; Aquarium Audio Products, Anacortes, WA) controlled by a sound-activated recording system (Syrinx software, www.syrinxpc.com; John Burt). Prior to the experiments, adult males were injected with 300 IU of human chorionic gonadotropin (hCG; Sigma, St. Louis, MO), which has been shown to elevate calling both intact and androgen-treated castrates (Wetzel and Kelley 1983; Yang et al. 2007). Animals were then recorded overnight to confirm their ability to produce vocalizations and to establish a baseline level of calling. They were then randomly assigned to undergo gonadectomy (castrates, $n = 26$) or sham operation (controls, $n = 13$). A previous study found that most calling is abolished in males 1 mo following castration (Wetzel and Kelley 1983). To address the long-term effects of castration on the vocal CPG, we began monitoring vocal behaviors ≥4 mo postsurgery. All animals were then recorded at least one night following injection with 300 IU hCG. Thirty of 39 animals (20 castrates, 10 controls; randomly chosen) were selected for additional vocal characterization (immediately before in vitro analyses). For these animals, vocal recordings were performed for three consecutive nights in the following manner: day 1: animals were recorded alone; day 2: animals were injected with 300 IU hCG and recorded alone; day 3: animals were given a 100 IU hCG booster and paired with an unreceptive female (known to be highly effective at eliciting male calling; Wetzel and Kelley 1983). Injections were given immediately before recordings that were initiated between 16:00 and 19:00. Vocalizations were recorded for ≥8 h each night. This intensive sampling strategy was used to capture residual (albeit sparse) vocal output by castrated animals.

Isolated brain preparation and fictive vocal recordings

Fictive vocal recordings in isolated brains were performed as previously described (Rhodes et al. 2007; Yamaguchi et al. 2008; Zornik et al. 2010). Briefly, animals were deeply anesthetized (subcutaneous injection of 0.5 ml 1.3% MS-222; Sigma, St. Louis, MO), placed on ice, and decapitated. Skulls were placed in ice-cold oxygenated saline (in mM: 96 NaCl, 20 $NaHCO_3$, 2 $CaCl_2$, 2 KCl, 0.5 $MgCl_2$, 10 HEPES, and 11 glucose, pH 7.8; 99% O_2-1% CO_2), brains were surgically removed, kept in a holding chamber (200 ml saline) for about 1 h, then moved to a recording chamber (20 ml saline) with constantly superfused saline (1–2 ml/min). To elicit fictive vocalizations, superfusion was suspended and 5-hydroxytryptamine (5-HT) was applied (1 ml, 0.6 mM; final concentration 30 μM). Nerve activity was monitored using a suction electrode attached to the most caudal root of cranial nerve (N.) IX–X that contains laryngeal motoneuron axons (Simpson et al. 1986). Signals were amplified (×1,000) and band-pass filtered (1 to 1,000 Hz) with a differential amplifier (model 1700; A-M Systems, Carlsborg, WA), digitized at 10 kHz (Digidata 1322A; Molecular Devices, Sunnyvale, CA), and recorded with Clampex software (Molecular Devices). In some cases, local field potential (LFP) recordings were made in a premotor CPG nucleus, dorsal tegmental area of medulla (DTAM). In these experiments, the pia dorsal to DTAM was removed and a 1 MΩ tungsten electrode (FHC, Bowdoin, ME) was placed in DTAM using previously determined coordinates (Zornik and Kelley 2008; Zornik et al. 2010). Signals were amplified (×1,000) and filtered (0.1 to 1,000 Hz) with a differential amplifier (model 1800; A-M Systems) and acquired as described earlier. Fictive vocalizations were recorded for 3 min following 5-HT application; superfusion was then reinstated at a fast rate (~10 ml/min) and fictive calling was recorded for another 3 min during washout. The responsiveness of the brains to 5-HT does not appear to decrease over time for the initial five or more trials, but rather varies randomly from one trial to another (Zornik and Yamaguchi, personal observations). In these experiments, 5-HT was applied at least three times (with 1-h intervals between each application) to each preparation, and only data from the most active trial were used for further analyses.

Contractile characteristics of laryngeal muscles

Laryngeal muscle tension rise and relaxation times are significantly faster in males than in females (Potter et al. 2005; Tobias and Kelley 1987). As a result, incomplete relaxation leads to decreased transient tension, or tetany, at rates >20 Hz in females; in contrast, male larynges do not exhibit tetany at stimulation rates ≤70 Hz (i.e., transient tension remains high). To test whether larynges were demasculinized by gonadectomy, we compared laryngeal muscle contraction in response to single pulses and trains of pulses as previously described (Potter et al. 2005). We isolated larynges from castrated and control males after brain isolation (Tobias and Kelley 1987). We pinned each larynx to a recording dish, exposed the muscles and nerves, and recorded tension transients following nerve stimulation. The distal portion of the laryngeal nerve (near the nerve insertion point) was drawn into a suction electrode connected to a stimulator (Master 8; AMPI, Jerusalem, Israel) and stimulus isolation unit (Iso-Flex; AMPI). Stimulus trains (20 pulses) at 10–100 Hz (10-Hz intervals), generated by the stimulator, were repeated three times at each rate. Tension transients were detected with a force transducer (model 1030; UFI, Morro Bay, CA) attached to the laryngeal muscle tendon just dorsal to its insertion into the arytenoid disc. Signals were amplified (strain gauge amplifier model 2001; UFI), digitized (Digidata 1440A; Molecular Devices), and recorded with Clampex (Molecular Devices).

Vocal recording analysis

All control animals produced vocalizations during all three recording sessions. We determined the total call duration during a 1-h period when animals tended to be most vocally active (21:00–22:00). The vast majority of calls produced by control animals were stereotyped advertisement calls. These consist of click trills that alternate between around 35 and 60 Hz, lasting about 300 and 700 ms, respectively, with fast trills sometimes preceded by low-amplitude, low rate (~20–40 Hz), introductory phases (Zornik et al. 2010). We confirmed previous studies showing that gonadectomized animals called very little. However, intensive sampling of vocalizations using a voice-activated recording system in this study revealed that gonadectomized animals produced a variety of calls, including relatively normal advertisement-

like calling, and short bursts of clicks that did not resemble any of the normal male calls. To obtain a unified value of total call duration for both control and gonadectomized groups, we defined uninterrupted bouts of calling as any series of clicks with silent intervals <500 ms. The total time spent calling was a sum of the durations of all calling bouts within the designated 1-h period. Because advertisement-like calls were very rare in castrated males, produced by only three castrated males with varying structure in each animal, the overall structure of these calls was not compared quantitatively with control male vocalizations.

Analysis of fictive vocalizations

A single compound action potential (CAP, produced by synchronous firing of laryngeal motoneurons) recorded from the laryngeal nerve (N.IX–X) precedes each vocal click (Yamaguchi and Kelley 2000); therefore the pattern of CAPs represents a readout of vocal production by the CPG (Rhodes et al. 2007). Of the three (or more) attempts, the 5-HT application inducing the most robust fictive calling was used for further analysis. To evaluate the general activity level induced by 5-HT application, we measured the number of fictive vocal CAPs produced within the most active 1-min period. CAPs recorded from N.IX–X were identified in Clampfit (Molecular Devices) using the threshold search function. Based on the onset time for each CAP, we calculated the inter-CAP interval and instantaneous CAP rate (reciprocal of the interval).

In addition to the general characteristics described earlier, we quantitatively compared the structure of CPG output between castrates and controls. In particular, we compared the rates of introductory, fast, and slow trills separately. An introductory phase with low-amplitude slow-rate CAPs is typically followed by fast trill with stereotyped high-amplitude fast-rate CAPs and slow trills with medium-amplitude slow-rate CAPs (see example in Fig. 3). Introductory phase and fast trill CAPs can be distinguished in intact brains based on the distribution of values calculated from the product of rate and amplitude of all CAP intervals across multiple trills (Zornik et al. 2010). However, because fictive trills of castrates were less stereotyped, the same methods could not be applied to distinguish between these two trill types. Therefore introductory phase and fast trill CAPs were pooled and analyzed as a combined unit (herein referred to simply as fast trills). Slow trills can also be easily distinguished from introductory phases and fast trills because of the abrupt switch from fast trill to slow trill. The transition from slow trill to fast trill can also be unambiguously identified because slow trill CAPs are larger in amplitude than introductory phase CAPs. Data were collected from the 5-HT application trial that induced the largest number of fictive vocalizations; for each trill type, up to ten samples (at least five) were used for analysis. To quantify trill CAP rates, all instantaneous CAP rates (for each trill type) were plotted in a frequency histogram (bin = 1 Hz), which was fitted with a one-term Gaussian curve (in Clampfit). The resulting mean (μ) was defined as the average trill rate for that preparation. Fast or slow trill means calculated from incomplete fictive calls were pooled with means calculated from biphasic fictive calls.

Analysis of larynx physiology

The rising slope (defined by fitting a straight line between pulse onset and peak tension) and half-relaxation time (time from the peak tension to half-relaxation) were measured following single nerve stimulus pulses. Trains of 20 stimulus pulses were used to measure the degree of transient tension at each stimulus rate (10–100 Hz, 10-Hz increments). Percentage transient tension values were calculated as: the difference between the peak tension value and the lowest tension level between two pulses (or trough) divided by the peak tension and multiplied by 100 {[(peak − trough)/peak] × 100}. Average values were calculated from the last ten complete intervals in each train and averaged across the three replicates of each rate, to arrive at a single mean percentage transient tension for each preparation at each rate.

In addition to contractility, the neuromuscular synapse in the larynx is also sexually distinct. Male synapses are weak and facilitate within a train of stimuli, whereas female synapses are strong and facilitate little (Tobias et al. 1995); accordingly, peak tension of laryngeal muscles increases progressively within a train of stimuli as rising number of muscle fibers are recruited in males but not in females. In this study, we quantified the progressive increase in laryngeal muscle tension as an indirect measure of synaptic strength. To this end, we calculated the "potentiation index," defined as the amplitude of peak tension of the final stimulus pulse divided by the peak of the first pulse (during a 10-Hz train) for each preparation (Tobias and Kelley 1987).

Statistics

Statistical analyses were performed using StatView (SAS Institute, Cary, NC) and Prism (GraphPad, San Diego, CA) software. We used the unpaired Mann–Whitney *U* test to compare features between castrate and control animals: the duration of in vivo calling (total time spent calling), in vitro fictive vocalization parameters (maximum number of CAPs in 1 min, fast and slow trill mean rates), laryngeal muscle properties (percentage transient tension, rise slope, time to half-relaxation, potentiation index), and morphological traits (body mass and length). To determine whether amounts of calling varied over the course of three nights of vocal recordings (under three distinct conditions) we performed the Friedman test for both castrates and controls. Because no differences were found between the three recording conditions, calling durations were also averaged over the three nights and compared between control and castrate groups using the Mann–Whitney *U* test. Differences in fraction of animals or brains producing particular call patterns (e.g., fast trill, slow trill, or biphasic calls) were determined with Fisher's exact test (2 × 2 contingency table: castrate vs. control; call vs. no call). Linear regression analyses (independent variable: time after surgery) were used to assess whether the amount of time elapsed after castration correlated with vocal or morphological properties. Repeated-measures ANOVA was used to determine whether laryngeal transient tension responses were different between castrates and controls (treatment groups as between factors, stimulus rates as within factor, percentage transient tension at ten rates as dependent variable). Data are reported as means ± SD unless otherwise noted.

RESULTS

Infrequent and disorganized vocalizations in gonadectomized male frogs

Prior to castration or sham surgery, total time spent calling during a 1-h period (21:00–22:00) was measured for all animals; durations of calling in castrate (1,117 ± 700 s; $n = 26$) and control (746 ± 590 s; $n = 13$) groups were not statistically different ($U = 123$, $z = -1.37$, $P = 0.17$; Mann–Whitney *U*; Fig. 1*A*). Thus there was no systematic difference in the propensity for calling prior to the surgery between the two groups. Between 4.9 and 18.7 mo after surgery (10.0 ± 3.8 mo for castrates, 10.5 ± 4.9 mo for controls), vocal recordings were obtained once again from each subject. A previous study showed that calling was substantially reduced 1 mo after castration and virtually eliminated after 1 yr (Wetzel and Kelley 1983). We quantified the amount of calling (sampled over a 1-h period between 21:00 and 22:00) following the initial hCG injection and compared these with values obtained prior to surgery. The duration of calling in control frogs (1,131 ± 604 s; $n = 13$) did not differ significantly from the pretreatment

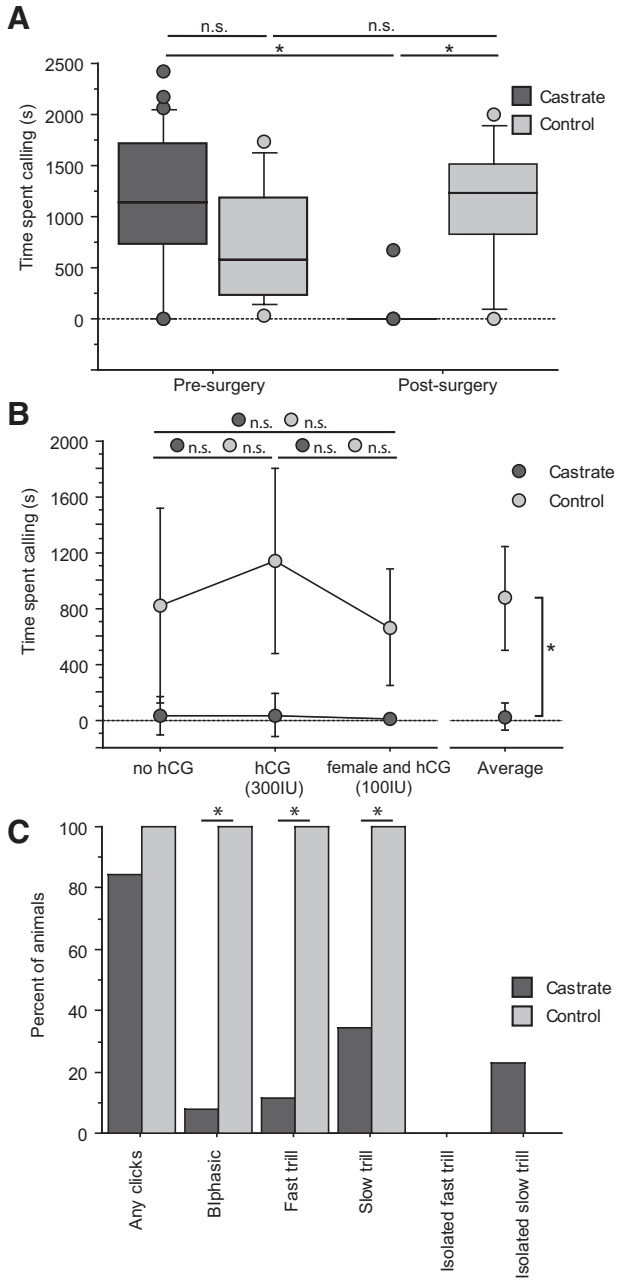

FIG. 1. Castration drastically reduced calling behavior in male frogs. *A*: box plots of time spent calling for castrate and control groups before and after surgery. Prior to treatment, the groups do not exhibit significantly different amounts of calling. Following surgery, controls continued to call at preoperation levels, but castrates were nearly silenced. Box plot formatting here and in all subsequent figures: horizontal lines represent median values, boxes show upper and lower quartiles, whiskers indicate 5% and 95% confidence intervals, and dots represent outliers. *B*: amount of calling did not vary over 3 nights of recordings with 3 distinct conditions in control and castrate groups. However, the average duration of calling over the 3 nights was vastly lower in castrated males. *C*: bar graph showing the percentage of animals in each group that produced advertisement call trills. All controls produced both fast and slow trills on every night of recordings. Most castrated males produced at least some vocal clicks on at least one night of recordings. Compared with controls, significantly fewer castrated animals produced biphasic calls (fast and slow trills), fast trills, and slow trills. All males that produced fast trills also exhibited slow trills, but some castrates (but no controls) that produced slow trills never generated fast trills. *$P < 0.0001$; n.s., not significant.

levels ($U = 33$, $z = -1.29$, $P = 0.20$; Mann–Whitney U; Fig. 1*A*). In contrast, the duration of calling by castrates (26 ± 132 s; $n = 26$) was drastically reduced compared with pregonadectomy levels ($U = 30$, $z = -5.56$, $P < 0.0001$; Mann–Whitney U; Fig. 1*A*). Accordingly, the amount of calling in castrates was significantly lower than that of controls postsurgery ($U = 15.5$, $z = -4.57$, $P < 0.0001$; Mann–Whitney U; Fig. 1*A*). These results parallel earlier findings by Wetzel and Kelley (1983) that castration virtually eliminates calling in adult male frogs.

Twenty castrates and ten controls were recorded over three consecutive nights under three different conditions (night 1: no hCG; night 2: hCG; night 3: with female and hCG booster; see METHODS) immediately before in vitro experiments commenced. The amount of calling (measured over a 1-h period between 21:00 and 22:00) did not vary across nights within each group (castrates: $\chi^2 = 0.78$, $P = 0.68$, $n = 20$; controls: $\chi^2 = 4.2$, $P = 0.12$, $n = 10$; Friedman test), but was well over an order of magnitude smaller in castrates than that in controls ($U = 1$, $z = -4.36$, $P < 0.0001$; Mann–Whitney U; Fig. 1*B*), indicating that the amount of calling was most significantly affected by gonadectomy and not by either hCG or the presence of a female (Fig. 1*B*).

Next, we examined the components of the advertisement calls that were affected by gonadectomy. All control animals produced normal biphasic advertisement calls containing alternating fast and slow trills (see example in Fig. 2*A*). Although calling was extremely rare in castrated males, our intensive recordings involving a sound-activated technique allowed us to obtain some vocal recordings (in many cases only a few isolated clicks) from 22 of 26 castrated males (85%) (Fig. 1*C*). Compared with controls, however, castrates were significantly less likely to produce biphasic advertisement-like calls with regularly alternating fast and slow trills (100% vs. 8%, respectively; $P < 0.0001$; Fisher's exact test; Fig. 1*C*). Overall, a larger proportion of castrates produced slow trills (35%) than fast trills (12%), but these were both significantly less common compared with controls ($P < 0.0001$ in each case; Fisher's exact test; Fig. 1*C*). Interestingly, all castrates that produced fast trill-like calls ($n = 3$) also exhibited slow trill-like activity as in controls. In other words, no castrates produced isolated fast trills (Fig. 1*C*), suggesting castrates that retain the ability to generate fast trills are also capable of generating slow trills. In contrast, 6 of 26 castrates (23%) that produced slow trills never produced fast trills, indicating that castrates with the ability to generate slow trills are not necessarily capable of producing fast trills. These isolated slow trills (i.e., slow trills not preceded by fast trills) produced by castrates were not observed in controls (Fig. 1*C*), nor in any previous studies of *X. laevis* vocalizations (e.g., Tobias et al. 1998, 2004; Wetzel and Kelley 1983). These results indicate that fast trill production may be more sensitive to castration than slow trill production.

As described earlier, two gonadectomized males produced biphasic advertisement-like calls. One of these castrated males (Fig. 2*B*) produced a pattern of alternating fast and slow trills with normal slow trill structure; however, fast trill clicks exhibited a lower sound intensity compared with that of normal calls (compare Fig. 2, *A* and *B*). In the second animal, slow and fast click rates were both present, but there were silent gaps inserted at the transition from fast to slow trills that are absent

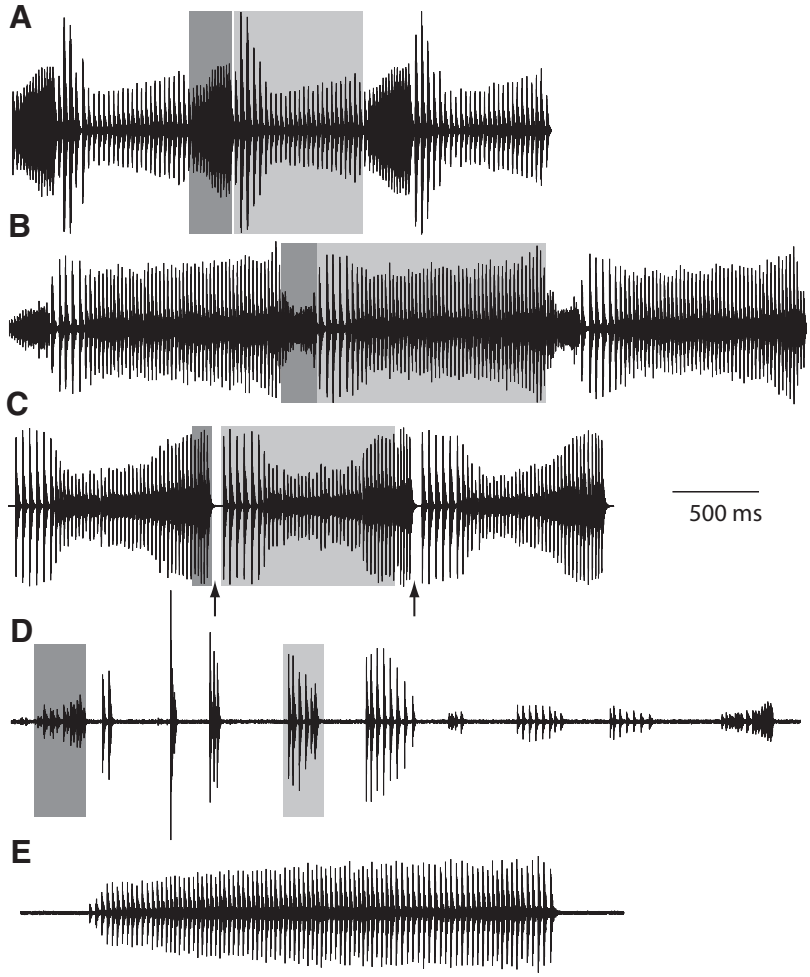

FIG. 2. Abnormal vocalizations in castrated males. *A*: example sound oscillogram of 3 consecutive advertisement calls produced by a control male. The middle call is highlighted with dark and light gray boxes, indicating fast trill and slow trill, respectively. *B*: example of a degraded fast trill generated by a castrated male. Note that the regular pattern of alternating fast and slow trills remains. However, fast trills appear to be shortened and clicks are of lower sound amplitude. *C*: another example of degraded advertisement calls made by a castrated male. In this example, an abnormal silent period is present during the transition from fast to slow click rates (arrows). *D*: a third castrate produced both fast- and slow-like trills, but without the typical biphasic pattern of regularly alternating trills. *E*: an example of an isolated slow trill—a long, slow trill-like call, uninterrupted by fast trills—observed only in castrates.

in control males (Fig. 2*C*, compare with Fig. 2*A*). The elapsed time after surgery of these two animals was 12.7 and 8.4 mo, respectively, which was not the shortest among all the castrates. Thus the ability to produce advertisement-like calling remained in a small percentage of the castrated population independent of the amount of time elapsed from the gonadectomy. A third castrated male produced both fast-like and slow-like trills (Fig. 2*D*), but fast trills were short in duration and low in sound amplitude; the normal pattern of alternating trills was completely disrupted in this animal, with seemingly random trill production. Isolated slow trills produced by other castrates were also qualitatively distinct from slow trills produced by controls, occurring with seemingly random duration, sometimes longer than a single biphasic call (Fig. 2*E*). Thus the sparse remaining advertisement-like calls produced by castrated males all showed some degree of abnormality.

Larynges are partly demasculinized after castration

We sought to test whether the abnormal acoustic morphology observed in the calls produced by castrates resulted from demasculinized larynges incapable of producing male-like advertisement trills (fast and/or slow). We isolated the larynges of each experimental animal and measured the ratio of transient tension to total tension during stimulus trains. With faster stimulus trains, incomplete relaxation between stimulus pulses (and decreased transient tension) translates into higher fused tension and eventual loss of click production (Tobias et al. 1987). We applied trains of 20 pulses from 10 to 100 Hz in 10-Hz intervals. Responsiveness to repetitive stimulation differed significantly between castrate ($n = 24$) and control ($n = 12$) larynges [$F_{(1,34)} = 50.5$, $P < 0.0001$; repeated-measures ANOVA: Fig. 3*B*]. At stimulus frequencies of ≤30 Hz, transient tension in castrate larynges did not differ from control preparations ($U = 134$, $z = -0.336$, $P = 0.78$). However, castrates showed significantly reduced transient tension compared with controls at rates of 40 Hz (castrate, 95.4 ± 6.3% vs. control, 99.1 ± 0.3%; $U = 41$, $z = -3.456$, $P = 0.0005$; Mann–Whitney *U*) and higher ($P < 0.0001$ for ≥50 Hz). In response to a single stimulus pulse, we found that the time course of muscle contraction differed between the castrates and controls. The slope of muscle tension following a single stimulus pulse was significantly smaller in castrates compared with controls (castrate = 17.3 ± 3.4, control = 23.2 ± 3.3; $U = 26$, $z = -3.315$, $P = 0.0009$; Fig. 3*C*). This indicates that laryngeal muscles take longer to reach peak tension in castrated preparations compared with controls. Likewise, the average time to half-relaxation in castrate larynges (6.3 ± 2.9 ms) was significantly longer than that in control tissue (2.6 ± 1.0 ms; $U = 24$, $z = -3.395$, $P = 0.007$; Fig. 3*C*). Thus the reduced transient tension of castrate laryngeal muscles during stimulus trains is likely a product of increases in both rise and relaxation times.

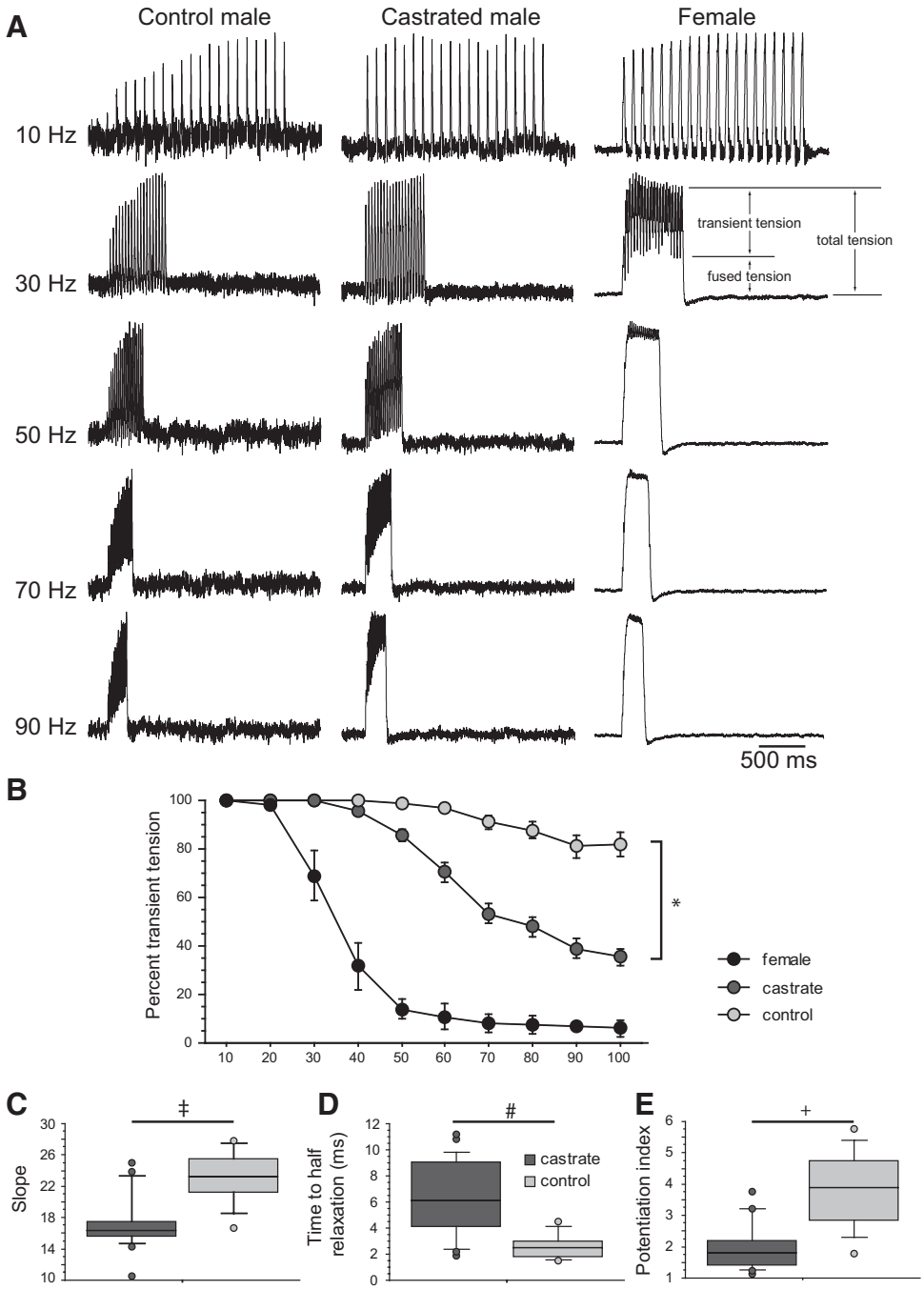

FIG. 3. Differences in contractile properties of laryngeal muscles between control, castrate, and female frogs. *A*: examples of tension transients in laryngeal muscles in response to trains of nerve stimulation at varying rates. Note that decreased transient tension (i.e., fused tension) is observed in control larynges with stimulation rates at ≥70 Hz; castrate larynges, in contrast, show sustained tension ≥50 Hz. Female laryngeal muscles show fused tension at ≥30 Hz. *B*: summary of average transient tension at all rates tested for controls, castrates, and females (values are means ± SE). *C*: box plot graphing the slopes of laryngeal muscle contraction (peak tension/time to peak tension) for castrate and control larynges; castrate muscles have significantly lower slopes, indicating slow contraction rates. *D*: box plot of time to half-relaxation; castrate male larynges take longer to relax compared with control muscles. *E*: potentiation index (peak tension of the final stimulation pulse divided by the peak tension of the first pulse) is greater in control males, suggesting a strengthening of the castrate neuromuscular junction, a female-typical trait. $*P < 0.0001$, $‡P = 0.0009$, $\#P = 0.007$, $+P = 0.0001$.

Sound production by the *Xenopus* larynx requires the snapping apart of two arytenoid cartilage discs during bilateral muscle contraction (Yager 1992). Therefore the laryngeal muscles must sufficiently relax between each click, allowing the discs to regain contact before the next contraction. For example, female laryngeal muscles stimulated at fast rates become tetanized and therefore generate only a single click at the beginning of a train of nerve stimulus pulses (Tobias and Kelley 1987). Therefore a potential consequence of decreased transient tension is that castrate larynges may not have been capable of generating fast trills. If the laryngeal muscles of castrates are not capable of producing fast trills, however, how is it that three castrates in our study generated fast-trill like vocalizations in vivo? We examined the average transient tension generated by larynges from the three castrates that produced fast trill-like calls. These animals exhibited maximum fast trill rates of 71, 59, and 63 Hz in vivo (as shown in Fig. 2, *B*, *C*, and *D*, respectively) and the corresponding fused tension values were 65% (at 70 Hz), 49%, and 60% (both at 60 Hz), respectively. Thus reduced transient tension in laryngeal muscles did not preclude the production of at least a few consecutive clicks at fast trill rates. A likely explanation is that clicks >50 Hz were produced early in a fast trill until sufficient fused tension built up to prevent the arytenoid discs from regaining contact; this hypothesis is consistent with the observation that two of these castrates produced only a few (<5) consecutive clicks at rapid rates (see Fig. 2, *C* and *D*). This suggests that demasculinized larynges of most castrates could

have produced at least truncated fast trills. The fact that most animals did not produce any fast trill clicks was likely due to a lack of neuronal signals descending from the CPG to the muscles.

Neuromuscular synapses in male larynges are weak and facilitation occurs during repeated nerve activity; in contrast female synapses are strong and do not facilitate (Tobias and Kelley 1987; Tobias et al. 1995). Although we did not directly measure synaptic strengths in this study, we assessed the progressive recruitment of muscle fibers in response to repeated stimulation by calculating a muscle potentiation index (Tobias and Kelley 1987; see METHODS). We found that the average potentiation index in castrate larynges (1.9 ± 0.7) was significantly smaller than that in control larynges (3.9 ± 1.2; $U = 19$, $z = -3.817$, $P = 0.0001$; Fig. 3*C*). These results support the hypothesis that all aspects of laryngeal function, including synaptic strength, require the continued presence of testicular secretions to maintain masculinized properties.

Castration decreases in vitro activity levels of the vocal CPG

In response to 5-HT application, fictive vocal activity was elicited in both castrate and control male brains. The average number of CAPs produced in the most active 1-min period in castrates (288 ± 371 CAPs, $n = 26$) was lower than that in controls (865 ± 638, $n = 13$; $U = 72$, $z = -2.89$, $P = 0.035$; Mann–Whitney *U*; Fig. 4*A*). These results indicate that castration decreased function of hindbrain vocal pathways.

Distinct effects of castration on CPG production of fast and slow trill

We next examined the trill composition of the fictive advertisement calls produced by brains from controls and castrates. Biphasic fictive advertisement calls that included alternating fast and slow trills were more common in brains from controls (7 of 13, 54%) compared with those from castrates (5 of 26, 19%), but this effect was not statistically significant ($P = 0.06$; Fisher's exact test; Fig. 4*B*). Similar to in vivo vocal recordings, fast trills were more common in controls (77%) than in castrates (27%; $P = 0.006$; Fisher's exact test; Fig. 4*B*). In contrast to in vivo results, however, the portion of castrates producing fictive slow trills was similar to that of controls (54% of controls, 42% of castrates; $P = 0.52$; Fisher's exact test; Fig. 4*B*).

The probability of fast trill production by castrates was lower than that of slow trills both in vivo and in vitro. Because some animals produced vocal patterns only in vivo, but others only in vitro (and still others were active in both cases), we computed the overall "vocal capacity" of castrates by totaling the number of animals that produced each trill type in at least one of the two recording conditions (in vivo, in vitro, or both). This analysis revealed that 8 of 26 castrated animals (31%) were capable of producing fast trill-like activity in vivo and/or in vitro (Fig. 4*C*). Slow trill-like activity in vivo and/or in vitro was observed in 17 of 26 (65%) castrates (Fig. 4*C*). Thus the vocal CPG was capable of forming fast trill-like calls in at least one third of animals, whereas two thirds of gonadectomized male CPGs were capable of generating slow trill-like vocal patterns.

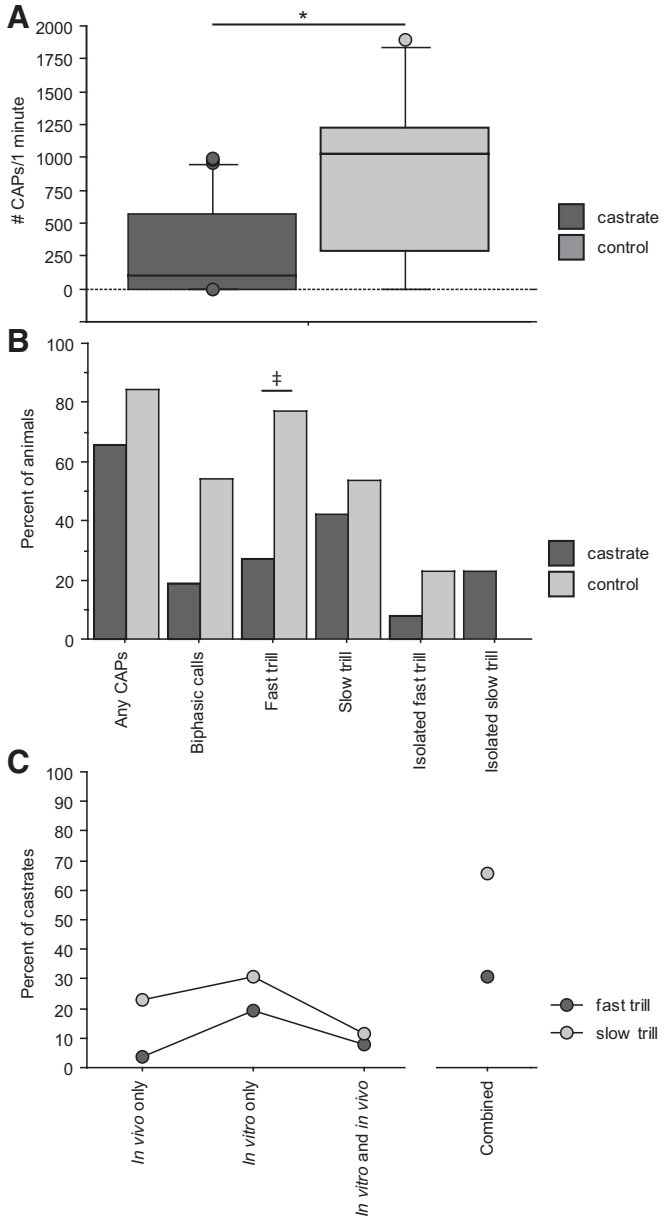

FIG. 4. Fictive calling by castrate and control brains. *A*: box plot showing the number of compound action potentials (CAPs) recorded in the laryngeal nerve during serotonin (5-HT)-induced fictive calling in the isolated brain. During the most active 1-min period, brains from castrated males produced significantly fewer CAPs than control brains. *B*: bar graph depicting the percentage of brains from castrates and control animals producing vocal-like nerve activity. The majority of preparations from both groups produced at least some vocal-like CAPs. Compared with controls, significantly fewer brains from castrates produced fast trills, but significant differences were not observed for biphasic calls or slow trills. Unlike in vivo results, some animals from both groups produced only fast trills. Similar to in vivo results, however, some isolated brains from castrates generated only isolated slow trills, whereas no controls exhibited this pattern of fictive vocalization. *C*: plot showing the proportion of individual castrates that produced vocal trills in vivo only, in vitro only, or both in vivo and in vitro. Note that both fast and slow trills were more common in vitro compared with in vivo. Combined results (percentage of animals producing a trill in vivo and/or in vitro) revealed that twice as many castrate brains retained the ability to produce slow trills than those capable of generating fast trills. *$P < 0.004$, ‡$P < 0.006$.

Unlike in vivo vocal recordings, some control (23%) and castrate (8%) brains produced only isolated fictive fast trills (Fig. 4*B*). Interestingly, some castrate brains (23%) produced only isolated slow trills, but this was not seen in control brains (Fig. 4*B*). The results are consistent with the idea that the isolated slow trills observed in vivo reflect the neuronal signals generated by a degraded (slow trill only) CPG and were not due to the low-pass filtering of the neuronal signals by demasculinized laryngeal muscles. Together, the findings that *1*) brains from castrates were not less likely to produce fictive slow trills than controls, *2*) castrate brains were twice as likely to produce fictive slow trills than fast trills, and *3*) several brains from castrates produced only isolated slow trills, suggest that the CPG elements generating slow trills may be less affected by castration than those underlying fast trills.

In many cases, fictive fast and slow trills of castrated males were qualitatively similar to those of controls (compare Fig. 5, *A* and *B*). However, some fictive fast trills appeared to be truncated, with silent periods inserted during the fast-to-slow trill transition (Fig. 5*C*). Abnormal patterns, such as introductory phase followed by slow trill (lacking normal fast trill), were also common (see example in Fig. 5*D*). In addition, isolated slow trill-like calls were common, often occurring in bouts interrupted by short silent periods between trills (see example in Fig. 5*E*). Unlike in vivo recordings, we obtained sufficient fictive vocal recordings to quantitatively compare activity patterns of CPGs from castrate and control males. We first compared fictive fast trill CAP rates (see METHODS) between the two groups. Fast trills were significantly slower in the castrate group (39 ± 8 Hz; $n = 6$) compared with controls (51 ± 6 Hz; $n = 10$; $U = 6$, $z = -2.603$, $P = 0.009$; Fig. 6*A*). Frequency histogram plots show a leftward shift in fast trill frequencies in the castrate group compared with controls (Fig. 6*B*). Slow trills from castrate brains (23 ± 5 Hz; $n = 11$) were also significantly slower compared with controls (29 ± 5 Hz; $n = 6$; $U = 11$; $z = -2.211$; $P = 0.03$; Mann–Whitney U test). Thus the ability of the vocal CPG to generate normal fast and slow trill rates requires the continued presence of gonadal secretions in adulthood.

Vocal pathways degrade after castration with distinct temporal patterns

Next, we examined how the parameters measured earlier deteriorated over time. To this end, we first examined how the amount of calling activity in vivo declined after gonadectomy by plotting the average amount of calling against the time after castration. Linear regression results revealed that the time after castration did not correlate with the amount of calling for either castrates (coefficient = 4.91, $R^2 = 0.02$, $P = 0.49$, $n = 26$) or controls (coefficient = 2.82, $R^2 = 0.001$, $P = 0.94$, $n = 13$). Rather, the amount of calling in castrates was already decreased to the minimum level by the time postcastration testing started 4.9 mo after surgery and

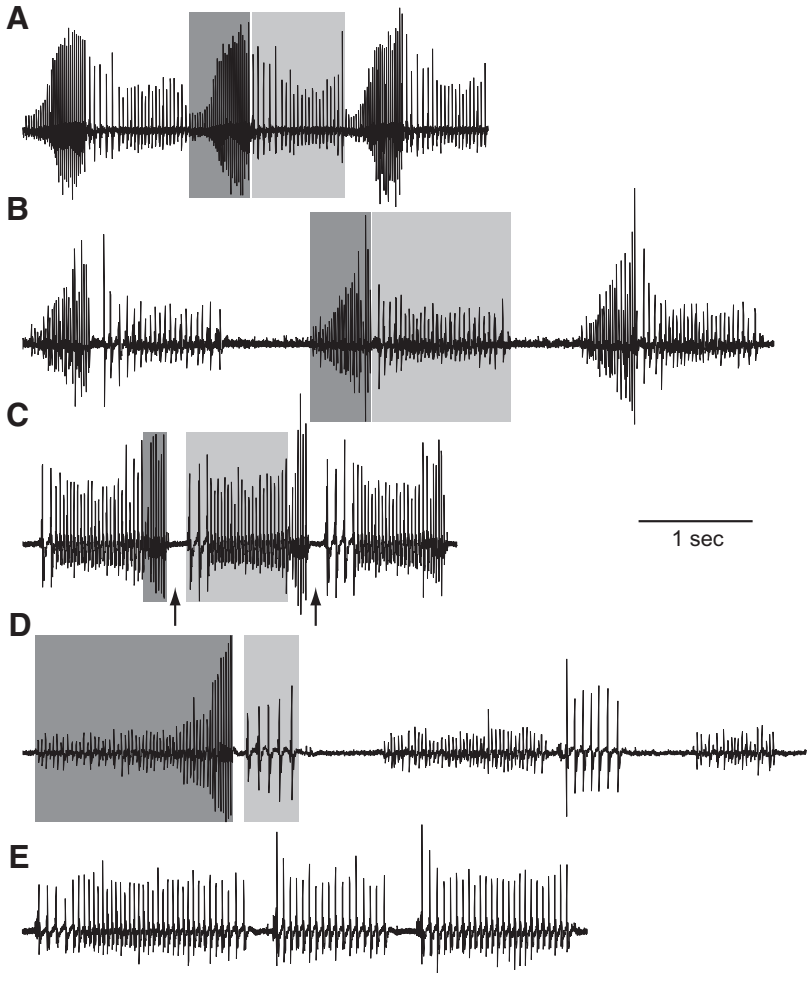

FIG. 5. Abnormal fictive vocalizations in castrated males. *A*: example nerve recording of 3 fictive advertisement calls after 5-HT application in an isolated brain from a control male. Fictive calls represent CAPs recorded in the laryngeal nerve, each of which would be translated into a vocal click in vivo. Dark and light gray boxes indicate fictive fast and slow trills, respectively. *B*: fictive vocal recordings in a castrate brain showing alternating fast and slow trills. *C*: fictive vocalization in which fast trills are truncated, leaving a "silent" gap between fast and slow trills (arrows). *D*: nerve recording during abnormal fictive vocalizations in a castrate brain. In this example, a relatively normal fast trill (with long introductory phase) is followed by a brief slow trill. Later, an introductory phase transitions to slow trill, skipping the fast trill portion of the call. *E*: example nerve recording showing 3 consecutive "isolated slow trills." "Silent" gaps replace intervening fast trills in these fictive vocalizations.

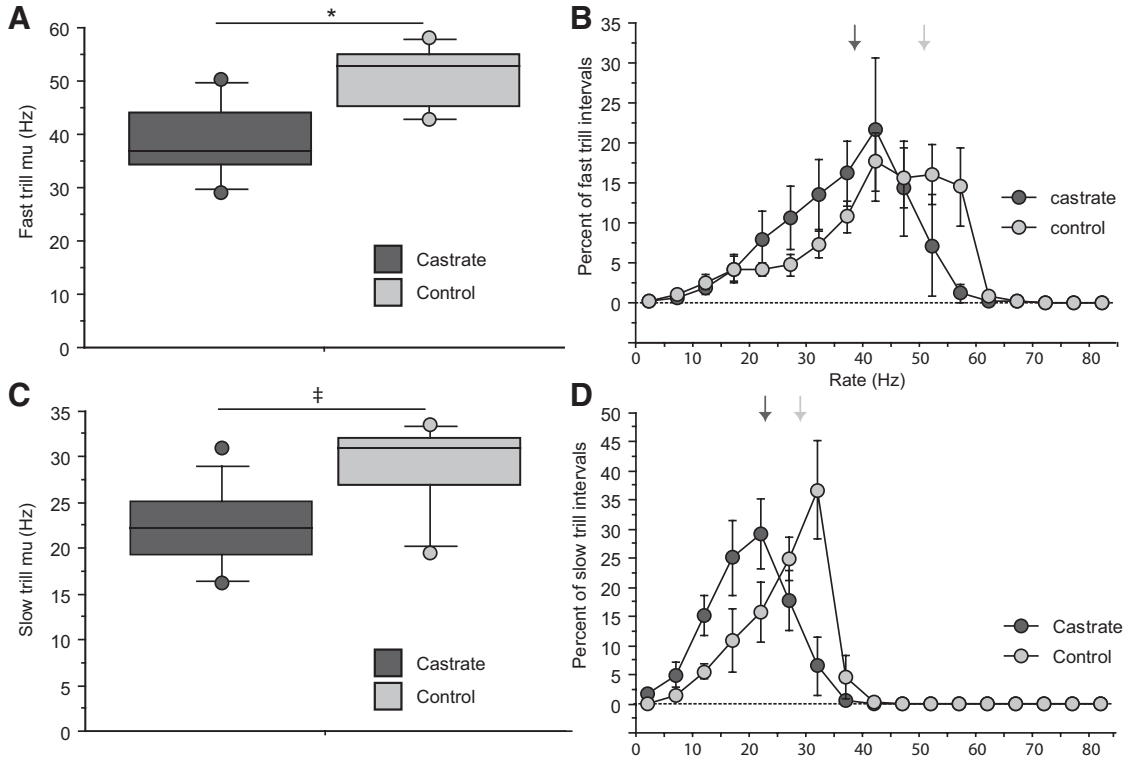

FIG. 6. Fictive vocalizations produced by castrate central pattern generators (CPGs) are quantitatively distinct from those produced by control CPGs. *A*: box plot of mean rates calculated from Gaussian fits to fictive fast trill interval histograms for each individual, revealing that fast trill rates are reduced in brains from castrates. *B*: average values for fast trill rate histograms; arrows indicate the average rate for each treatment. *C*: box plot of control and castrate fictive slow trills (including normal and isolated slow trills in castrates) showing a significant reduction in castrate rates. *D*: average histograms showing rate distributions of CAP from control and castrate slow trills. Arrows indicate the average mean rate for each treatment. $*P = 0.009$, $‡P = 0.03$.

vocalizations in controls remained constant at presurgical levels (Fig. 7*A*; see RESULTS). Thus we conclude that the effect of castration on the amount of calling was complete by 4.9 mo.

We next examined whether the function of the vocal pathways deteriorated progressively over time throughout our experiment or abruptly during the first 5 mo following castration (before experimentation began) and maintained at a constant level afterward, as was the case for the amount of in vivo calling. Linear regression analyses were performed on the following parameters: percentage transient tension in laryngeal muscles at 70-Hz stimulation, the number of CAPs produced by isolated brains, and the rate of fast and slow trills produced by isolated brains. The analyses revealed a negative correlation between time after castration and transient tension (coefficient = -3.12, $R^2 = 0.417$, $P = 0.0007$, $n = 24$, Fig. 7*B*), the number of CAPs (coefficient = -43.1, $R^2 = 0.20$, $P = 0.02$, $n = 26$, Fig. 7*C*), and fast trill rate (coefficient = -5.5, $R^2 = 0.738$, $P = 0.03$, $n = 6$, Fig. 7*D*), but no significant relation between slow trill rate and the time postsurgery (coefficient = -0.6, $R^2 = 0.274$, $P = 0.10$, $n = 12$, Fig. 7*E*). In contrast, there was no significant relation between time after sham operation and percentage transient tension (coefficient = -0.97, $R^2 = 0.247$, $P = 0.10$, $n = 12$), number of CAPs (coefficient = -0.19, $R^2 = 0.02$, $P = 0.64$, $n = 13$), fast trill rate (coefficient = -0.64, $R^2 = 0.338$, $P = 0.08$, $n = 10$), or slow trill rate (coefficient = 0.98, $R^2 = 0.31$, $P = 0.26$, $n = 6$) in control animals.

Thus unlike the amount of calling in vivo, larynx contractility and CPG function gradually degraded between 5 and 18 mo postcastration. Transient laryngeal tension decreased by

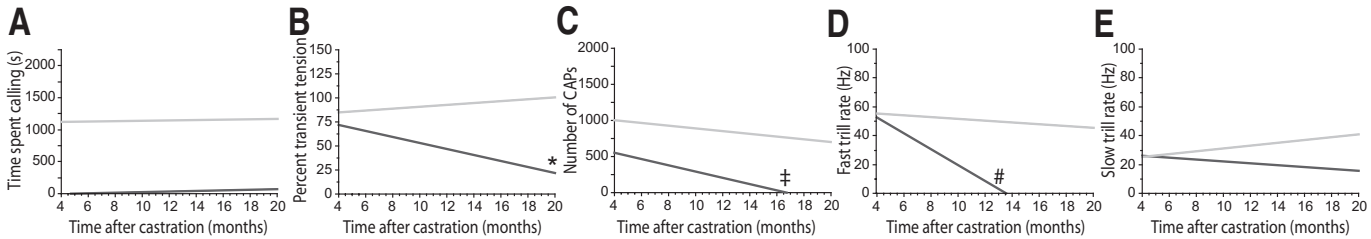

FIG. 7. Linear regressions show time course of castration-dependent degradation of vocal parameters. *A*: linear regression results fit to control (light gray) and castrate (dark gray) data do not show significant correlations between time spent calling and time after treatment. Thus it is concluded that decreases in calling caused by castration were complete by 5 mo postsurgery. *B*: percentage transient tension decreased progressively in castrates, whereas controls showed no significant relation between tension and time after surgery. *C*: the number of CAPs produced by castrate brains decreased with time, whereas those produced by control brains remained constant over time. *D*: fictive fast trill rates of castrates, but not of controls, showed a sharp and significant decrease over time after surgery. *E*: slow trill rates of neither controls nor castrates changed over time after surgery. $*P = 0.0007$, $‡P = 0.02$, $\#P = 0.03$.

about 3% each month after castration, isolated brains produced progressively fewer CAPs (by about 40 fewer CAPs for each month elapsed after castration) and gradually reduced fast trills rates (~5.5 Hz slower for each elapsed month after castration). Of these parameters, fictive fast trill production appeared to be most sensitive to castration. Of the six castrate brains that generated fictive fast trills, all came from animals castrated for <9 mo (range = 4.9–8.4 mo). Within this time period, fast trill rates rapidly decayed from near normal to about 60% of control rates. This rapid decline and lack of fast trill production after 9 mo indicate that CPG circuits generating fast trills are especially sensitive to castration compared with other vocal components.

Neural correlates of calls recorded from a CPG premotor nucleus are degraded in castrate brains

We recently identified a local field potential (LFP) wave in a premotor CPG nucleus—the dorsal tegmental area of the medulla (DTAM, a parabrachial nucleus homologue; Zornik and Kelley 2008; Zornik et al. 2010)—that correlates with onset and offset of fictive trills (Zornik et al. 2010). In intact males, an upward baseline shift in the LFP coincides with the onset of fast trills, whereas the downward part of the wave correlates with the offset of fast trills and the onset of slow trills (Fig. 8*A*; Zornik et al. 2010). Some fictive advertisement calls lacked slow trills but the DTAM wave was the same regardless of the presence of slow trills. Thus the LFP wave in DTAM marks the timing of trill transition in normal male brains (Zornik et al. 2010).

In this study, we recorded LFPs in DTAM in four controls and four castrates (of 4 and 17 attempts, respectively). In all four control brains, we observed the typical pattern of slow baseline waves coinciding with fast trill onset and offset (see example in Fig. 8*B*). Of four castrated male brains that produced fictive calls during LFP recordings, fictive fast trill-like activity was observed in two brains. In these preparations, a slow baseline coincided with the onset and offset of fictive fast trills; there were no slow trills that followed fast trills in these brains (Fig. 8*C*). In the two other brains, only isolated slow trills were produced during DTAM LFP recordings. Interestingly, when isolated slow trill activity was observed, only a downward wave (from baseline) was present in DTAM (see example in Fig. 8*D*). Thus fictive fast and slow trill production in castrate brains were accompanied by upward or downward LFP waves, respectively, as they are in intact frogs, supporting the hypothesis that the waves are required for normal trill production. Isolated downward waves were observed only in castrate brains, as were isolated slow trills, indicating that upward waves may be more sensitive than downward waves to castration.

Morphological differences between castrate and control animals

Finally, we analyzed the body length and mass of control and castrate males. Sexually mature adult male *X. laevis* are much smaller than females (Kobel et al. 1996). However, the physiological mechanism underlying this sexual dimorphism has not been investigated. In this study, we found that castrating adult males leads to rapid increase in body mass. Although castrates and controls showed increases in length and mass following surgery, castrates were significantly longer (7.8 ± 0.6 cm) and heavier (56.7 ± 13.2 g) than controls (7.2 ± 0.5 cm and 47.2 ± 8.5 g; $U = 78.5/95$, $z = -2.696/-2.205$, $P = 0.007/0.03$, respectively, Mann–Whitney *U*; Fig. 9, *A* and *C*). Linear regression analyses showed that castrated males increased their body mass and length between 5 and 18 mo after castration (linear regression of size increase vs. time after surgery: length: coefficient = 0.09, $R^2 = 0.201$, $P = 0.04$; mass: coefficient = 2.1, $R^2 = 0.221$, $P = 0.03$) but control animals did not (length: coefficient = −0.005, $R^2 = 0.002$, $P = 0.90$; mass: coefficient = −0.119, $R^2 = 0.019$, $P = 0.71$; Fig. 9, *B* and *D*). Thus we conclude that testicular secretions inhibit somatic growth and castration results in significant growth in male *Xenopus*.

DISCUSSION

Hormonal activation: assaying multiple components of the vocal pathway

Androgens influence neural and muscular tissues underlying male vertebrate reproductive behaviors (Arnold and Breedlove 1985; Bass and Zakon 2005; Forger 2009; Zornik and Yamaguchi 2008). For instance, castrating adult male rats, which reduces copulatory behaviors (Whalen and Luttge 1971), alters motoneuron size and dendritic branching in the spinal nucleus of the bulbocavernosus (SNB; Breedlove and Arnold 1981; Kurz et al. 1986). Because the SNB controls copulatory muscles, these changes may contribute to behavioral losses. Such hypotheses are difficult to prove, however, because hormones regulate many brain regions and muscles. The *Xenopus* vocal system is well suited to dissecting the hierarchical roles of hormones because two in vitro preparations—the isolated larynx (Tobias et al. 1987) and the isolated brain (Rhodes et al. 2007)—allow direct testing of laryngeal muscle and vocal CPG functionality.

Vocal activation in male *Xenopus* is androgen dependent. Wetzel and Kelley (1983) found that castration eliminated male calling within 1 yr and androgen treatment reinstated calling within 3 to 5 wk. In another study, castration paired with 18 mo of androgen treatment did not decrease calling or alter vocalizations (Watson and Kelley 1992). Thus castration-induced vocal deterioration is caused by decreased androgens, not by other testicular secretions. Each CPG-generated CAP induces one click in vivo (Yamaguchi and Kelley 2000) and fictive vocalizations are extremely similar to actual calling (Rhodes et al. 2007). One implication of these findings is that the CPGs of androgen-replaced castrates (in Watson and Kelley 1992) must have been unchanged compared with controls. Likewise, normal calling duration and acoustic structure between intact and androgen-replaced castrated males indicate that androgen replacement alone is sufficient to prevent changes in vocal initiation centers and larynges of castrated males. Although we did not perform androgen replacement in this study, we can conclude that castration-induced changes in the brain and larynx are most likely caused by androgen deprivation.

Consistent with previous studies, the amount of calling was negligible 5–18 mo postcastration. There are several possible (not mutually exclusive) explanations for these results. First,

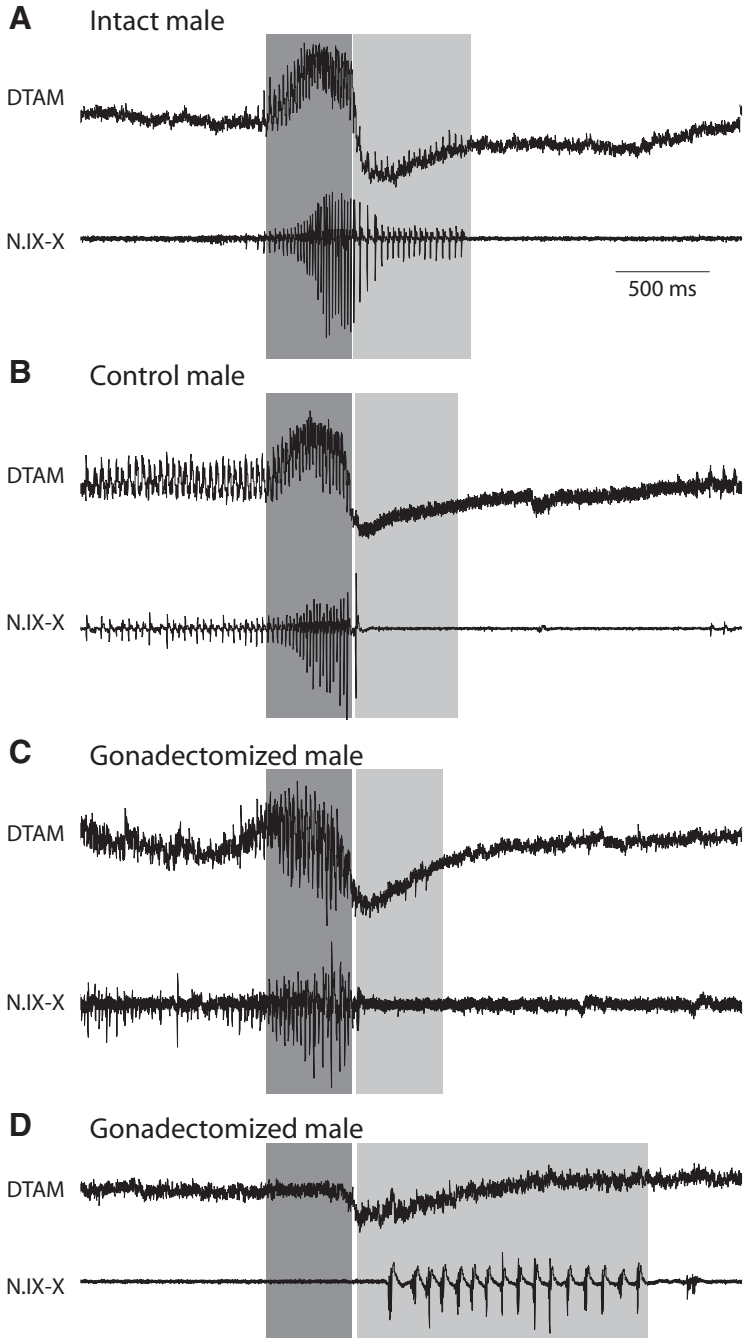

FIG. 8. Neuronal population activity underlying vocal patterns is degraded in castrate CPGs. *A*: local field potential (LFP) recording (*top trace*) in the premotor nucleus dorsal tegmental area of medulla (DTAM) of an intact male during a single advertisement call recorded in the vocal nerve (*bottom trace*). Note that fast, high-frequency activity in the LFP accompanies each vocal CAP in the nerve. Furthermore, an upward LFP baseline wave coincides with the onset of fictive fast trill, whereas a sharp downward shift is seen during the transition to fictive slow trill. Dark and light gray boxes mark fast and slow trills, respectively. *B*: similar recording in a sham-operated control male. A long introductory phase is present before the onset of fast trill (with accompanying baseline wave). In this example, only a single "slow trill" CAP is present, but the typical downward shift in the LFP baseline remains. *C*: LFP recording from DTAM of a castrate brain reveals a normal LFP wave that accompanies the onset and offset of fast trill. *D*: in a castrate brain that produced isolated slow trills, the upward DTAM wave was missing, but a downward shift preceding the isolated slow trill was present.

vocal initiation centers could not be activated in castrates. Second, laryngeal muscles may be demasculinized, preventing the translation of nerve signals into sound. Third, CPG degradation might prevent vocal pattern production. The availability of in vitro larynx and brain preparations in *Xenopus* provided a novel opportunity to tease apart these possibilities and revealed that castration affects all three levels, albeit at different timescales.

Effects of castration on laryngeal muscle properties

Watson and Kelley (1992) found that long-term castration partially demasculinized laryngeal muscles, a roughly 15% decrease in transient tension at 71-Hz nerve stimulation. We also discovered significant differences between castrate and control larynges >30 Hz; like Watson and Kelley, we concluded that the demasculinized larynx alone does not account for the scarcity of vocalizations in castrates (see following text).

What properties caused the decreased transient tension? Mature male laryngeal fibers are entirely fast twitch, whereas females exhibit a mixture of twitch types (Sassoon et al. 1987). Fiber type is organized in males during development and muscle fibers remain fast twitch after castration (Sassoon et al. 1987). The contraction rate of muscle fibers in *X. laevis* depends on the myosin heavy chain isoforms expressed (Län-

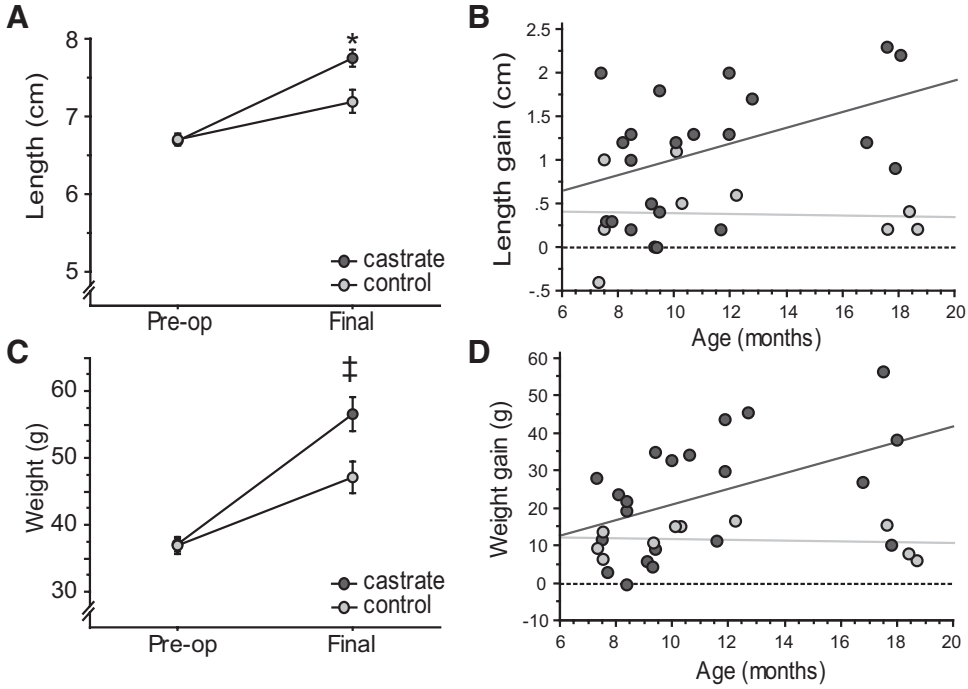

FIG. 9. Morphological differences between castrate and control males. *A*: castration resulted in a greater increase in body length compared with controls. *B*: linear regression analyses showed that this effect depended on the amount of time after gonadectomy (coefficient = 0.09 cm/mo, R^2 = 0.221, P = 0.04). Control animals, however, did not become longer during the postsurgery period (coefficient = −0.005 cm/mo, R^2 = 0.002, P = 0.90). *C*: castrates were also heavier than controls at the time of experimentation. *D*: linear regression analyses revealed that the amount of time between surgery and experiments accounted for the growth in castrates (coefficient = 2.1 g/mo, R^2 = 0.221, P = 0.03). However, no time-dependent growth was observed in controls (coefficient = 0.12 g/mo, R^2 = 0.019, P = 0.71). *P = 0.007, ‡P = 0.03.

nergren 1987). Male laryngeal muscle fibers all strongly express a larynx-specific myosin (LM), whereas a subset of female fibers expresses LM. Because LM expression is androgen dependent (Catz et al. 1992) and reduced or eliminated in juvenile castrates (Catz et al. 1995), LM expression may remain androgen dependent in adulthood, leading to castration-dependent down-regulation.

Motor initiation centers and the vocal CPG require intact testes

Despite demasculinized laryngeal muscles, some castrates produced fast trills in vivo, indicating that castrate larynges were capable of transducing at least some fast trill nerve signals into clicks. Accordingly, we conclude that the scarcity of vocalizations was due to the lack of neuronal signals descending from the CNS to the larynx. What CNS components are dysfunctional in castrates: motor initiation centers or the vocal CPG?

To address this question, we used a fictively calling brain preparation. Fictive calling is elicited by 5-HT binding to 5-HT_{2C}-like receptors in the hindbrain (Yu and Yamaguchi 2010). Thus the preparation allowed us to bypass putative upstream motor initiation centers, such as central amygdala and preoptic area (which concentrate estradiol and are connected, directly or indirectly, to the hindbrain vocal pathways; Brahic and Kelley 2003; Kelley 1981; Morrell et al. 1975; Wetzel and Kelley 1985), and to activate the CPG directly. Unlike in vivo results, the proportion of animals that produced biphasic advertisement-like activity in vitro was not significantly different between groups. This result suggests the scarcity of in vivo calling by castrates is mostly due to deactivated initiation centers; this would silence castrates in vivo, but not when the CPG is directly activated in vitro. The time course of the vocal decline is rapid. Wetzel and Kelley (1983) found that calling was nearly eliminated 1 mo after gonadectomy and we found that in vivo calling was vestigial by 5 mo postcastration and did not show further decline. Thus castration seems to affect motor initiation centers within a time window of weeks to months, most likely due to steroid hormone deprivation.

In addition to motor initiation centers, vocal CPG function was also affected by castration. The number of 5-HT–induced CAPs and the rate of fictive fast and slow trills recorded in vitro were decreased in castrates. The decline in these variable occurred gradually between 5 and 18 mo postcastration. This reduction in vocal output indicates that *1*) the excitability of the CPG was reduced, *2*) the neurons responsible for activating the CPG are less responsive to 5-HT, or *3*) both. Because fictive vocal initiation requires activation of 5-HT_{2C} receptors, future experiments that specifically modify 5-HT_{2C} receptor signal pathways may help distinguish between these scenarios. Taken together, these results suggest that androgens may provide trophic maintenance of CPG function over the lifetime of the animal. Unlike changes in motor initiation, however, these castration-dependent changes occur slowly, indicating that androgens may function on different vocal pathways with distinct timescales.

Distinct effects of castration on fast and slow trills

What is the basis for CPG slowing in castrates? Some insight comes from the sexually dimorphic vocal motoneurons. Male motoneurons exhibit sexually dimorphic levels of two currents—hyperpolarization-activated cation current (I_h) and low-threshold potassium current (I_{KI})—that appear to permit male-specific firing patterns (Yamaguchi et al. 2003). In electric fish, Liu et al. (2007) identified androgen-dependent expression of ion channel variants directly affecting behavior. Likewise, castrate and control CPG neurons may exhibit distinct ionic conductances underlying rhythm generation.

The percentage of castrate brains producing fast trills was lower than that of controls, but the fraction of brains generating

slow trills was similar. Twice as many castrate CPGs produced slow trills than fast trills. What CPG components account for this difference? One possibility is the parabrachial nucleus homologue DTAM (Zornik and Kelley 2008; Zornik et al. 2010), which concentrates androgens (Kelley 1980) and exhibits a local field potential (LFP) wave in which an upward baseline deflection corresponds to fast trill onset, whereas a downward wave precedes slow trills (Zornik et al. 2010). Isolated downward waves accompany isolated slow trills, both of which were observed only in castrates. Thus circuitry generating the upward and downward components of the LFP wave may be distinct. Neurons underlying the upward wave may be more androgen dependent than those producing downward waves, resulting in decreased fast trill generation compared with slow trills. Because only a few recordings were obtained (precluding quantitative analyses), these results remain preliminary. Nevertheless, our results are consistent with the hypothesis that the circuitry underlying fast and slow trills may be distinct.

Castration and body size

Female *X. laevis* are significantly larger than males (Kobel et al. 1996). Castrates grew faster than controls, suggesting that androgens may prevent skeletal and muscular growth (except in the larynx). We cannot rule out a role for other gonadal secretions, but androgen-dependent sexual dimorphisms are reported in many species (Ball and Balthazart 2004; Forger 2009; Kelley 1986). If androgens control size dimorphisms, how might they inhibit growth rather than enhance it? In lizards, testosterone controls sexual size dimorphisms in species with either male- or female-biased differences (Cox et al. 2009). Similarly, electric organ discharges (EODs) of electric fish are sexually dimorphic and androgen dependent; in some species females exhibit faster EODs, whereas others show the opposite pattern (Dunlap and Zakon 1998). Thus androgen secretion and receptor expression are elevated in males of most species, but the downstream effects of androgens are not fixed. Such flexibility provides an evolutionary mechanism for generating diversity in sexual dimorphisms.

The Xenopus vocal CPG: a model for hormone-dependent neural plasticity

Testosterone masculinizes adult female vocalizations (Hannigan and Kelley 1986; Potter et al. 2005). The present study demonstrated that castration of adult males alters vocal CPG function, likely due to extended periods of androgen deprivation. Thus hormone dependence of *Xenopus* vocal pathways appears to be open-ended. Hormone-induced neural plasticity in adulthood is found in many species. For example, song control neurons of seasonally breeding songbirds exhibit steroid-dependent changes in size and functional properties across breeding seasons (Meitzen et al. 2009; Nottebohm 1981; Park et al. 2005). Similarly, androgen masculinizes EODs in adult electric fish (Dunlap et al. 1998) and the sexual dimorphism of the posterodorsal nucleus of the medial amygdala can be reversed with androgen manipulation in adult rats (Cooke et al. 1999). Androgen-induced neural plasticity could account for disadvantaged recovery of human female stroke survivors (Petrea et al. 2009) and testosterone-enhanced recovery in a rat model for stroke (Pan et al. 2005). Thus understanding mechanisms underlying hormone-dependent plasticity in adult vertebrates may have significant clinical ramifications. The *X. laevis* isolated brain preparation holds promise for further research because it allows us to relate discrete cellular changes to the function of a complex network.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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