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Generating Sexually Differentiated Vocal Patterns: Laryngeal Nerve and EMG Recordings from Vocalizing Male and Female African Clawed Frogs (*Xenopus laevis*)

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Male and female African clawed frogs (*Xenopus laevis*) produce sexually dimorphic vocalizations; for males these include advertisement, amplectant, and growling calls, whereas female calls include ticking. Previous studies have shown that the vocal organ, the larynx, of the sexes differs in physiological properties that parallel vocal differences. However, it was not clear whether these characteristics are sufficient to explain sex differences in vocal behavior. To examine the contribution of the CNS to generating vocal patterns, we developed a preparation in which both laryngeal nerve activity and electromyograms can be recorded from awake, vocalizing frogs. Recordings reveal that the CNS of the two sexes produces patterned activity that closely matches each vocalization whereas the larynx faithfully translates nerve activity into sound. Thus, the CNS is the source of sexually differentiated vocalizations in *Xenopus laevis*. Furthermore, detailed analyses of compound action potentials recorded from the nerve lead us to hypothesize that neuronal activity underlying different male call types is distinct; some calls are likely to be generated by synchronous firing of motoneuron populations of either constant size or progressively larger sizes, whereas others are generated by asynchronous activity of motoneurons, a pattern shared with vocal production in females. We suggest that these distinct neuronal activity patterns in males may be subserved by two populations of motor units in males that can be distinguished by the strength of the neuromuscular synapse.

Key words: vocalizations; nerve recordings; electromyograms; Xenopus laevis*; sex differences; compound action potentials*

Males and females of many species produce sexually dimorphic vocalizations to coordinate reproduction. These sexually differentiated vocal behaviors are often accompanied by sex differences in the CNS and in the vocal organ (for review, see Kelley and Tobias, 1999). However, how sex differences in the CNS and the effector organ shape the production of sex-specific behaviors is not yet clear. For example, it has been difficult to determine the relative contributions of the CNS and the peripheral organ to generating a vocal behavior, because the mechanism of sound production is usually complex involving coordinated contraction of multiple muscles during expiration. Thus, our understanding of the mechanism of sexually differentiated vocal production remains primarily anatomical (DeVoogd, 1990; Ball et al., 1994), although some success in assigning sex-specific acoustic features to brain nuclei or motor nerves has been achieved in songbirds (Simpson and Vicario, 1990). Here, we examine the functional contributions of the CNS and the vocal organ to producing sexually differentiated vocalizations using African clawed frogs (*Xenopus laevis*), a vertebrate with a remarkably simple mechanism of vocal production.

Male and female clawed frogs produce a variety of vocalizations made up of clicks that are repeated in distinctive, sex-specific temporal patterns (Kelley and Tobias, 1999). Male-specific vocalizations include advertisement calls, growling, and amplectant calls (see Fig. 1*A*). Female-typical vocalizations include ticking given by nongravid females in response to male clasp attempts (see Fig. 1*B*). The calls of the two sexes differ fundamentally in temporal structure and amplitude profiles. Male calls consist of clicks that cover a wide range of repetition rates (8 Hz in amplectant calls to 80 Hz in growling), whereas female calls consist of clicks at relatively slow repetition rates (3–20 Hz). The amplitude of some male calls, but no female calls, is systematically modulated.

Clicks are generated in the larynx when laryngeal muscles contract and separate a pair of cartilaginous disks (Tobias and Kelley, 1987; Yager, 1992). Thus, the rate at which laryngeal muscles contract and relax is directly translated into the click rate. The larynx of the sexes shows striking differences that mirror vocal dimorphism. For example, male laryngeal muscle is made up of entirely fast twitch fibers, whereas female muscle fibers are mostly slow twitch (Sassoon et al., 1987), so that the male larynx can produce rapid clicks but the female larynx cannot. Moreover, laryngeal neuromuscular synapses in females are mostly strong, whereas most synapses in males are weak and require facilitation to produce muscle action potentials (Tobias and Kelley, 1987, 1988). Facilitation parallels amplitude modulation of click trains in isolated male larynges (Ruel et al., 1998).

To examine the functional contributions of the CNS and the larynx to generating sexually dimorphic vocalizations, we developed a method to record the collective output of laryngeal motor neurons from the laryngeal nerve together with the activity of laryngeal muscles that underlies click production. We also determined whether the firing patterns of laryngeal motoneurons, including firing synchrony and recruitment, differ in the sexes.

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MATERIALS AND METHODS

Animals. Seventeen male and 8 female adult *Xenopus laevis* were obtained commercially (Nasco, Fort Atkinson, WI; *Xenopus* One, Ann Arbor, MI). Three males and 3 females were used for electromyographic (EMG) recordings, 12 males and 5 females were used for laryngeal nerve recordings, and 2 males were used for simultaneous EMG and nerve recordings. *Xenopus* normally do not come into breeding condition, when calls are produced, in captivity. Thus, human chorionic gonadotropin (HCG; Sigma, St. Louis, MO) was administered (600 IU/animal) to induce reproductive vocalizations in males.

Eliciting vocal behaviors. EMG and nerve recordings were obtained while frogs were freely swimming and vocalizing in a polycarbonate tank. A frog of either the same or opposite sex was introduced into the recording tank to enhance vocal activity.

Sound recordings. Sound was antialias filtered at 10 kHz and recorded using a hydrophone (Cornell Bioacoustics Laboratory) and a tape deck (Vetter Instrumentation Recorder Model B; Dagan DAT recorder DAS-75) or digitized directly using MacLab (AD Instruments; sampling rate, 10 kHz). The taped recordings were later digitized at a sampling rate of 10 kHz.

Electrophysiology. Chronic electrodes were surgically implanted into frogs that were anesthetized with ethyl *m*-aminobenzoate methane sulfonic acid (MS-222; 0.013%, 1 ml; Sigma). Laryngeal muscle and nerve were accessed via a small incision (2 cm) on one side of the body caudal to a forelimb. The laryngeal nerve contains only motor neuron axons; electron microscopic studies reveal that there are no muscle spindles or other proprioceptors in laryngeal muscle (Sassoon et al., 1986). Thus, by recording from the laryngeal nerve, we can determine motor output from the CNS without the confounding effect of reafferent sensory feedback. After electrodes were successfully implanted, the opening was sutured leaving ~1 cm of electrode leads protruding from the caudal end of the incision. Frogs usually recovered from surgery within 2 hr, and the recordings were made 6 hr to 2 d after surgery. Frogs did not attempt to displace the electrode leads.

Teflon-coated silver wire (0.125 mm bare diameter; A-M systems, Everett, WA) was formed into bipolar electrodes and used for both EMG and nerve compound action potential (CAP) recordings. Both nerve and EMG electrode tips were separated by 0.5 mm. To secure the electrodes, we glued (Krazy Glue; Elmer's Products, Columbus, OH) the lead wires to the laryngeal cartilage ~0.2 mm distant from the tips. EMG electrodes, insulated to their tips, were inserted into the dorsal, caudal laryngeal muscle. Nerve electrodes were hooks with ~0.2 mm of exposed silver wire at the tips. The laryngeal nerve was electrically isolated from the rest of the body using either silicon (World Precision Instruments, Sarasota, FL) or dental cement coating (Kerr Laboratory, Romulus, MI) that encased both the nerve and electrode tips. Nerve recordings obtained using both dental cement and silicon were identical, and these data were pooled for further analyses.

To examine the effects of electrode tip spacing, we used an electrode with three tips separated by 0.5 mm to record laryngeal nerve activity from one male and one female. This configuration allowed us to record nerve activity using 1 and 0.5 mm electrode tip distance as well as to compare two different combinations of 0.5 mm spacings.

Differential outputs from the electrode were amplified (Grass Preamplifier P15; Warner Instrument Differential Amplifier DP-301) and recorded along with acoustic recordings of the vocalizations either onto a tape recorder or digitized directly as described above. Digitized EMG and nerve recordings were bandpass filtered (60–800 Hz pass band; Igor Filter Design Laboratory, Wavemetrics, Lake Oswego, OR) to remove 60 Hz and high-frequency noise.

Nerve recordings revealed systematic differences in CAP duration across vocalizations (see below). The duration of the CAP can reflect the degree of synchronous motoneuron activity. To examine this question directly, we simultaneously activated all laryngeal motor neuron axons in an *in vitro* preparation and compared nerve CAPs with those recorded *in vivo*. Three female and four male adult frogs were anesthetized using MS-222 and perfused with oxygenated saline [75 mM NaCl, 25 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM KCl, 0.5 mM $MgCl_2$, and 11 mM glucose, pH 7.3 (Luksch et al., 1996)]. A bipolar electrode was placed on the laryngeal nerve as in the *in vivo* preparation described above. The dorsal surface of the brain was exposed by removing the skull. The fourth rootlet of cranial nerve IX–X, which contains all the laryngeal motoneuron axons (Simpson et al., 1986), was cut, and its distal end was drawn into a suction electrode. The nerve was stimulated with 10-μsec-long bipolar pulses at a voltage 50% greater than that required to evoke a maximum CAP amplitude (approximately +6 V). Recordings were made at 21.4°C, the temperature of *in vivo* recordings. For each animal, 60 recordings were averaged to measure CAP duration.

Compound action potentials. The duration and the peak amplitude of CAPs and EMGs were measured. All the recordings were first rectified and then smoothed by 50 points to remove noise, and the measurements were made using an on-screen cursor (Igor Pro; Wavemetrics).

The CAP duration from the nerve was defined as the time during which the recording trace was above the mean noise level. The durations of 30 CAPs subserving each call type were averaged from each individual used for *in vivo* and *in vitro* recordings and were then subjected to statistical analysis. Advertisement calls of males consist of alternating fast and slow trills (Fig. 1*A3*). These two components differ in both sound amplitude and click repetition rate and were therefore treated as different call types for further analysis.

To determine whether the click repetition rate of vocalizations dictates the CAP duration, we measured five exemplars of inter-CAP intervals and CAP durations subserving each call type from each individual. For statistical analysis, mean duration and interval were used. Inter-CAP interval was defined as the time between the onset of the CAP of interest and the onset of the following CAP.

Peak amplitudes of nerve CAPs and EMGs were measured using Igor Pro. Thirty nerve CAPs and EMGs subserving each call were sampled, and the coefficient of variation of each individual frog was calculated to assess the call-dependent variation in CAP and EMG peak amplitude. To illustrate the increase in CAP and EMG amplitude during the fast trill, CAPs and EMGs accompanying three episodes of fast trilling were sampled from all males, and the peak amplitudes of CAPs and EMGs underlying seven consecutive clicks were measured using Igor. Each data point was standardized to the first EMG or CAP amplitude within a trill, and this ratio, here called the potentiation factor, was used for further statistical analysis.

To compare the degree with which the nerve and muscle potentiate during the fast trill of advertisement calling, five fast trills were sampled from two males with EMG and nerve electrode implants, and potentiation factors for CAPs and EMGs were calculated.

Statistical analysis. We examined whether the CAPs that accompany each call type differ in duration using an ANOVA. The following five *post hoc* comparisons were performed using the Mann–Whitney *U* test with the family confidence interval for the comparisons set at 95% with sequential Bonferroni adjustment [i.e., statistical significance required to reject H_0 is set at 0.01, 0.013, 0.017, 0.025, and 0.05 for the most significant to the least significant five comparisons (Rice, 1989; Wright, 1992)]: growling versus fast trill, fast trill versus slow trill, slow trill versus amplectant call, amplectant call versus fast tick, and fast tick versus slow tick. The Wilcoxon signed rank test was used to compare fast and slow trills of advertisement calls and fast and slow ticks of females because each pair of data sets was obtained from the same individual (i.e., paired). To examine the relation between the inter-CAP interval and the CAP duration, a regression analysis was performed after the independent variable, the inter-CAP interval, was log transformed.

To determine the relation between the CAP duration and the electrode tip distance, CAPs obtained from a male and a female implanted with a three-tip nerve electrode were subjected to an ANOVA. A one-factor ANOVA was used for female data (electrode combination, three levels), and a two-factor ANOVA was used for male data (electrode combination, three levels; call types, four levels). A two-factor ANOVA was also used to determine whether variation in the CAP duration recorded *in vivo* was influenced by sex (two levels) or call type (five levels).

To compare the variability of the peak amplitude of CAPs and EMGs, a one-factor ANOVA (call types, four levels) was performed on the coefficient of variation, and four *post hoc* comparisons were made using the Mann–Whitney *U* test with the family confidence interval for the comparisons set at 95% with sequential Bonferroni adjustment.

To determine whether the increase in EMG amplitude is greater than the increase seen in CAP amplitude during fast trills, potentiation factors obtained from seven consecutive EMGs or CAPs were averaged for five trills sampled from each of two males with simultaneous recordings of EMG and CAP and were compared using a two-factor ANOVA with animals and nerve/EMG as the factors.

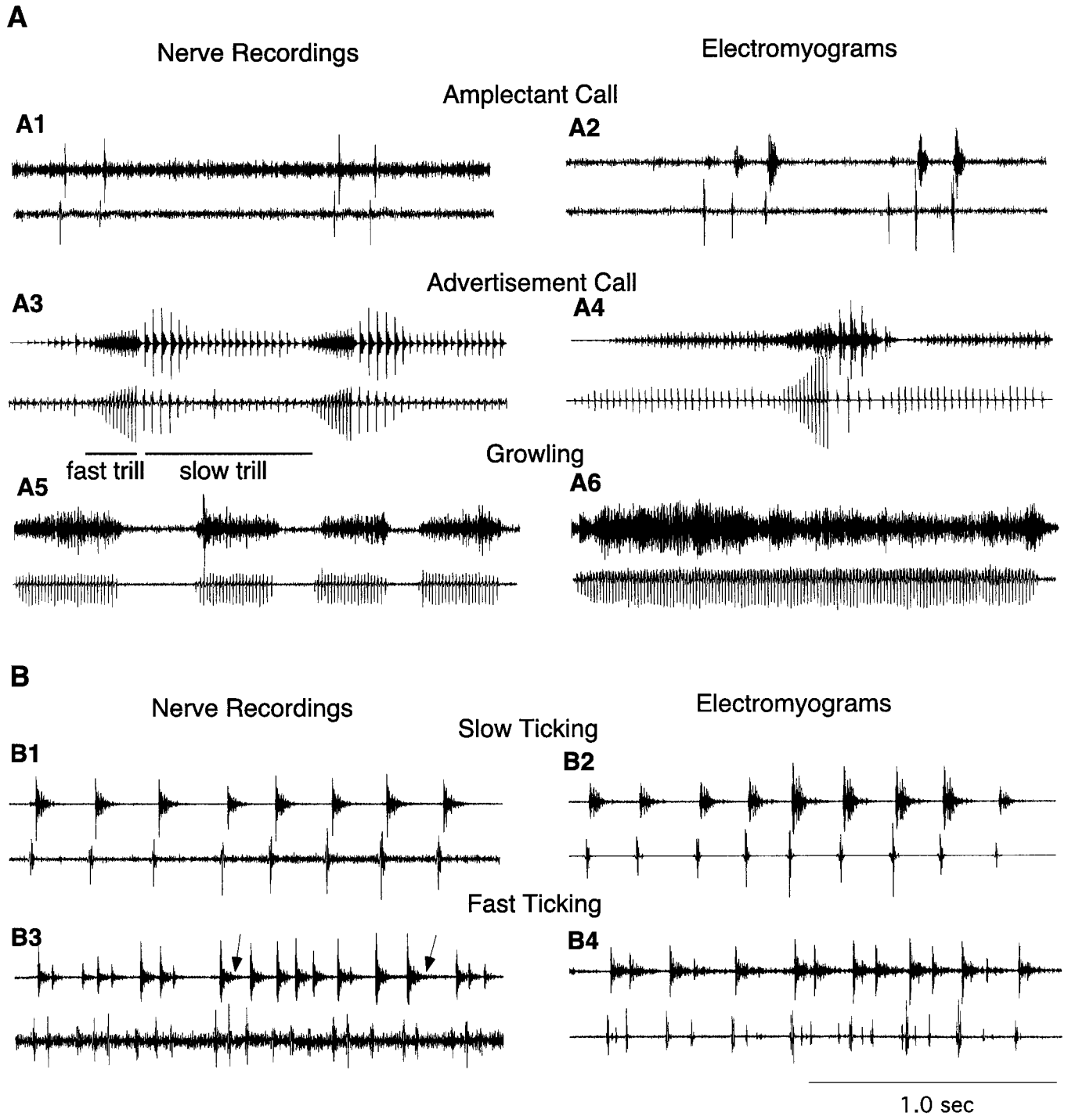

Figure 1. Nerve and EMG recordings along with sound amplitude waveforms of male and female vocalizations. The *top trace* of each *panel* is the amplitude waveform (sound amplitude over time); the *bottom trace* is either the nerve or the EMG recording (voltage over time). *Left,* Nerve recordings. *Right,* EMG recordings. *A,* Male vocalizations. *A1, A2,* Nerve (*A1*) and EMG (*A2*) recordings during an amplectant call. *A3, A4,* Nerve (*A3*) and EMG (*A4*) recordings during an advertisement call. Fast and slow trills are shown. *A5, A6,* Nerve (*A5*) and EMG (*A6*) recordings during growling. *B,* Female vocalizations. *B1, B2,* Nerve (*B1*) and EMG (*B2*) recordings during slow ticking. *B3, B4,* Nerve (*B3*) and EMG (*B4*) recordings during fast ticking. *Arrows* in *B3* indicate where a click sound was expected in response to nerve activity. The variation in the duration of the clicks seen in the sound amplitude waveform is caused by the variable recording environment under water.

RESULTS

Temporal patterns of nerve and EMG recordings

Electrode implantation did not abolish the ability of frogs to produce vocalizations. Males produced three calls (Fig. 1*A*, amplectant calls, advertisement calls, and growling), and females produced one call, ticking, at slow and fast rates (Fig. 1*B*). Nerve and EMG recordings accompanying ticking were sampled from all 5 and 3 females with nerve and EMG electrodes, respectively. Nerve recordings during amplectant calling, advertisement calling, and growling were obtained from 5, 11, and 6 males, respec-

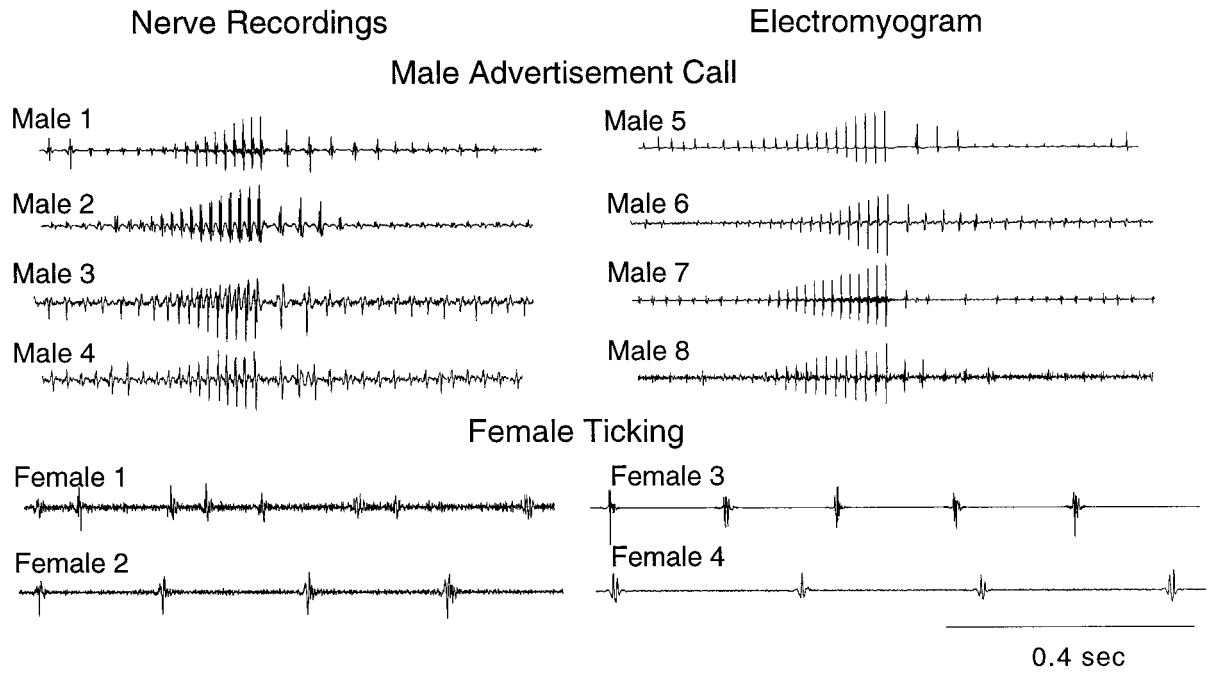

Figure 2. Nerve and EMG recordings are stereotyped across individuals. *Left*, Nerve recordings of advertisement calls recorded from four males and ticking recorded from two females. *Right*, EMG recordings of advertisement calls recorded from four males and ticking recorded from two females.

tively, and EMG recordings for the three calls were sampled from 2, 5, and 5 males, respectively.

The temporal structures of nerve and electromyographic recordings are strikingly similar to the call types being produced and thus quite distinct for the two sexes (Fig. 1). Each click in every vocalization is typically preceded by discrete laryngeal nerve and muscle activity. Because the temporal structure of each call type is highly stereotyped across different individuals, the overall temporal patterns of nerve and EMG activity were very similar from one individual to another in all calls examined (>5 calls/individual), as illustrated for advertisement calling and ticking in Figure 2. Thus, the call actually being produced can be reliably determined by inspection of electrophysiological recordings from the larynx or its motor nerve.

The larynx does very little to modify the temporal patterns of neuronal activity that descend from the CNS. Although an episode of discrete nerve activity that fails to produce sound is observed occasionally in both sexes, this is a rare event. For example, in males, the initial nerve activity for advertisement calls sometimes fails to produce audible clicks (Fig. 1*A3*, see *beginning* of *traces*). Typically, the failure occurs during the first call of a prolonged calling bout that can contain several hundred calls. In females, nerve compound action potentials also sometimes fail to produce clicks (Fig. 1*B3*, failures to produce sound indicated by *arrows*). These failures were infrequent (<4% of all clicks) and occurred for both fast and slow ticking. When these failures were examined with respect to inter-CAP intervals, failures were equally likely to follow short and long intervals (Wilcoxon signed rank test, $Z = -1.604$; $p = 0.109$). This result rules out the possibility that the female larynx acts as a low-pass filter for CNS output that includes some high-frequency neuronal activity. We conclude that the overall temporal patterns of vocalizations of *Xenopus laevis* originate in the CNS and that the larynx primarily translates sexually distinct nerve activities into sound faithfully.

Having established that the motor output from the CNS is differentiated to match sex-specific vocal patterns, we next examined, in detail, the shape of CAPs recorded in males and females. In particular we focused on the duration of CAPs that reflects the degree of firing synchrony of laryngeal motoneurons and on the peak amplitude of CAPs that allows us to estimate the size of the active motoneuron population (Jacklet, 1988).

The duration of nerve compound action potentials and click repetition rates

The durations of CAPs in the laryngeal nerve are similar in males and females [Fig. 3*A*, $F_{(1,38)} = 2.16$; $p = 0.15$] but do show systematic variation across call type [Fig. 3*A*, $F_{(5,34)} = 7.01$; $p < 0.001$]. CAPs that accompany growling are significantly shorter than those underlying the fast trill of advertisement calling. CAPs of fast trills are significantly shorter than those of slow trills. In contrast, CAPs subserving the slow trill and amplectant calls, amplectant calls and fast ticks, and fast ticks and slow ticks did not differ significantly in duration (Fig. 3*A*).

The exact shape of the nerve activity recorded using bipolar electrodes could depend on the placement of the electrode and on the distance between the electrode tips so that the observed variation in CAPs could be an artifact of electrode configuration. To rule out this possibility, we recorded laryngeal nerve activity from an electrode with three tips separated by 0.5 mm in one male and one female (Fig. 4). The CAP durations measured using two different electrode tip distances with three different electrode combinations (two combinations with a 0.5 mm tip distance and one with a 1 mm tip distance) revealed that CAP durations consistently vary across call types [Fig. 4*B*, male, $F_{(2,290)} = 133.5$; $p < 0.001$]. Within each call type, the CAP duration measured using three electrode tip combinations did not differ [Fig. 4*B*, male, $F_{(1,290)} = 0.797$; $p = 0.373$; female, $F_{(2,87)} = 0.49$; $p = 0.614$]. Thus, call type-dependent variation in CAP duration cannot be explained by the electrode configuration.

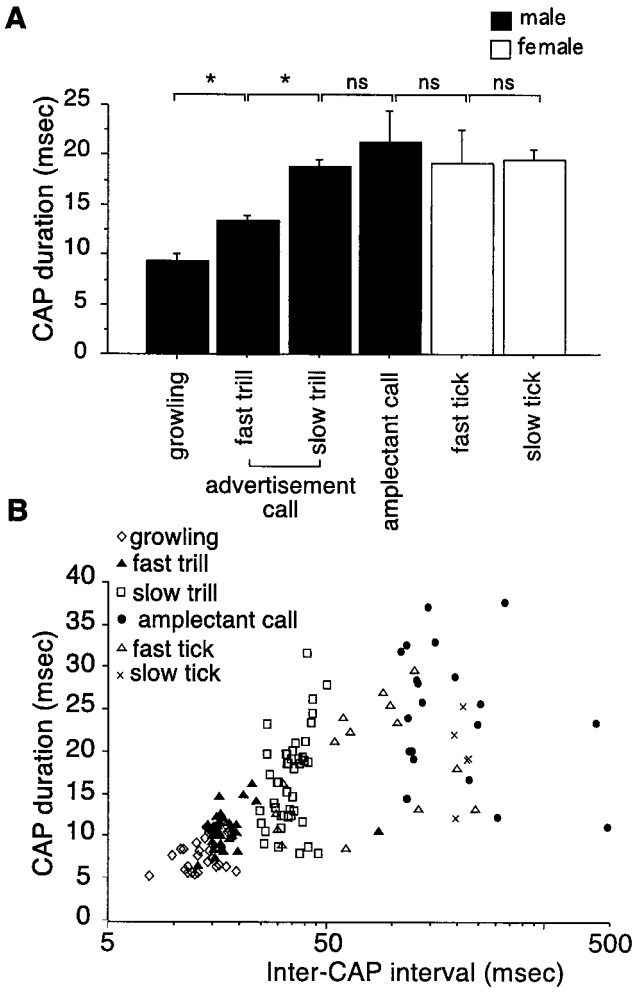

Figure 3. CAP durations vary across call types. *A,* Cell plots of CAP durations for male and female vocalizations. Each *vertical bar* is a mean CAP duration, and each error bar is a SE. The mean ± SE CAP duration for each call type was 9.3 ± 0.8 msec for growling, 13.4 ± 0.5 msec for the fast trill, 18.8 ± 0.8 msec for the slow trill, 21.3 ± 3.2 msec for amplectant clicks, 19.8 ± 1.6 msec for the female slow tick (3–8 Hz), and 19.1 ± 3.5 msec for the female fast tick (8–20 Hz). Statistically significant comparisons are marked with *asterisks*, and nonsignificant comparisons are marked as *ns*. Test statistic and p values of five comparisons are listed as follows: growling versus fast trill, $Z = -2.69$ and $p < 0.01$; fast trill versus slow trill, $Z = -2.8$ and $p < 0.01$; slow trill versus an amplectant call, $Z = 0$ and $p > 0.99$; an amplectant call versus fast tick, $Z = -0.11$ and $p = 0.91$; and fast tick versus slow tick, $Z = -0.15$ and $p = 0.88$. The Wilcoxon signed rank test was used for fast and slow trill and fast and slow tick comparisons because the data were obtained from the same individuals (i.e., paired comparison), whereas the Mann–Whitney U test was used for other comparisons. *B,* Scatter plots of the CAP duration against the inter-CAP interval (logarithmic scale). Five data points obtained from each individual for each call type are presented. Notice that the association between the CAP duration and the inter-CAP interval is very tight at the lower range of ICIs but not at the higher range.

The different durations of CAPs may be related to interclick intervals (ICI) for different call types. For example, nerve CAPs that generate fast clicks such as growling must be short because contraction and relaxation of laryngeal muscle have to be complete within one interclick interval (i.e., before the next cycle of click generation begins). To determine the nature of the relation between CAP duration and ICI, a regression analysis was performed. ICIs were estimated using inter-CAP intervals. Inter-CAP intervals were log transformed and subjected to simple linear regression analysis. The result of this analysis indicates that ICIs significantly explain variations in CAP duration [$R^2 = 0.583$; $F_{(1,29)} = 540.984$; $p < 0.01$]. Examination of the regression plot (Fig. 3*B*) reveals that the association between CAP duration and ICI is very tight for calls with fast-click repetition rates but looser for calls with slow-click repetition rates. We conclude that rapid calls, characteristic of males, require short CAPs whereas slower calls, in both sexes, can be produced by either short or long CAPs.

Nerve CAPs and the firing synchrony of laryngeal motoneurons

Differences in CAP duration reflect differences in the degree to which motor neurons fire synchronously and the conduction velocity of motor axons. To examine these contributions, we produced simultaneous firing of all laryngeal nerve axons by electrical stimulation of the nerve *in vitro* and compared results with CAPs recorded during vocalization *in vivo*. In the *in vitro* preparation, a stimulus pulse was applied to the nerve at the point where laryngeal axons exit the medulla, and CAPs were recorded at the same point as in *in vivo* recordings. In males, the average CAP duration resulting from synchronous activation of laryngeal axons was 7.7 ± 1.5 msec (mean ± SE), a value that is not significantly different from CAP durations recorded *in vivo* during growling in males (9.3 ± 0.8 msec; Mann–Whitney U test, $Z = -1.043$; $p = 0.297$). Thus, the short CAP durations associated with growling reflect nearly synchronous activity of laryngeal motoneurons. The long CAP durations associated with slow male calls, such as amplectant calls, are likely to be subserved by asynchronous activity of motoneurons. In females, CAP durations resulting from induced synchronous firing of axons in the laryngeal nerve were similar to those of males (6.5 ± 0.2 msec) and briefer than any associated with ticking (either fast or slow), suggesting that long CAPs in females represent asynchronous firing of motoneurons.

The peak amplitude of the nerve CAP, EMG, and sound amplitude

The peak amplitude of the nerve CAP also displays call-dependent variation (Fig. 5*A*). The peak CAP amplitude subserving growling had a significantly lower coefficient of variation compared with that of CAPs of other call types, indicating that CAP amplitude during growling is relatively monotonous whereas CAP amplitudes underlying other call types (the fast trill, the slow trill of advertisement calling, amplectant calling, and ticking) are highly variable (Fig. 5*A*). Although the peak amplitudes of CAPs associated with the slower call types appear to vary randomly, those associated with the fast trill portion of the advertisement call increase progressively (Fig. 5*D*). These amplitude profiles of CAPs are preserved when recordings were made with different configurations of electrodes as described above; the amplitude of CAPs subserving growling shows the smallest variation (Fig. 5*C*), whereas those underlying the fast trill show progressive increases in amplitude (Fig. 5*F*) regardless of the electrodes used.

Because the CAPs associated with growling and the fast trill (calls with the most rapid click rates) are all short in duration, peak amplitudes most likely represent summed APs that arrive together, with little phase cancellation, at the electrode. Accordingly, the peak amplitude of short CAPs approximately estimates the population size of axons producing the CAP. We can infer, then, that growling is generated by activity of a motoneuron population of constant size and that the fast trill is produced by a

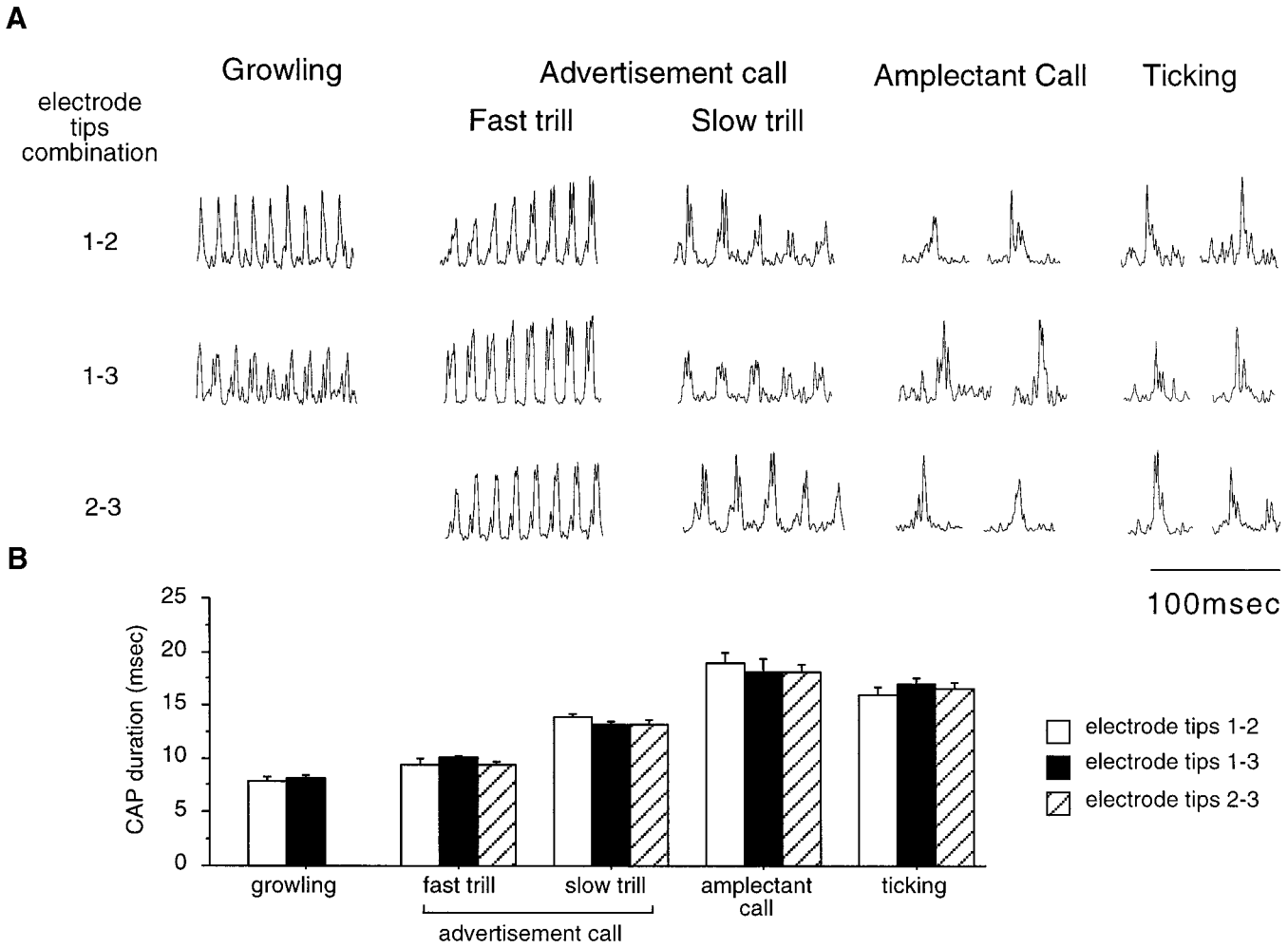

Figure 4. Call-dependent variation in the CAP duration is not caused by electrode configuration. *A,* Nerve recordings using a three-tipped electrode. Electrode tip combinations 1 and 2 and also 2 and 3 are separated by 0.5 mm; combination 1 and 3 is separated by 1 mm. CAPs of all male and female vocalizations using three electrode tip combinations are shown. The original recordings were rectified and smoothed by 50 points. Growling was not recorded with the electrode tip combination 2 and 3. *B,* Cell plot of CAP durations of each call type using three different combinations of electrode tips. The CAP duration varies across different vocalization types but does not depend on the electrode tip combination.

population of motoneurons of progressively larger size, with more being active at the end of the trill than at the beginning. CAPs with variable amplitude, in contrast, are always long in duration. The irregularity of peak amplitudes is probably the result of APs produced by the asynchronous firing of motoneurons that arrive at the electrode at different times with significant phase cancellation. The population size of axons producing these long CAPs therefore cannot easily be estimated from their peak amplitudes.

Peak amplitudes of EMGs did not show call-dependent variation (Fig. 5*B*), although this is likely to be caused by a smaller sample size. Despite statistical insignificance, the trend was that CAPs with stereotyped amplitudes (growling and fast trills) generate EMGs that closely match the CAP amplitude profiles; the EMG amplitude is relatively constant during growling (Fig. 5*B*) and increases progressively during fast trills (Fig. 5*E*). The constant amplitude of the CAPs and EMGs indicates that the larynx does not modify the amplitude information conveyed from the CNS during growling. Increasing EMG amplitudes during the fast trill, in contrast, could reflect amplification of neuronal activity by facilitation at the neuromuscular synapse. To examine this possibility, we compared EMG amplitude increases with nerve CAP amplitude increases. The peak amplitudes of EMG and CAP recordings during fast trills were measured from two males in which simultaneous nerve and EMG recordings were obtained (Fig. 6). During the fast trill of the advertisement call, the amplitude increase of EMGs is greater than the amplitude increase of nerve CAPs [$F_{(1,16)} = 7.652$; $p = 0.014$]. This difference may represent the potentiation of laryngeal neuromuscular synapses in response to a rapid series of nerve action potentials, although we cannot rule out the possibility that, as the trill progresses, motor units with larger sizes are recruited. The former interpretation would signify a role of the larynx as an amplifier of neuronal signals during fast trills.

Sound amplitude in the fast trill, in turn, parallels CAP and EMG amplitude; the fast trill becomes progressively louder throughout (Fig. 1*A3,A4*). Thus, the CNS can encode amplitude information for some call types in males.

DISCUSSION

The CNS generates sexually differentiated vocal patterns

The temporal structure of laryngeal nerve and muscle activity recorded from vocalizing male and female *Xenopus* is remarkably

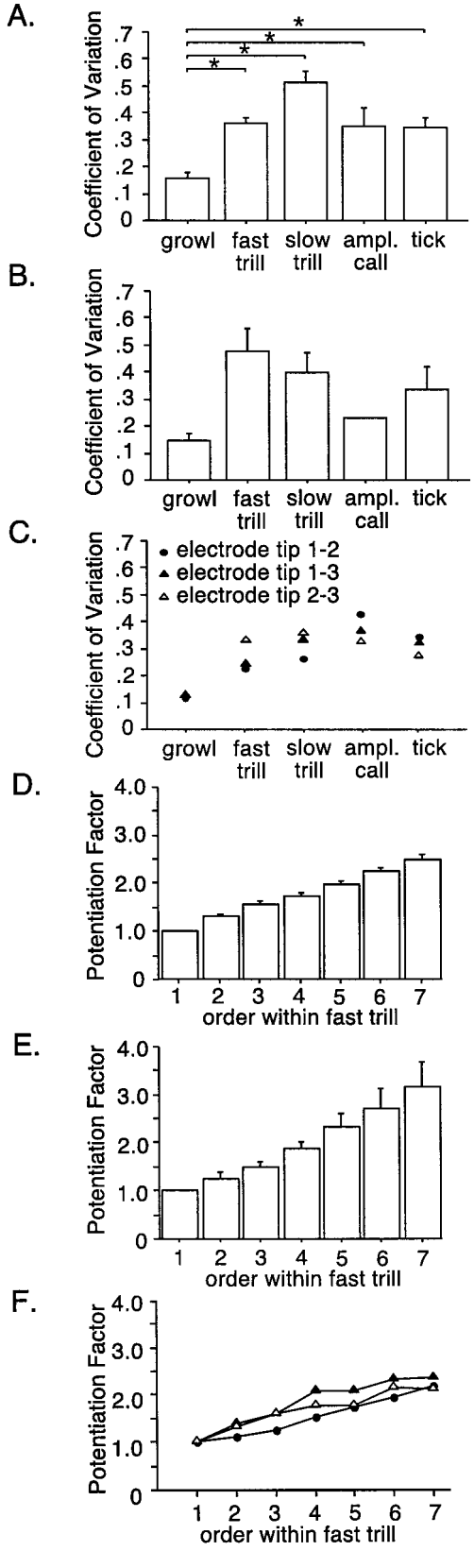

Figure 5. Call-dependent variation in the amplitude of CAP and EMG as indicated by the coefficient of variation (CV). *A,* A cell plot of the CV derived from the peak CAP amplitude across call types. Variation in the CV is significantly explained by call types [$F_{(4,34)} = 10.487$; $p < 0.0001$]. The CV for growling (*growl*) is significantly different from that of fast trill ($Z = -3.317$; $p = 0.0009$), slow trill ($Z = -3.317$; $p = 0.0009$), the amplectant call (*ampl. call*; $Z = -1.922$; $p = 0.05$), and ticking (*tick*; $Z = -2.739$; $p = 0.007$). *B,* A cell plot of the CV derived from the peak EMG amplitude across call types. Variation in the CV is not significantly explained by call types [$F_{(4,12)} = 2.504$; $p = 0.0979$]. *C,* A scatter plot showing the CV of peak CAP amplitude measured using a three-tip electrode configuration. *D,* A cell plot of the CAP potentiation factor underlying fast trill of the advertisement call. *E,* A cell plot of the EMG potentiation factor underlying fast trill of the advertisement call. *F,* A scatter plot of the CAP potentiation factor during fast trill of the advertisement call, using three configurations of electrodes. See *C* for electrode tip combinations. *Asterisks* indicate statistically significant comparisons.

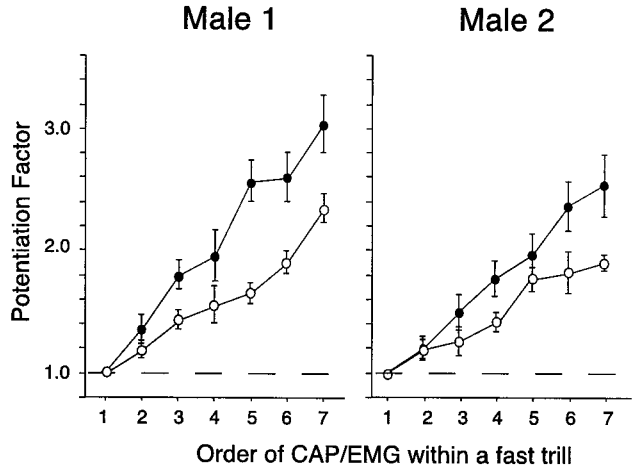

Figure 6. Potentiation factors for nerve CAPs and for EMGs. The increase in the peak amplitude of nerve CAPs (*open circles*) and EMGs (*filled circles*) during the fast trill of advertisement calls is illustrated for two males in which both nerve and EMG recordings were made simultaneously. Each data point was standardized to the first peak amplitude of the fast trill. Each plot indicates the mean and SE of five trills sampled from each male. Notice that the potentiation factor for the EMG is greater than that for the nerve CAP.

similar to the vocalizations themselves and thus is sex specific. In addition, the CNS generates neuronal activity that produces sound amplitude modulation, another sex-specific feature of vocal behaviors. Temporal patterns generated by the CNS are modified very little by the larynx in either sex, whereas amplitude information could be further amplified by the larynx in males. Thus, the CNS plays a dominant role in generating sex-specific temporal and amplitude profiles of vocalizations, whereas the larynx translates the nerve activity into sound. In wave-generating weakly electric fish, the pacemaker nucleus in the CNS regulates the overall frequency of the electric organ discharge (EOD), whereas the electrocytes determine the exact shape of each EOD waveform (Meyer, 1984; Ferrari et al., 1995; Zakon, 1996). In this system, and in the *Xenopus* vocal system, both the CNS and the periphery of the two sexes are functionally differentiated. In contrast, pulse-generating electric fish, such as some mormyrids, produce male- and female-typical pulse shapes using only functional differences in electrocytes. The input to the electrocytes is the same in the two sexes, and the electrocytes act as peripheral filters to produce specific male and female discharge patterns (Bass and Hopkins, 1983; Hagedorn and Carr, 1985; Freedman et al., 1989). This exclusive sexual differentiation of the periphery paired with a shared CNS pattern generator may be rare.

The pattern generator

During the fast trill of advertisement calling in males, increasing numbers of motoneurons are recruited while their collective firing

rate remains constant. We do not know whether a particular motoneuron, after being recruited, participates in the entire series of CAPs. However, this pattern of neuronal participation would be the simplest mechanism to generate CAPs observed during the fast trill. If this assumption is correct, the observation provides clues to the mechanism by which firing rates are generated by the male CNS. One way of producing gradual recruitment of motoneurons is to have weak, facilitating presynaptic inputs. In such a circuit, motoneurons act as capacitors; an increase in the presynaptic firing rate enhances the probability that each motoneuron will fire and thus increases the total number of motoneurons activated. In this circuit, however, more motoneurons cannot be recruited unless the overall firing rate increases. We do not observe such an increase. Thus, our data suggest an alternative mechanism in which the firing rate of motoneurons is controlled independent of the recruitment of motoneurons. Control may be achieved by distinct pattern generators as in other motor systems that control rhythmic movement (Roberts et al., 1998). The recruitment of motoneurons could also be controlled by interneurons that change motoneuron-firing thresholds or that modify the strength of their presynaptic inputs.

How the CNS generates sex-specific temporal patterns is not known in *Xenopus*. In other species of anurans, such as *Rana pipiens*, the pretrigeminal nucleus of the dorsal tegmental area of the medulla (DTAM) acts as a vocal pulse generator (Schmidt, 1974, 1976). In both *Xenopus* and other anurans, DTAM innervates laryngeal motoneurons directly (Wetzel et al., 1985). Whether DTAM is involved in generating the vocal patterns remains to be addressed.

Firing activity of motor neurons

Close examination of nerve recordings revealed that overall patterns of laryngeal motoneuron activity differ depending on the call types produced. This variation in patterned neuronal activity appears to be dictated by the click repetition rates of each call type. Previously, we deduced that a population of male laryngeal motoneurons fires nearly synchronously to produce fast call types such as growling and fast trills. To produce slow call types such as amplectant calls, male motoneurons fire asynchronously. Female laryngeal motoneurons always fire asynchronously to produce ticking. Thus the male, but not the female, CNS is equipped with a mechanism(s) to synchronize activity of a population of motoneurons that generate fast call types.

Synchronization of motoneuron activity could be achieved at the level of the motoneurons themselves. One potential cellular mechanism is electrical coupling. The degree of electrical coupling among some motoneurons is sexually differentiated in an androgen-dependent manner. For example, the motoneurons of the spinal nucleus of the bulbocavernosus in rats are dye-coupled, and androgen treatment increases gap junction expression (Matsumoto et al., 1988). The firing of axons innervating the clasping muscles of *Xenopus laevis* is more synchronous in HCG-injected males, with elevated androgen levels, than in uninjected males (Erulkar et al., 1981). Similarly, it is possible that, in response to elevated levels of circulating androgen, electrical coupling in a subpopulation of laryngeal motoneurons in male *Xenopus* is increased and that motoneurons fire synchronously and generate fast clicks.

Separate populations of motor units may be involved in producing different call types

The call-dependent variation in the degree of synchronous firing and the patterned recruitment of laryngeal motoneurons in males raise the possibility that different populations of motor units are involved in generating different types of male calls. Previous studies suggest the presence of at least two types of motor units in the male laryngeal motor system that are distinguishable by the strength of the neuromuscular synapse. These different motor units may be responsible for generating different call types.

Laryngeal synapses can be divided into physiologically distinct types (Tobias and Kelley, 1988). Type II synapses (predominant in male larynx; ~70%) produce only subthreshold muscle potentials in response to a single nerve shock regardless of voltage; these synapses require repetitive nerve activity for muscle action potential production because of facilitation (Ruel et al., 1998). Type III synapses (predominant in female larynx; ~75%) reliably produce muscle APs in response to any suprathreshold nerve shock. In the male larynx, ~21% of synapses are type III. All muscle fibers innervated by a single motoneuron are likely to have the same type of synapses so that the collective output of the motoneuron can be integrated. Thus, we propose that males have two kinds of motor units, a weak motor unit and a strong motor unit.

These two types of motor units with different synaptic strength are, in turn, expected to show functional differences in their ability to produce sound; weak motor units act as a high-pass filter of neuronal activity and translate only high rates of firing by motoneurons into muscle contraction, whereas strong motor units can translate both low and high rates of neuronal activity. Our nerve recordings show that some male calls, such as amplectant calls, are subserved by CAPs repeated at rates too slow to induce facilitation at the weak laryngeal synapse. Thus, we suggest that slow call types in males are produced by strong motor units. Other call types with faster click rates could be subserved by either or both strong and weak motor units because their click repetition rates are fast enough for facilitation and because strong synapses can reliably follow fast rates of nerve activity (Tobias and Kelley, 1988).

If the two types of motor units are in fact responsible for different call types, their firing synchrony might also be different. Because amplectant calls are generated by asynchronous activity of motoneurons, we suggest that strong motor units involved in generating amplectant calls fire asynchronously. Female ticking is also subserved by asynchronous activity of motoneurons, and the majority of female motor units are strong. This observation reinforces the suggestion that strong motor units are activated asynchronously to produce clicks at slow rates regardless of sex. Strong and weak motor units responsible for faster call types, in contrast, may be equipped with a mechanism that synchronizes their firing activity. Further examination of the motoneurons associated with strong and weak motor units may reveal differences in intrinsic properties such as the maximum firing frequency, permitting an examination of functional relations between the activity of motoneurons, their muscle fibers, and vocal production.

In conclusion, we have established an informative experimental preparation in which the output of the CNS and the activity of the peripheral organ can be systematically observed while the animals vocalize. These observations reveal that the sexually distinct calls of male and female *Xenopus laevis* are generated by the CNS, whereas the neuromuscular periphery translates the neuronal activity to sound with the capacity to further amplify amplitude information in males. Further analysis of compound action potentials allowed us to deduce separable neuronal activities underlying vocalization type; fast call types of males are likely to be

generated by nearly synchronous firing of motoneurons that are recruited systematically, whereas slow call types of males and females appear to be generated by asynchronous firing. We hypothesize that different call types of males are likely to be generated by different types of motor units; weak and strong motor unit types in males produce male typical fast call types, and a strong motor unit in both sexes produces slow call types. Direct tests of this hypothesis, as well as anatomical and physiological characterization of laryngeal motoneurons, will now be a focus of our inquiries.

REFERENCES

Ball GF, Castro JM, Bernard DJ (1994) Sex differences in the volume of avian song control nuclei: comparative studies and the issue of brain nucleus delineation. Psychoneuroendocrinology 19:485–504.

Bass AH, Hopkins CD (1983) Hormonal control of sexual differentiation: changes in electric organ discharge waveform. Science 220:971–974.

DeVoogd TJ (1990) Recent findings on the development of dimorphic anatomy in the avian song system. J Exp Zool Suppl 4:183–186.

Erulkar SD, Kelley DB, Jurman ME, Zemlan FP, Schneider GT, Krieger NR (1981) Modulation of the neural control of the clasp reflex in male *Xenopus laevis* by androgens: a multidisciplinary study. Proc Natl Acad Sci USA 78:5876–5880.

Ferrari MB, McAnelly ML, Zakon HH (1995) Individual variation in and androgen-modulation of the sodium current in electric organ. J Neurosci 15:4023–4032.

Freedman EG, Olyarchuk J, Marchaterre MA, Bass AH (1989) A temporal analysis of testosterone-induced changes in electric organs and electric organ discharges of mormyrid fishes. J Neurobiol 20:619–634.

Hagedorn M, Carr C (1985) Single electrocytes produce a sexually dimorphic signal in South American electric fish, *Hypopomus occidentalis* (Gymnotiformes, Hypopomidae). J Comp Physiol [A] 156:511–523.

Jacklet JW (1988) Circadian pacemaker neurons: membranes and molecules. J Physiol (Paris) 83:164–171.

Kelley D, Tobias ML (1999) The vocal repertoire of *Xenopus laevis*. In: Neural mechanisms of communication (Hauser M, Konishi M, eds), pp 9–35. Cambridge, MA: MIT.

Luksch H, Walkowiak W, Munoz A, ten Donkelaar HJ (1996) The use of *in vitro* preparations of the isolated amphibian central nervous system in neuroanatomy and electrophysiology. J Neurosci Methods 70:91–102.

Matsumoto A, Arnold AP, Zampighi GA, Micevych PE (1988) Androgenic regulation of gap junctions between motoneurons in the rat spinal cord. J Neurosci 8:4177–4183.

Meyer JH (1984) Steroid influences upon discharge frequencies of intact and isolated pacemakers of weakly electric fish. J Comp Physiol [A] 154:659–668.

Rice WR (1989) Analyzing tables of statistical tests. Evolution 43:223–225.

Roberts A, Soffe SR, Wolf ES, Yoshida M, Zhao F-Y (1998) Central circuits controlling locomotion in young frog tadpoles. Ann NY Acad Sci 360:19–34.

Ruel TD, Kelley DB, Tobias ML (1998) Facilitation at the sexually differentiated laryngeal synapse of *Xenopus laevis*. J Comp Physiol [A] 182:35–42.

Sassoon D, Segil N, Kelley D (1986) Androgen-induced myogenesis and chondrogenesis in the larynx of *Xenopus laevis*. Dev Biol 113:135–140.

Sassoon D, Gray G, Kelley D (1987) Androgen regulation of muscle fiber type in the sexually dimorphic larynx of *Xenopus laevis*. J Neurosci 7:3198–3206.

Schmidt R (1974) Neural correlates of frog calling. Trigeminal tegmentum. J Comp Physiol [A] 92:229–254.

Schmidt R (1976) Neural correlates of frog calling. Isolated brainstem. J Comp Physiol [A] 108:99–113.

Simpson HB, Vicario DS (1990) Brain pathways for learned and unlearned vocalizations differ in zebra finches. J Neurosci 10:1541–1556.

Simpson HB, Tobias ML, Kelley DB (1986) Origin and identification of fibers in the cranial nerve IX-X complex of *Xenopus laevis*: Lucifer yellow backfills *in vitro*. J Comp Neurol 244:430–444.

Tobias ML, Kelley D (1987) Vocalizations of a sexually dimorphic isolated larynx: peripheral constraints on behavioral expression. J Neurosci 7:3191–3197.

Tobias ML, Kelley DB (1988) Electrophysiology and dye-coupling are sexually dimorphic characteristics of individual laryngeal muscle fibers in *Xenopus laevis*. J Neurosci 8:2422–2429.

Wetzel D, Haerter U, Kelley D (1985) A proposed efferent pathway for mate calling in South African clawed frogs, *Xenopus laevis*: tracing afferents to laryngeal motoneurons with HRP-WGA. J Comp Physiol [A] 157:749–761.

Wright SP (1992) Adjusted p-values for simultaneous inference. Biometrics 48:1005–1013.

Yager DD (1992) A unique sound production mechanism in the pipid anuran *Xenopus borealis*. Zool J Linn Soc 104:351–375.

Zakon HH (1996) Hormonal modulation of communication signals in electric fish. Dev Neurosci 18:115–123.