Sexually Distinct Development of Vocal Pathways in *Xenopus laevis*

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ABSTRACT: Deterministic rules, rather than experience, are thought to regulate the development of simple behaviors in vertebrates and invertebrates. We revisited this issue through examination of the sexually distinct vocalizations of African clawed frogs (*Xenopus laevis*), a reproductive behavior used by sexually mature males and females. We discovered that, as expected for simple behavior, female vocalizations develop through deterministic rules. The rare calls of juvenile females are indistinguishable from those of adult females. The vocal pathways of juvenile females, as measured by the contractile properties of the laryngeal muscles (the vocal muscles) and the laryngeal motoneuron somata (vocal motoneurons) size, are the developmental default and do not differentiate as they mature. Male *Xenopus*, in contrast, produce extensive vocalizations with rudimentary acoustic structure before reaching sexual maturity. Moreover, the functional properties of the vocal central pattern generator mature before muscle fibers and motoneuron size are fully masculinized. The results suggest that neuronal activity during development may be important in organizing the contractile properties of the muscle fibers in male, but not in female *Xenopus*. © 2010 Wiley Periodicals, Inc. Develop Neurobiol 70: 862–874, 2010

Keywords: central pattern generator; vocalization; sexual differentiation; development; vocal ontogeny

INTRODUCTION

Considerable variation exists in the extent to which behavioral development relies on experience during ontogeny. For example, the vocalizations of many primate species or hand movements in human infants develop as a result of extensive motor practice during development (Konczak et al., 1995; Elowson et al., 1998), whereas the pecking behavior of precocious birds (e.g., chicks of Galliforms) or the courtship behavior of male Drosophila are nearly perfect the first time they are performed (Hutchinson and Taylor, 1962; Ruedy and Hughes,

2009). Such difference in the developmental requirement is considered to be dictated by the precociousness of the animal at birth. Accordingly, behaviors of highly precocious animals, which are generally thought to be simple fixed action patterns, are considered to be "hard-wired" (i.e., genetically programmed), which develop independent of experience. In this study, we revisited this issue using the vocal behavior of African clawed frogs, *Xenopus laevis*.

The mechanism of sound production in *Xenopus* vocalizations is one of the least complex among vertebrates. The calls (comprised of clicks) are produced underwater independent of the respiratory system, reducing the number of muscles involved in behavioral expression. Each click is produced by the contraction of a single pair of laryngeal muscles, and the contractile properties of these muscles determines the frequency at which clicks may be produced (Tobias and Kelley, 1987; Yager, 1992). Adult male and female calls are sexually distinct, and used mainly in the context of reproduction

(Tobias et al., 1998, 2004). Given the precociousness of *Xenopus* and the reproductive nature of the vocalizations, we predicted that *Xenopus* froglets are mostly silent during development, but generate vocalizations in complete forms once they reach sexual maturity, as in the courtship behavior of Drosophila. In this study, we first tested this hypothesis by obtaining intensive vocal recordings from male and female *Xenopus* froglets. We discovered that, as predicted, the female vocal behavior is primarily expressed in adulthood. Male froglets, however, vocalize extensively long before becoming large enough to clasp sexually mature females, and the acoustic morphology of these juvenile vocalizations were significantly different from those of adult *Xenopus*.

The discovery of sexually distinct vocal development in *Xenopus* provided us with an excellent opportunity to explore how the neuromuscular pathways underlying behavior differentiate. In particular, we hypothesized that female neuromuscular pathways for vocalizations are the developmental default, whereas male vocal pathways differentiate significantly as they mature, as demonstrated in most sexually differentiated behaviors (Kelley and Brenowitz, 2002). Furthermore, we examined the relative rate at which different elements of the vocal pathways (e.g., muscle fibers, neurons, and neuronal circuits) develop in males. Specifically, we wished to determine whether these elements differentiate at the same rate throughout the development, or whether one component occurs first and leads the others. This is of particular interest because relative rate of maturation will provide us with opportunity to understand the mechanisms by which the vocal pathways are configured in response to androgen in males. To this end, we examined the function of the central vocal pathways, laryngeal muscles, and the morphology of laryngeal motoneuron somata during development. Neuronal circuit function is difficult to evaluate directly in most systems. However, we could examine the vocal circuit activity because serotonin application to isolated brains evokes fictive vocalizations that can be recorded in the laryngeal nerve (Rhodes et al., 2007). The results confirmed our prediction that the female vocal pathways are the developmental default. Male vocal pathways, in contrast, differentiated during development, with the functional maturation of the central vocal pathways leading the maturation of the laryngeal muscles, and the morphology of constituent neurons. We suggest that "babbling" in *Xenopus* froglets may represent activity-dependent developmental program reserved for males.

METHODS

Animals

Seventy-three juvenile *Xenopus laevis* (mass ranged from 1.22 to 24.50 g) were purchased from NASCO (Fort Atkinson, WI). Animals were kept in 10l aquaria on a 12:12 light: dark cycle and at 18°C. All care and procedures adhered to National Institutes of Health standards for animal welfare. Individual variation in the development of *Xenopus* is enigmatic. After fertilization, an individual may metamorphose into a froglet within a month whereas its sibling raised in the same aquarium at the same temperature with identical access to food may remain a tadpole for another year (Yamaguchi pers. obs). Thus, instead of age, we used body mass in this study as a relative index to assess vocal maturation.

Sound Recording

Two separate series of recordings of juvenile *Xenopus* were made, once in 2004 and once in 2005. In both series, sound recordings were made with a hydrophone (H2, Aquarian Audio Products, Shoreline, WA) suspended ~2 cm below the surface in the center of a plastic 4l aquarium. All recordings were made at 19–22°C, in near-darkness. Vocalizations were collected with a voice-activated recording system (Syrinx software, John Burt, www.syrinxpc.com). None of the animals used in these two recording series were treated with human chorionic gonadotropin (HCG), which stimulates release of gonadal hormones.

During the first series, animals were sexed by laparotomy seven days prior to the beginning of the recording session, then a male-female *Xenopus* pair was introduced into the recording aquarium. Recording sessions continued until five sound files containing 10 or more clicks had been recorded, or for 14 days. The vocalizations of *Xenopus* consist of a series of clicks. Click sound is generated by the larynx when a pair of laryngeal muscles contract and pull apart arytenoid discs that result in implosion of air (Tobias and Kelley, 1987; Yager, 1992). Thus, repetitive contraction followed by relaxation of the laryngeal muscles directly translates into a train of clicks. The contractile properties of the laryngeal muscles are sexually distinct - in adult females the muscles are tetanized when stimulated at frequency >20 Hz (and thus cannot produce clicks faster than 20 Hz) whereas the male muscles only tetanize when it is stimulated at >80 Hz (Tobias et al., 1987). On the basis of laryngeal muscle analyses of the juvenile froglets, we determined that clicks repeated >20 Hz could be only produced by males, whereas clicks <20 Hz could be produced by either member of the pair. We positively identified female vocalizations only when slow clicks (<20 Hz) overlapped with rapid (male) clicks (>20 Hz); other slow clicks were not used for further analyses because we could not be certain of the caller identity. During this series, vocal recordings were obtained from six males and one female.

Because it was clear from the first recording series that male juveniles can produce clicks at rates faster than those

of both juvenile and adult females (20 Hz), the second series of recordings focused on the vocalizations in males. Pairs of juvenile animals (unsexed prior to recordings, lest laparotomy reduce their tendency to vocalize) of similar mass (within ±0.5 g) were selected at random and placed into the recording aquarium. Recordings were made from each pair until 10 calls consisting of five or more clicks had been recorded. After the recordings were obtained, the animals were sexed by laparotomy. When one member of the pair was a male and the other was a female, all the vocalizations that contained clicks >20 Hz were considered to be produced by the male and used for further analyses, whereas all the clicks <20 Hz were not analyzed because the identity of the caller could not be determined. When both members of the pair were males, then all the vocalizations regardless of the click rates were considered to be produced by a juvenile male of the mass that is an average of the pair, and used for further analyses. Out of 20 pairs of animals tested, recordings were obtained from eight pairs of animals (three male-male pairs, five male-female pairs).

In a separate experiment, effects of HCG on the vocalizations of juvenile males were examined. In adult *Xenopus*, HCG induces vocal production in males and oviposition in females. In this experiment, three juvenile males (sexed by laparotomy) were treated with 0.5 mL of HCG (50IU) via subcutaneous injection, and the vocalizations were recorded over the following 15 to 18 days using the voice-activated recording technique described above. In one of these males, a second booster shot (0.5 mL) was given 16 days after the first shot. We used sound recordings obtained from adult males and females from our previous study (Potter et al., 2005) for comparison.

Analysis of Vocal Behavior

Five sound files collected from each individual were used for the analyses. Instantaneous click rate was calculated as a reciprocal of inter-click interval using Raven software (version 1.1; Cornell Lab of Ornithology, Ithaca, NY). To evaluate the overall rate at which clicks are repeated by each individual, a frequency histogram was plotted with a bin size of 1 Hz [Fig. 1(A–G)]. To quantitatively characterize the click rates produced by each animal at the upper end of the spectrum, maximum instantaneous click rates (the highest rate at which a pair of clicks is produced), and maximum sustained click rates (the highest rate at which ten consecutive clicks are produced) were calculated.

In Vitro Nerve Recordings of 5-HT-Induced Activity

Four juvenile males were used to obtain serotonin-induced fictive vocalizations *in vitro*. Methods of recording fictive vocalizations from isolated brains in *Xenopus* have been described previously (Rhodes et al., 2007). Briefly, frogs were anesthetized with ethyl 3-amino benzoate methanesulfonic acid (MS-222, 0.15 mg/g body weight, Sigma Aldrich) injected subcutaneously, placed on ice, and decapitated. The

brain was dissected away from the skull while submerged in oxygenated (99% O_2/1% CO_2) ice-cold saline (in m*M*): 96 NaCl, 20 NaHCO$_3$, 2 CaCl$_2$, 2 KCl, 0.5 MgCl$_2$, 10 HEPES, and 11glucose, pH 7.8. The brain was continually superfused with oxygenated saline for the following hour in a recording chamber and brought to room temperature. A suction electrode placed on the most caudal (fourth) rootlet of the nerve IX-X (this rootlet contains the axons of the laryngeal and glottal motoneurons; Simpson et al., 1986) was used to obtain population activity of the motor axons. The signal was amplified (differential amplifier 1700, A-M Systems, Carlsborg, WA), high-pass filtered (1 Hz), digitized at 10 kHz (Digidata 1322A; Molecular Devices, Sunnyvale, CA), and recorded on a personal computer using AxoScope software (Molecular Devices). 5-HT was applied by replacing half of the saline in the recording chamber with 60 μM 5-HT dissolved in oxygenated saline to achieve the final concentration of 30 μM. 5-HT remained in the chamber for up to 30 min (superfusion of saline was suspended during this time). Nerve activity was recorded throughout 5-HT exposure.

Analysis of *In Vitro* Nerve Recordings

All fictive advertisement calls obtained from two juvenile males (five and six calls from each individual) were used for analyses. For comparison, fictive advertisement calls obtained from five adult male brains (10 fictive calls from each individual) were also used. Compound action potentials (CAPs) corresponding to laryngeal motoneuron activity were identified in Clampfit using a threshold search (threshold set at 3σ of background noise), and instantaneous CAP rates were calculated. We measured the number of CAPs included in fictive fast trills and compared between juvenile and adult males. The onset of fictive fast trill was defined to be marked by at least five consecutive increases in instantaneous CAP rates and amplitude, and the offset to be marked by sudden drop in CAP rates by over 10 Hz.

Contractile Properties of Laryngeal Muscles

Eighteen juvenile males and 12 juvenile females were used to characterize the contractile properties of the laryngeal muscles. Animals were anesthetized by injecting MS-222. The larynx was isolated in oxygenated saline (described above) and pinned to a Petri dish coated with Sylgard (Dow Corning, Midland, MI). Laryngeal muscles and nerves were exposed, and the laryngeal nerve was stimulated via a suction electrode, with a train of pulses generated by a Master 8 (AMPI, Jerusalem, Israel) stimulator, and delivered to the electrode using an IsoFlex (AMPI) stimulus isolation unit. The methods of recording contractile properties of laryngeal muscles were described elsewhere (Potter et al., 2005). Briefly, semi-isotonic tension was measured using a force transducer (model 1030, UFI, Morro Bay, CA; frequency response ±500 Hz) attached to the tendon of the laryngeal muscle. The stimuli used were single 1ms square pulses or trains of pulses at frequencies ranging from 10 to 70 Hz in
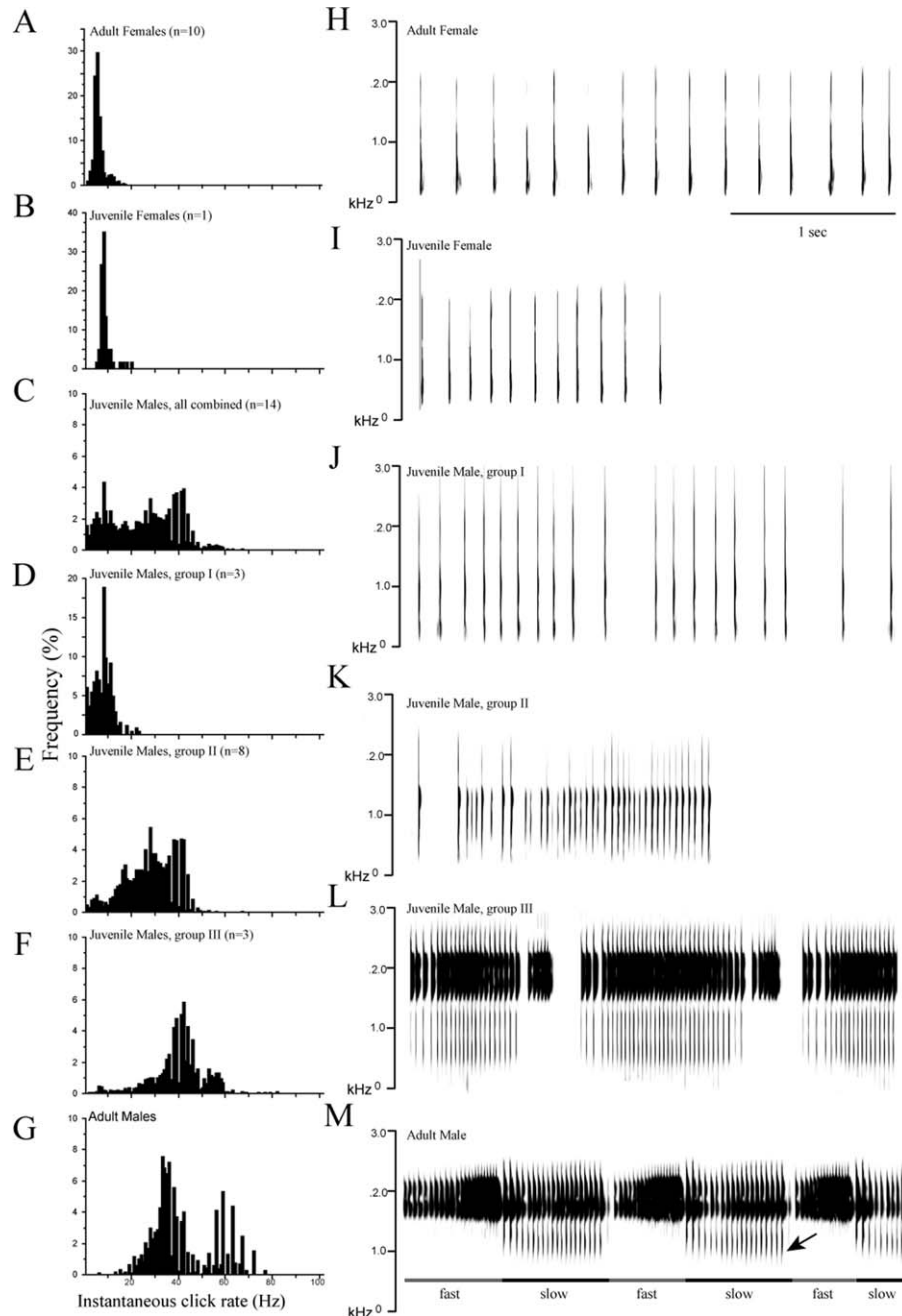
Figure 1 Vocal development in male and female *Xenopus*. A–G: Normalized frequency histograms of instantaneous click rates obtained from adult females (A), juvenile females (B), juvenile males (all animals combined, C), Group I juvenile males (D), Group II juvenile males (E), Group III juvenile males (F), adult males (G). H–M: Sound spectrograms of calls recorded from an adult female (H), juvenile female (I), Group I juvenile male (J), Group II juvenile male (K), Group III juvenile male (L), and adult male (M).

20-Hz increments. The range of stimuli was chosen to match the natural rates of clicks produced by the animals. Force output was amplified using a strain gauge amplifier (UFI model 2001, Morro Bay, CA), and digitized using a Digidata 1322A (Molecular Devices, Sunnyvale, CA) with a sampling rate of 1 kHz. All experiments were carried out at $22 \pm 0.5°C$.

Muscle Physiology Data Analyses

The temporal profile of twitch tension elicited from a laryngeal muscle was characterized by two parameters: slope to peak tension and half-relaxation time. Slope was measured by fitting a straight line from the onset of twitch

tension to the peak, and half-relaxation time was defined as the time between the peak twitch tension and its return to 50% of peak value [Fig. 5(B)]. When laryngeal muscles were stimulated rapidly, a sustained contraction was generated above fusion frequency. Development of significant fused (tetanic) tension retracts and holds apart the arytenoid discs, and prevents the *Xenopus* larynx from producing a click (Tobias and Kelley, 1987; Yager, 1992). We measured the ratio of fused tension relative to the total tension [i.e., the sum transient and fused tensions; Fig. 5(A)]. A low fused tension ratio translates to faithful production of clicks at the stimulus frequency, whereas a high ratio indicates failure to produce repetitive clicks. All measurements were made using Clampfit (Molecular Devices, Sunnyvale, CA) and Igor (Wavemetrix, Lake Oswego, OR).

Retrograde Labeling of Laryngeal Motoneurons

For morphometric analyses of laryngeal motoneurons, data from juvenile males ($n = 12$) and females ($n = 12$) were obtained and compared to previously obtained data from adult males and females. Methods of measuring the volume of laryngeal motoneuron somata have been described previously (Potter et al., 2005). Briefly, the brainstem was isolated (described above), and laryngeal motoneurons were retrogradely labeled with 5% Neurobiotin (Vector Laboratories, Burlingame, CA) via the fourth rootlet of nerve IX-X in oxygenated saline ($4°C$) for 12 h. The brain was fixed overnight in 4% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), embedded in gelatin (Fisher, Fair Lawn, NJ), and sectioned transversely at 80 μm with a Vibratome (series 1000, Technical Products International, St. Louis, MO). Labeled motoneurons were visualized using 0.5% Fluorescein Avidin D (Vector Laboratories).

Morphometric Analyses of Laryngeal Motoneurons

Morphometric measurements are made using the same technique described previously (Potter et al., 2005). Briefly, an Olympus laser confocal microscope controlled by Fluoview software was used to acquire Fluorescein labeled laryngeal motoneuron somata (Argon laser intensity = 20%, confocal aperture = 2, excitation wavelength = 488 nm). Z-stacks (1-μm increments) through the somata were acquired using an 40$\times$ oil objective. Motoneuron soma volume was estimated by constructing a projected image of the soma using the Sync Measure 3D plug-in for ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij/), determining the outer perimeter of the soma in the *XY*-plane of the projected image, and counting the pixels inside of the perimeter. Each pixel was assigned a volume of 2.4 $\times$ 2.4 $\times$ 1 μm^3, and all were summed to arrive at an estimate of total soma volume. Eight to 29 μm from each individual were used to measure volume (mean $\pm$ s.e. = 15.9 $\pm$ 1.3).

Statistical Analyses

All statistical analyses were done using StatView software (SAS Institute, Cary, NC). For analyses of fused muscle tension, repeated measure ANOVA (developmental stage as independent variable with two levels and transient tension measured in response to four different frequencies as dependent variable) was used. Mann-Whitney U test was used to determine if slope, half-relaxation time, maximum instantaneous click rates, maximum sustained click rates, and volume of laryngeal motoneuron somata differ between adult and juvenile males and females. Juvenile males were categorized into three groups (I, II, and III) based on the acoustic morphology of vocalizations produced (see Results). Kruskal-Wallis test was used to determine if the body mass of juvenile males categorized in Group I, II, and III males differ. To determine if body mass can significantly explain individual variability observed in the contractile properties of muscles (slope, half-relaxation time, transient tension at 50 Hz and 70 Hz), click rates (maximum instantaneous and sustained click rates) and motoneuron size, regression ANOVA with logarithmic model with body mass as independent variable was used.

RESULTS

Vocal Behavior of Juvenile *Xenopus*

Juvenile females vocalized very little, and we obtained vocal recordings from only one of four individuals [mass = 32.8g, Fig. 1(I)]. Click rates generated by this individual were similar to those of adult females; mean click rate (8.2 Hz) and maximum click rate (19.2 Hz) were similar [Fig. 1(A,B,H,I)]. Thus, although juvenile females vocalize very little, when the vocal pathways are activated, the motor program produced by the neural circuit is similar to those of adult females.

Juvenile males, in contrast, generated rudimentary vocalizations extensively. We obtained vocalizations from 12 males that ranged in mass from 2.5 to 18.3g. Click rates ranged from 1 to 60 Hz [Fig. 1(C,J,K,L)]; frequency histograms of instantaneous click rates showed broad distribution without any salient peak, and were distinct from adult males and females [Fig. 1(A,C,G)].

Because there was sufficient individual variation in vocal repertoires, we divided juvenile males into three groups. The first group (Group I) only produced female-like ticking [<20 Hz, Fig. 1(J)] and click rates overlapped with adult and juvenile females [$n = 3$; Fig. 1(A,B,D)]. The second group (Group II) produced trills that resemble slow trills of male advertisement calls [$n = 8$; 30–40 Hz; Fig. 1(E,K)]. Three of the eight males within this group also produced female-like ticking in addition to

slow trills. The third group (Group III) produced calls that resembled adult male advertisement calls [$n = 3$; Fig. 1(F,G,L,M)] that include alternating fast and slow trills, but acoustic morphology of calls was less stereotyped. The results indicate that, unlike juvenile females, juvenile male *Xenopus* produce rudimentary vocalizations during development.

We next examined the temporal morphology of juvenile male vocalizations. When maximum instantaneous and maximum sustained click rates (see Methods) of all juvenile male vocalizations were compared with those of adult males and females, both parameters of Group I males were the same as those of adult females (i.e., undifferentiated, $U = 10.5, 8.0, p = 0.999, 0.569$ for maximum instantaneous and sustained click rates, respectively). Values for Group II males were intermediate between adult males and females-significantly slower than those of adult males ($U = 7.0, 0.0, p = 0.0034, 0.0004$ for maximum instantaneous and sustained click rates, respectively), and significantly faster than those of adult females ($U = 0.0, 0.0, p = 0.0012, 0.0012$ for maximum instantaneous and sustained click rates, respectively). Maximum instantaneous click rates of Group III males resembled those of adult males ($U = 12.0, p = 0.612$), but the sustained click rates were significantly slower than those of adult males ($U = 0.0, p = 0.011$). Both parameters were significantly faster than those of adult females ($U = 0.0, 0.0, p = 0.017, 0.017$ for maximum instantaneous and sustained click rates, respectively).

Interestingly, the body mass of the juvenile males did not account for the types of vocalizations produced. The mass of Group III animals (ranging between 4.6 and 18.3 g) that produced calls most similar to those of adult males largely overlapped with those of Groups I (4.7 g) and II (ranging between 2.5 and 13.1 g) that generated rudimentary vocalizations (see Fig. 2), and there was no systematic difference across the groups in mass (Kruskal-Wallis test, $H = 2.28, p = 0.32$, Fig. 2). Accordingly, regression analyses (body mass as independent variable, maximum instantaneous or sustained click rates as dependent variable) showed no significant correlation ($F_{1,12} = 1.23, 1.48, p = 0.28, 0.25$, respectively, Fig. 2). We conclude that the maturity of the *Xenopus* vocal system does not correlate with body mass.

We next analyzed the vocalizations of Group III males in detail. Adult male advertisement calls are highly stereotyped, and consist of alternating fast and slow trills [Fig. 1(M), Fig. 3]. Clicks contained in fast and slow trills can be distinguished based on spectral properties; slow trill clicks contain a low frequency component that is absent in fast trill clicks
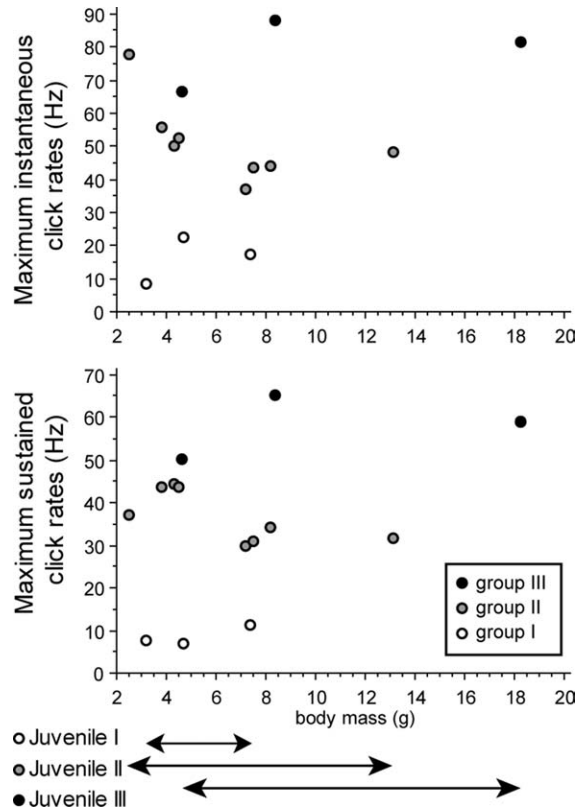

Figure 2 Body mass of juvenile males is a poor predictor of vocal maturity. Bivariate plots of maximum instantaneous click rates (top) and maximum sustained click rates (bottom) as a function of body mass. Note that there is no association between click rates and body mass. The range of body weight for each juvenile group is indicated with a horizontal bar with double arrows in the bottom.

[Fig. 1(M), arrow, Fig. 3]. Juvenile males in Group III also generated vocalizations that resembled advertisement calls with alternating fast and slow trills with distinct spectral properties [Fig. 1(L), Fig. 3]. However, the temporal profile of juvenile calls was abnormal. The advertisement-like calls of all Group III juvenile males showed silent gaps between fast and slow trills (Fig. 3, black arrows). In adult males, clicks accelerate and amplify progressively during fast trills before transitioning into slow trills. In Group III juvenile males, in contrast, the last section of the fast trills are replaced by silence before onset of slow trill (see Fig. 3).

Fictive Vocalizations in Male Juveniles

Is the inability of Group III juvenile males to generate complete fast trill due to constraints set by immature laryngeal muscles that cannot contract at rapid rates,
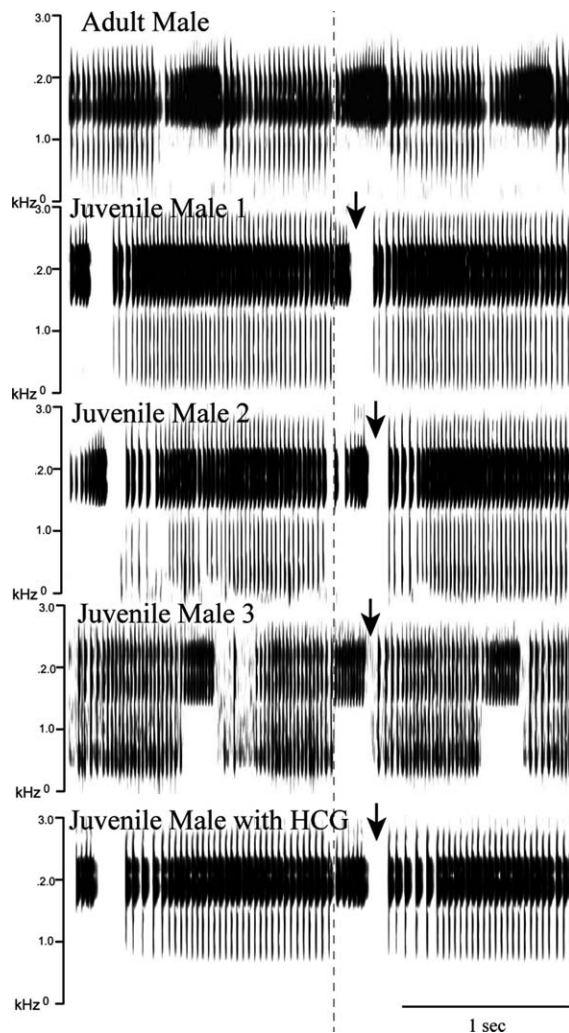
Figure 3 Sound spectrograms of advertisement calls produced by adult males and juvenile Group III males. Dotted line indicates the onset of fast trills. Arrows indicate the silent intervals found at the end of the fast trills in all juvenile males.

or by the central pattern generator that is unable to produce complete motor programs? To distinguish these possibilities, we examined the temporal morphology of fictive vocalizations generated by juvenile male brains. Adult male brains *in vitro* generate fictive advertisement calls that can be recorded via the laryngeal motor nerve in response to serotonin application (5-HT; Fig. 5, Rhodes et al., 2007). We examined the fictive vocalizations generated by juvenile male brains using the same procedure.

In pilot experiments, juvenile male brains did not respond to 5-HT ($n = 3$). Therefore, we injected 0.5 mL HCG (50 IU) into subject ∼20 h prior to isolation of the brains. HCG is known to enhance vocal activity in adult male *Xenopus* (Wetzel and Kelley, 1983). Following HCG injection, no animals produced advertise-

ment-like vocalizations prior to isolation of the brains. In two of four HCG-injected males (body mass = 9.0, 15.4 g), however, fictive advertisement calls were induced in response to 5-HT application. Although the latency to evoke fictive vocalizations from the
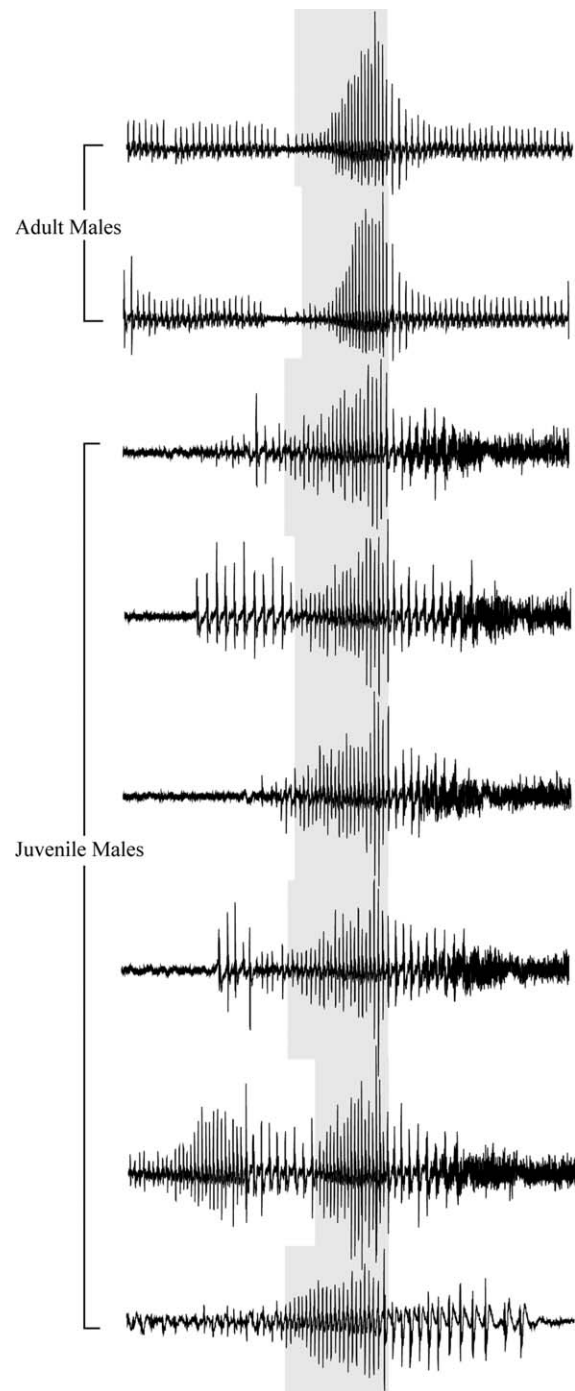

Figure 4 Fictive advertisement calls produced by adult and juvenile male brains. Shaded boxes indicate fast trills in each call. Note that there are no silent intervals (see Fig. 3). In fictive advertisement calls of juvenile males.

time of 5-HT application was much longer in both juvenile brains than in adult brains (25 min instead of 0.5–1 min), fictive advertisement calls of these animals contained complete fast trills that resemble those of adult males (see Fig. 4). The number of CAPs in a fast trill generated by juvenile male brains was similar to those produced by adult male brains (18.2 ± 1.3 and 17.6 ± 3.4 CAPs (mean ± s.e.) in adult ($n = 5$) and juvenile ($n = 2$) males, respectively), unlike vocalizations recorded *in vivo* in which fast trills are truncated (see Fig. 3). Moreover, in all juvenile fictive calls that contained both fast and slow trills, the transition between the two trill types took place within 30 ms. Thus, the motor program generated by the CPG of these juvenile males was adult-like; fast trill contained a similar number of CAPs, and fast trills smoothly transitioned into slow trills as in adult males.

Is it possible that HCG injected to the juvenile males 20 h prior to the isolation of the brain fully masculinized the function of the juvenile CPG? The time frame is certainly sufficient to increase the vocal activity in adult male *Xenopus* (Wetzel and Kelley, 1983). If exposure to elevated levels of androgen organizes the central vocal circuits within the matter of hours to bring it to functional maturity, we predicted that HCG treated juvenile males should also produce adult-like vocalizations within the same time frame. However, when HCG was given to a separate group of juvenile males ($n = 3$) and their vocalizations were recorded *in vivo*, one individual (mass = 5.5 g) never produced any advertisement-like calls for as long as 15 days following injection (0.5 mL), the second individual (mass = 6.7 g) produced advertisement-like calls for the first time on the 16th day following the injection (0.5 mL), and the third individual (mass = 12.2 g) did not generate advertisement-like calls until two days after the second booster shot (0.5 mL) was given 16 days after the first shot. Therefore, if HCG is to organize the central vocal pathways, it appears to take longer than 20 h. Moreover, the advertisement-like calls of the HCG-injected juvenile males still had the silent interval between the fast and slow trills (Fig. 3, bottom panel), as in intact Group III juvenile males. Although the two HCG-injected juveniles from which fictive vocalizations were recorded did not produce advertisement-like calls *in vivo*, it is likely their calls would have been incomplete because advertisement-like calls with silent intervals was universal, regardless of HCG treatment, across all juvenile males in this study ($n = 5$). The results indicate that motor programs generated by the CPG of juvenile males *in vitro* are functionally mature even though their vocalizations produced *in vivo* are not.

Laryngeal Muscle Properties

The contractile properties of laryngeal muscles dictate the click rates that animals can produce (Tobias and Kelley, 1987; Yager, 1992). We suspect that juvenile males generate advertisement-like calls with truncated fast trills because of the mismatch between the adult-like motor programs generated by the juvenile central vocal pathways and slower muscular contractile properties. Thus, we examined the physiological properties of juvenile laryngeal muscles. The contractile properties of juvenile female laryngeal muscles ($n = 8$, body mass range from 4.63 to 17.01 g, mean ± s.e. = 8.027 ± 1.76 g) were similar to those of adult females ($n = 5$). In response to repetitive stimulation, female juvenile muscles developed significant fused tension at frequencies <30 Hz in a manner similar to those of adult female muscles [Fig. 5(C), repeated measure ANOVA, developmental stage as independent variable with two levels, fused tension at four distinct frequency as repeated measure dependent variable, $F_{1, 7} = 0.09$, $p = 0.77$], and distinct from those of juvenile males [Fig. 5(C), $F_{1,20} = 43.98$, $p < 0.0001$] or adult males [Fig. 5(C) $F_{1, 19} = 504.98$, $p < 0.0001$]. When a single electrical pulse is delivered to the laryngeal nerve, the temporal profile of a twitch generated by the laryngeal muscles of juvenile females [measured as slope and half-relaxation time; Fig. 5(B)] were similar to those of adult females [Fig. 5(D,E); Mann-Whitney U test, $U = 15.0, 27.0$, $p = 0.08, 0.634$ for slope and half-relaxation time, respectively], and distinct from those of juvenile males [Fig. 5(D,E); $U = 9.0, 3.0$, $p = 0.0007, 0.0002$ for slope and half-relaxation time, respectively] and adult males [Fig. 5(D,E), $U = 0.0, 0.0$, $p < 0.0001, 0.0001$ for slope and half-relaxation time, respectively]. We conclude that female laryngeal muscles do not differentiate significantly during development; adult females retain juvenile laryngeal properties.

The contractile properties of juvenile male laryngeal muscles ($n = 14$, body mass range 3.82–12.97 g, mean ± s.e. = 8.36 ± 0.83 g), in contrast, fell between those of adult males and females. Vocalizations of the animals used for the laryngeal muscle study were not recorded, and thus, were not grouped into three categories described above. When the juvenile male larynx received repetitive stimulation, fused tension differed significantly from those of adult females [Fig. 5(C), repeated measure ANOVA, $F_{1, 19} = 39.84$, $p < 0.0001$], juvenile females [Fig. 5(C), see above], and adult males [$n = 5$, Fig. 5(C), $F_{1, 31} = 9.825$, $p = 0.004$]. Similarly, when a single electrical pulse was delivered to juvenile male laryngeal muscles, both
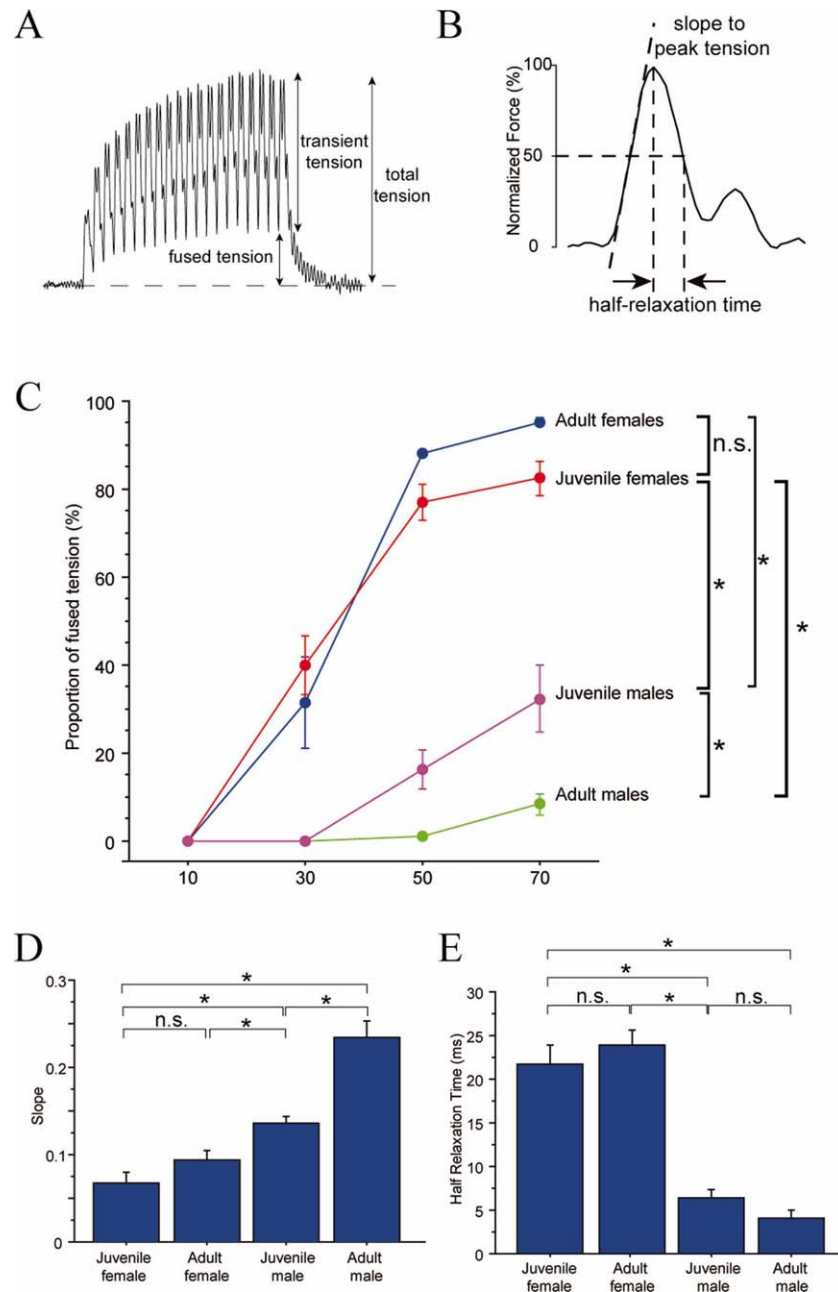
Figure 5 Contractile properties of adult and juvenile laryngeal muscles. A: Contractile force recorded from a laryngeal muscle in response to repetitive stimulation delivered to the laryngeal nerve. B: Slope to peak tension and half-relaxation time were measured from the tension profile elicited in response to a single shock delivered to the nerve. C: Proportion of fused tension produced in response to stimuli delivered at 10, 30, 50, and 70 Hz by larynges obtained from adult females, juvenile females, juvenile males, and adult males. D, E: Cell plots of tension slope (D) and half relaxation time (E). Error bars indicate standard errors. Asterisks in C, D, and E indicate significant differences, and "n.s." indicates not significant difference. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

slope and half-relaxation time of muscle contractions were significantly different from those of adult females [Fig. 5(D,E), Mann-Whitney U test, $U = 11.0, 0.0, p = 0,005, 0.0003$ for slope and half-relaxation time respectively] and juvenile females [Fig. 5(D,E), see above], and slope was significantly smaller than those of adult males [Fig. 5(D), $U = 2.0, p = 0.0022$]. The only exception was that the half-relaxation time of the

juvenile male muscles were similar to those of adult muscles [Fig. 5(E), $U = 15.5$, $p = 0.068$]. The mechanisms that mediate relaxation of the muscle fibers may reach maturity more rapidly than other features of the muscle physiology. Taken together, we conclude that contractile properties of juvenile male muscles were distinct from those of females, but not fully masculinized.

Some aspects of the contractile properties correlated with the body weight while the others did not. For example, half-relaxation time and fused tension at 50 Hz were explained by body weight (regression ANOVA with logarithmic model, body weight as an independent and half-relaxation time as a dependent variable, $F_{1, 12} = 8.20$, $p = 0.014$; $F_{1, 11} = 7.47$, $p = 0.020$), but slope and fused tension at 70 Hz were not ($F_{1, 12} = 0.002$, $p = 0.96$; $F_{1, 11} = 4.46$, $p = 0.058$). Thus, we conclude that the juvenile male larynx differentiates during development, but the rate of functional maturation does not necessarily correlate with body weight.

Laryngeal Motoneurons Somata Size

To assess how morphological features of the central vocal pathways develop relative to functional maturation, we measured laryngeal motoneuron somata volumes of 12 juvenile males (mean body mass $\pm$ s.e. = 9.37 ± 2.67 g, range = 1.22 to 24.5 g) and 12 juvenile females (10.48 ± 2.48 g, 1.36 to 24.35 g). The volume of juvenile motoneuron somata are similar in males and females [$U = 40.0$, $p = 0.065$, Fig. 6(A)], and are indistinguishable from those of adult females [$U = 18.0$, 26.0, $p = 0.206$, 0.673 for juvenile males and females, respectively, Fig. 6(A)]. Consequently, juvenile motoneurons of both sexes are significantly smaller than those of adult males [$U = 11.0$, 1.0, $p = 0.045$, 0.002 for juvenile males and females, respectively, Fig. 6(A)]. The results indicate that laryngeal motoneuron volume increases during development in juvenile males, but adult females retain the juvenile size.

Although body mass is a poor indicator of functional maturity of male laryngeal muscles and vocalizations, it is a good indicator of motoneuron size. The body mass of animals significantly accounts for the volume of motoneuron somata in juvenile males [regression ANOVA, body mass as independent variable, somata volume as dependent, logarithmic model, $F_{1, 15} = 12.544$, $p = 0.003$; Fig. 6(B)]. Such relation was not found in females [$F_{1, 15} = 3.222$, $p = 0.093$; Fig. 6(B)]; the motoneuron size in females remains small and constant regardless of body size. A significant proportion of the motoneurons of the lightest juvenile males (<3 g) are small in size

male: $Y = 7433.355 + 976.775 * \ln(X)$
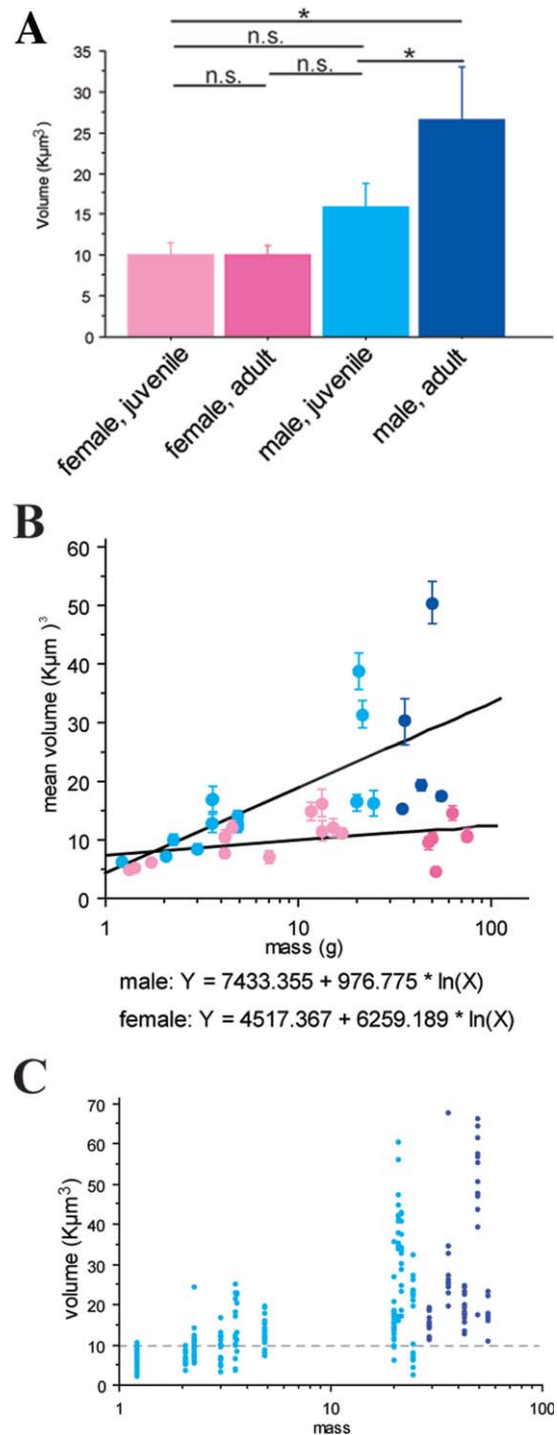
female: $Y = 4517.367 + 6259.189 * \ln(X)$

Figure 6 Size of laryngeal motoneuron somata. A: Cell plots of somata size obtained from adult and juvenile males and females. Error bars indicate standard errors. B: Regression plot of mean somata volume as a function of body mass. Four groups of animals (adult and juvenile males and females) are color coded as in A. C: Bivariate plot showing the somata volume as a function of body mass. Color coding scheme is the same as in A. Adult males do not have small sized motoneurons, indicated by dotted line.

[$<$10 K μm, Fig. 6(C), gray dotted line]. As body mass increases, a greater proportion of the motoneurons are large ($>$10 K μm) although small motoneurons are retained in varying proportions by most individuals. By adulthood, all male motoneurons are larger than 10 K μm.

DISCUSSION

We used an integrative approach to examine the development of *Xenopus* vocalizations, considering behavior, muscular physiology, and neurophysiology. We discovered that female juveniles vocalize very little, but when they vocalize, their calls are indistinguishable from those of adult females. We conclude that the vocal pathways of juvenile females are the developmental default and do not differentiate as they mature. Male juveniles, in contrast, produce rudimentary vocalizations extensively. When different elements of the vocal pathways were examined, we discovered that the central pattern generator for advertisement calls matures before both contractile properties of the laryngeal muscles and morphology of laryngeal motoneurons. The results suggest that in male *Xenopus*, "babbling" in the juvenile stage may be a built-in mechanism necessary for vocal pathway maturation whereas, in females, deterministic activity-independent developmental rules may govern vocal pathway organization.

Rudimentary Vocalizations in Juvenile Male *Xenopus*

Vocal practice is common in higher vertebrates. Human speech and song of songbirds (Oscines), for example, involves extensive vocal practice in infancy as part of a complex vocal learning process (reviewed by Doupe and Kuhl, 1999). Some nonvocal learners, including primates and bats, are also known to "babble" during development (Knörnschild et al., 2009). It is often deduced that such rudimentary vocalizations in infancy is necessary because of the temporal and spatial coordinations required for the motor program are complicated (Knörnschild et al., 2009). However, our finding that simple amphibian vocalizations develop through experience contradicts such a notion.

We divided juveniles males into three groups based on the types of vocalizations produced. Although we did not obtain time-lapse vocal recordings from the juvenile males in this study, the most

logical interpretation of the results is that these groups represent three stages of vocal development that juvenile males follow. Accordingly, we suggest that juvenile males begin by generating female-like slow clicks, then gain the ability to produce doublets of clicks at male-typical rates, and finally acquire the ability to string multiple clicks together at male-like rates.

Rudimentary vocalizations that we observed in juvenile males most likely represent neuronal activity generated by the laryngeal motoneurons and translated into sound by immature laryngeal muscles. What roles do these neuronal activities play in central vocal pathway development? During embryonic developmental stages, spontaneous activity is commonly observed in the nervous system across diverse taxa. These spontaneous activities, mediated by a set of ion channels expressed at a predetermined time in development, are important in regulating later developmental processes (reviewed by Moody and Bosma, 2005). For example, spontaneous activity of the mammalian retina during prenatal development regulates the patterning of retinogeniculate connections (Shatz and Stryker, 1988), and spontaneous calcium transients in granule cells of the cerebellum are required for later neuronal migration (Komuro and Rakic, 1998). Furthermore, spontaneous neuronal activity in developing motor systems such as spinal cord is known to elicit some movement in embryos (chicks, Hamburger et al., 1965; zebrafich, Saint-Amant and Drapeau, 1998; mice, Hanson and Landmesser, 2003). Such motoneuron activity and subsequent motility is thought to play a role in axon guidance and correct matching of pre- and post-synaptic partners (Hanson and Landmesser, 2004). Although "babbling" in *Xenopus* described in this paper occurred at a later developmental stage than the examples above, it is possible that immature neuronal activity organizes the neuromuscular apparatus controlling vocal behavior.

Mismatch in the Maturation Rate of the CPG and the Muscles

Our results suggest that the vocal CPG matures before the laryngeal muscles. One caveat in making this conclusion is that muscle properties and fictive vocalizations were not obtained from the same set of juvenile males used in the behavioral study. Thus, we cannot rule out the possibility that silent intervals observed in the advertisement-like calls of Group III males are due to immature CPGs that failed to generate complete fast trills, and not due to the immature

muscular properties. However, the fact that we never observed complete advertisement calls from any juvenile males while we only observed complete fictive advertisement calls from juvenile brains argues for early maturation of CPG relative to the muscles.

Early maturation of the vocal CPG likely regulates the differentiation of the laryngeal muscle fibers. It is well-documented that contractile properties of muscle fibers are partly dictated by the motoneuron firing rates (reviewed by Gundersen, 1998). Accordingly, it is conceivable that rapid trains of action potentials generated by the laryngeal motoneurons of immature males help differentiate the muscles into fast-twitch fibers. Androgen is known to bind to both laryngeal muscle fibers and motoneurons, and plays a role in differentiating the contractile properties of the fibers (reviewed by Kelley, 1986). Direct action of androgen may fully masculinize the function of laryngeal motoneurons, but laryngeal muscle fibers may require both electrical stimulation provided by the motoneurons and binding of androgen in order to become masculinized. Future experiments involving denervation of the laryngeal nerve in juvenile males should allow us to evaluate the importance of electrical activity in the maturation of muscle fibers during development.

Mature Vocalizations Are Expressed by the Vocal CPG Containing Immature Motoneurons

Laryngeal motoneuron somata volume measurements indicated that the motoneurons of juveniles were not morphologically mature. Although the size of motoneurons were not measured in animals used for the behavioral study and *in vitro* fictive study described above, two of three animals that produced advertisement-like calls *in vivo*, and one of two animals whose brains generated fictive advertisement calls *in vitro* had body mass <10 g (4.6 g, 8.4 g, and 9.0 g), leading us to predict that a significant proportion of their motoneurons must have been small (<10 K μm). Moreover, the fact that small motoneurons, never observed in adult males, were found in every juvenile male used in the morphological study strongly suggests that the vocal CPGs of Group III males contain some small motoneurons. Our results suggest that some juveniles with small body mass, with presumably smaller laryngeal motoneurons, are capable of producing advertisement-like calls. Accordingly, the results suggest that the entire motoneuron pool does not have to be fully masculinized in size to generate male-like vocal motor patterns. Instead, a minimum proportion of fully masculinized motoneurons may

be necessary to produce male-like vocal motor programs.

Production of advertisement-like calls by immature CPGs may be possible because only a proportion of mature motoneurons are required to produce advertisement calls, or because the motoneurons of immature size are endowed with intrinsic membrane properties comparable to those of adults. The size of somata largely dictates the passive membrane properties of neurons including input resistance and membrane capacitance (Fleshman et al., 1981). If the intrinsic properties of the small motoneurons are similar to those of adult motoneurons, then we may find a developmental homeostasis that maintains constant intrinsic properties during neuronal growth, perhaps by regulating the number of ion channels expressed at each developmental stage.

Morphological difference in adults and juveniles may still account for behavioral differences other than the temporal morphology of calls. Adult males typically repeat advertisement calls hundreds of times whereas juvenile males only produce one or two calls at a time. Such a difference in overall vocal activity may be ascribed to morphological differences between neurons adults and juveniles. Similarly, smaller motoneurons of juvenile males may dictate smaller motor unit size with less effective force generation, and thus may account for the silent intervals seen at the end of the fast trills.

Common Trajectories of Vocal Masculinization in Juvenile Males and Adult Females

The process of vocal development in juvenile males observed in this study closely parallels the vocal masculinization process of testosterone-treated adult females (Potter et al., 2005). In response to testosterone administration, adult females acquire male-typical vocalizations within 13 weeks. Interestingly, testosterone-treated females in the process of vocal masculinization also produce extensive "transitional" vocalizations that are not male- or female-specific. They start by accelerating click repetition rates, and then organize two trill types into alternating phases. Furthermore, the vocal CPG also seems to masculinize earlier than the laryngeal muscle fibers; fictive advertisement calls recorded from isolated brains are nearly complete whereas the vocalizations produced had silent interval at the end of the fast trills (Rhodes et al., 2007). Thus, elevated levels of testosterone may organize the vocal pathways in *Xenopus* by first masculinizing CPG function, which sets the activity-

dependent developmental process in motion regardless of age or sex of the animals.

In conclusion, we discovered that female *Xenopus* develop their calls by following deterministic developmental rules independent of activity, as presumed for the ontogeny of most simple behaviors, whereas male *Xenopus* develop their calls through extensive vocalizations during development. Accessibility of the *Xenopus* vocal systems to experimental approach will provide us with an opportunity to explore how activity during ontogeny organizes the development of the vocal pathways.

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