2.08 Hormones and Vocal Systems: Insights from *Xenopus*

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This chapter is a revision of the previous edition chapter by E.J. Yang, D.B. Kelley, volume 1, pp. 693–706, © 2009, Elsevier Inc.

2.08.1 Introduction

The stunning diversity of patterned movements that make up behavioral repertoires are governed by neural circuits at multiple levels and supported by specialized features of muscle effectors. In vertebrates, behaviors that are specific to each sex are also regulated, during development and in adulthood, by the endocrine signals that control reproduction. The complex nature of hormone action, with multiple targets and temporally distinct but overlapping sensitive periods, creates a major challenge in deciphering the causal relevance of hormone signaling at each level of control. To overcome this hurdle, investigations of the physiological, cellular, and molecular mechanisms at each level must be complemented with studies that integrate across mechanism and level of organization.

In this chapter, we review research on the sexually differentiated vocal behaviors of South African clawed frogs, *Xenopus laevis*. The *Xenopus* vocal system is unusual because *Xenopus* is entirely aquatic throughout its life cycle; vocalization is produced under water and is not powered by air movement (breathing). The essential involvement of breathing circuits in terrestrial vertebrates greatly complicates the study of hindbrain control of vocalization (McLean et al., 2013; Tupal et al., 2014). Vocalization without breathing in *Xenopus* provides many experimental advantages that include two powerful reduced experimental preparations: the isolated

Hormones, Brain and Behavior, Third Edition, 2017, 131–144

(*ex vivo*) larynx and the isolated brain. 'Fictive' vocal behaviors can be evoked in either preparation independently of the other (Tobias and Kelley, 1987; Rhodes et al., 2007). The *ex vivo* larynx preparation has shed light on how the periphery constrains sound production, as well as on how hormones support the production of sex-specific behaviors (Zornik and Kelley, 2011). The *ex vivo* brain preparation has provided important insights into the essential components of the vocal central pattern generator (CPG) and the neural circuits that generate different vocal rhythms (Rhodes et al., 2007; Yu and Yamaguchi, 2009, 2010; Yamaguchi et al., 2010; Zornik and Yamaguchi, 2012). *In vivo* studies of intact animals complement findings *ex vivo*. Lesion studies, combined with the *ex vivo* brain preparation, have provided insight into forebrain mechanisms that govern context-dependent social signaling (Hall et al., 2013). Finally, recent advances in understanding auditory processing will facilitate future integrative syntheses of sensory, sensorimotor, and motor levels of control (Elliott et al., 2011; Hall et al., 2016).

2.08.2 Mating and Vocal Behaviors

In *X. laevis*, the most extensively studied *Xenopus* species, mating occurs over a prolonged period (~6 months), and only a small percentage of females are sexually receptive at any given time (Tobias et al., 1998b). Because these fully aquatic frogs mate under water at night and typically live in turbid bodies of water with many conspecifics, vocal signals drive courtship as they are well suited to locating a suitable mate. Each call is made up of brief (~5–10 ms; Vignal and Kelley, 2007) sound pulses produced with different temporal patterns. Eight unique call types have been described (**Figure 1**), and each can be distinguished through a combination of the pulse rate, duration, and period (repetition rate) of each trill, as well as the pulse intensity (loudness) and frequency (pitch).

2.08.2.1 Vocalizations Depend on Social Context

2.08.2.1.1 Male Advertisement Calls

The male advertisement call (**Figure 1(a)**) is the most spectrally and temporally complex in the *X. laevis* repertoire. It can be divided broadly into two phases: the fast trill and the slow trill. Fast and slow trills alternate during calling bouts. Across the fast phase (~60 Hz; 200–300 ms duration), sound pulses increase in amplitude. For the slow phase, sound pulses are produced at ~30 Hz; several 'loud slow' pulses often precede the longer-duration (700–800 ms) slow trills. Fast trill sound pulses contain two dominant frequencies (DF1: ~1.9 kHz; DF2: ~2.2 kHz), while slow trill pulses contain a third, lower DF ~1.2 kHz (**Figure 2**).

During the peak reproductive season (August in Cape Town, South Africa), the male-specific advertisement call can be recorded using an underwater microphone for prolonged periods (>45 min; Tobias et al., 2004). In the lab, males also call for prolonged periods when alone. When paired with a conspecific, males switch from advertisement to answer calling (**Figure 1(b)**). In the laboratory, broadcasts of advertisement calls suppress calling by other males (Tobias et al., 2004) accounting for vocal dominance and the prevalence of calling by a single male in field recordings (Tobias et al., 2010). The male advertisement call also attracts gravid females (Picker, 1983) and evokes a specific call, rapping (**Figure 1(b)**; Tobias et al., 1998b).

2.08.2.1.2 Female Unreceptive Calls

During the breeding season, most female *X. laevis* are sexually unreceptive at any given time. When clasped by a male, nongravid females produce a slow series of sound pulses at a rate of 4–8 Hz called ticking (**Figure 1(c)**). Unlike male advertisement calling, the sound pulses in ticking are spectrally broad, with a single DF around 1.2 kHz (**Figure 2**). The temporal pattern is simple, consisting of an ongoing, monotonous trill, and pulses do not vary systematically in intensity as the call progresses (Tobias et al., 1998b). Nongravid females also extend their hindlimbs when clasped (Kelley, 1982). When males are exposed to playbacks of female unreceptive calls during advertisement calling, they exhibit temporary vocal suppression (Tobias et al., 2004; Elliott and Kelley, 2007).

2.08.2.1.3 Female Rapping and Male Answer Call

Female *X. laevis* also advertise for mates using the rapping call. Rapping can be evoked in gravid females by advertisement calls, and pairs of sexually receptive males and females produce rapping/advertisement calling duets (Tobias et al., 1998b). Rapping trill rates average ~12 Hz. Playbacks of rapping evoke advertisement and answer calling. The answer call, a modified advertisement call, contains elongated fast trills, shortened slow trills, and enhanced intensity modulation (**Figure 1(b)**).

2.08.2.1.4 Amplectant Call

Upon clasping a female or another male (amplexus), males generate a low-intensity amplectant call consisting of two to three sound pulses at ~150 ms intervals (**Figure 1(d)**; Picker, 1980; Zornik and Kelley, 2008). These sounds coincide with male forelimb contraction and ventrally directed head movements, potential signals for synchronizing egg and sperm release.

2.08.2.1.5 Male–Male Interaction Calls

All of the six call types given by males can be recorded from male/male pairs; three (chirping, growling, and male ticking) are directed exclusively toward males (Tobias et al., 2004). Chirping, a brief (~5 pulses) 60–80 Hz burst repeated approximately four times per second (**Figure 1(e)**), can be recorded as one male approaches another and is reliably produced by the clasping male (Tobias et al., 2004) as well as in response to playbacks of synthetic male-like calls (Vignal and Kelley, 2007). Chirping could be a male–male aggressive signal, though as noted above, advertisement calls suffice for vocal suppression.

In male/male pairs, the clasped male produces growling, a rapid, variable-length trill (**Figure 1(e)**; 40–80 Hz Tobias et al., 2004). Unlike the sound pulses of other male calls, growling sound pulses have a lower DF around 1 kHz (Tobias et al., 2004).

Male ticking is a rare call, and it is not clear whether it is elicited by amplexus, and if so, whether the clasper or claspee produces the call (Tobias et al., 2004). The sound pulse rate in male ticking is ~4 Hz, as in female ticking (**Figure 1(c)**),

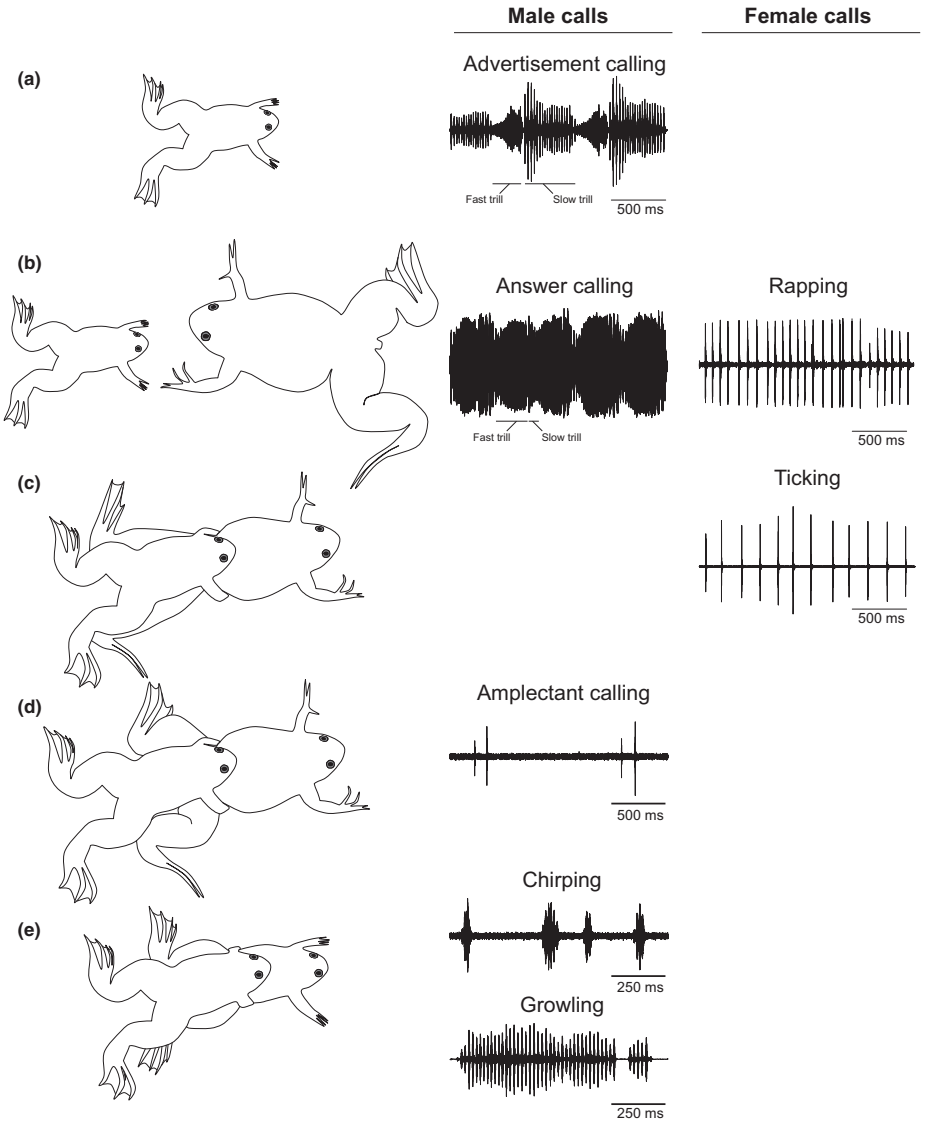

Figure 1 The social context-dependent *Xenopus laevis* vocal repertoire. (a) Male *X. laevis* produce advertisement calls in isolation, as well as in the presence of conspecific females and males. Advertisement calls are temporally complex. Sound pulses occur in alternating trills: fast trill pulses are produced at a rate of ~60 Hz, while slow trill pulse rates average ~30 Hz. Sound pulse intensity changes within each call. Calls produced by a single animal can continue uninterrupted for up to 45 min. (b) When sexually receptive females are about to oviposit, they often generate a call, rapping, consisting of an ~12 Hz pulse rate that attracts males. When males hear rapping, they approach the female (or loudspeaker playing an artificial call) and generate a modified version of the advertisement call, answer call. Answer calling consists of elongated fast trills and truncated slow trills. (c) When unreceptive females are clasped by a male, they extend their hindlimbs and generate a slower, ~4 Hz, call: ticking. Ticking suppresses male calling. Ticking has also been recorded from male/male pairs (Tobias et al., 2004). While the sound pulse rate (~4 Hz) does not differ from female ticking, sound pulses frequencies (1.7–2.3 kHz) in male ticking are higher than in female ticking (~1.2 kHz). (d) When females are reproductively active, they respond to male clasps with flexion of the hindlimbs, which promotes fertilization during oviposition. During this prolonged amplexus, males generate a low-intensity, low pulse rate call: amplectant call. (e) Males produce all call types in the presence of other males. Two call types most commonly associated with male–male interactions are chirping and growling, which are believed to represent agonistic signals. Chirping appears to be largely associated with dominant males, while growling is produced by clasped males. Pulse intervals for both calls are fast (40–80 Hz), with distinctive temporal patterns; chirps are brief and repeated multiple times per second, each growling trill tend to last several hundred milliseconds. Reproduced from Zornik, E., Kelley, D.B., 2011. A neuroendocrine basis for the hierarchical control of frog courtship vocalizations. Front. Neuroendocrinol. 32, 353–366.

but spectral frequencies of male pulses are higher (~1.8 kHz) than in females (~1.2 kHz).

The distinctive features of different calls given by the sexes allow the social context in which a recording was obtained to be determined from the calls alone. For example, a laboratory recording that includes advertisement calling and growling indicates a male/male pair in which one is clasping the other (and thus sexually active). A recording that includes rapping and answer calling indicates interactions between a sexually active female/male pair. Support for the reliability of these

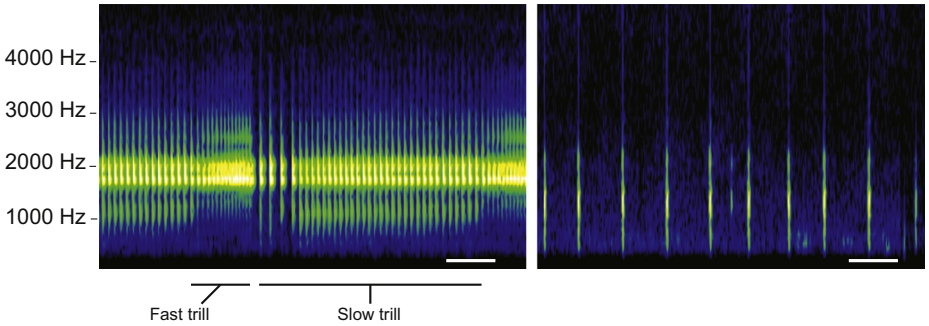

Figure 2 Spectrograms of sexually differentiated *Xenopus laevis* calls. Left, male advertisement calls are spectrally complex. Fast trill sound pulses contain two dominant frequencies (DFs) at ~1.9 and ~2.2 kHz. Slow trill sound pulses contain a lower frequency component ~1.2 kHz. Right, female ticking sound pulses have less finely tuned frequency ranges, with a single DF at ~1.2 kHz. Scale bars: 250 ms.

inferences comes from the ability to reproduce the outcomes of social interactions in isolated frogs using sound broadcasts. Rapping broadcasts, for example, stimulate male answer calling (Vignal and Kelley, 2007) while intense advertisement calling produces prolonged vocal suppression in males (Tobias et al., 2010). The broadcast paradigm has been useful in functional dissection of forebrain influences on vocal responses, described below (Hall et al., 2013).

2.08.2.2 Clasping, Reproductive Status, and Social Hierarchies

2.08.2.2.1 Female Receptivity

Nearly a century of experimental work has probed the role of steroids in female *X. laevis* reproduction. Early studies found that anterior pituitary extracts could induce ovulation in *X. laevis* females, while hypophysectomy lead to dramatic ovarian retrogression (Hogben et al., 1931). These results set the stage for the widespread use of *Xenopus* as a test for pregnancy; injection of urine containing human chorionic gonadotropin (hCG, a hormone produced by the placenta) leads to egg ripening and oviposition in *Xenopus* (Elkan, 1938). Although little is known about the relation between hormonal fluctuations in adult female *X. laevis* to their reproductive cycles in nature, one study found a strong positive correlation between hypothalamic GnRH (also known as LHRH) and breeding season (King and Millar, 1979). Thus, it is likely that seasonal elevation of GnRH leads to gonadotropin (LH)-induced oocyte maturation and oviposition, two events that can be recapitulated by exposure to hCG. Although it was initially assumed that hCG injections promote oocyte maturation via progesterone signaling, recent experiments indicate that plasma progesterone is an order of magnitude lower than androgens and that androgens instead appear responsible for oocyte maturation (Lutz et al., 2001).

Both hCG and GnRH induce a receptive posture – flexion of the hindlimbs – in intact females clasped by males (**Figure 1(d)**; Kelley, 1982). Ovariectomy eliminates this behavior, which can be reinstated by injections of progesterone and estradiol together, although each hormone by itself does not induce the receptive response (Kelley, 1982). These results do not rule out a role for androgens in female responses to clasping, as progesterone has been shown to be rapidly metabolized to the androgen androstenedione (Lutz et al., 2001). Injection of GnRH into ovariectomized females treated with progesterone and estradiol increased the likelihood of clasping by males and led to prolonged amplexus, an effect not present after hCG administration. Thus GnRH might be acting directly on brain targets rather the pituitary to elicit receptive behaviors.

Prostaglandin E_2 (PGE2) injection has also been shown to increase female receptivity (Weintraub et al., 1985). PGE2 injections led to dramatic and rapid (30 s–3 min) reduction of unreceptive leg extensions. Because these effects occur in both intact and ovariectomized females, it is likely that a PGE2 acts directly on neuronal targets to mediate these behavioral changes. Similar rapid actions of prostaglandins have been described in fish (Stacey and Goetz, 1982; Juntti et al., 2016).

2.08.2.2.2 Male–Female Clasping

Clasping is a critical component of *Xenopus* mating behavior; in the laboratory, amplexus can be maintained for several days (Kelley, 1982). Male clasping is androgen-dependent. Castration abolishes clasping, while T or DHT implants reinstate the behavior (Kelley and Pfaff, 1976). Although intact females do not clasp males, gonadectomized females treated with either T or DHT do clasp males, and clasp durations do not differ from hormone-replaced males. The finding that DHT can induce clasping in both sexes suggests androgens are sufficient to induce clasping; E on the other hand does not induce clasping.

2.08.2.2.3 Male–Male Clasping

Group-housed male *X. laevis* are often observed clasping each other, sometimes for prolonged periods. A recent study suggests that these male–male interactions do not necessarily reflect aggression or failure to discriminate sex, but may instead represent an alternative mating strategy (Rhodes et al., 2014). In triad experiments, in which two males were tested with one female, most males preferentially clasped females; however, a subset of males preferentially directed clasping toward other males. Males that are less likely to clasp a male in male–male pairs are more likely to 'win' clasps of females in subsequent male–male–female triads. Conversely, males that preferentially clasped the other male in the male–male

pair rarely gained access to the female, but continued to clasp the other male. Thus clasping in this study does not appear to represent a means of demonstrating or establishing reproductive dominance. Together, these results support the hypothesis that some males use an alternative mating strategy in which they gain physical proximity to gravid females via clasping an amplexing male; such physical proximity could allow fertilization of some eggs as they are laid by the female.

2.08.2.2.4 Vocal Dominance

The finding that, in any given pond, only one or a few males produce advertisement calls each night prompted laboratory investigations into the mechanisms regulating calling. Tobias et al. (2004) showed that pairing two males led to vocal silencing of one. Transitive, linear vocal dominance hierarchies can be inferred from tests of multiple male pairs and remain stable for up to 2 weeks (Tobias et al., 2010). Physical contact is not required to establish vocal dominance, and the amount of male clasping did not match vocal dominance patterns. Playback of male advertisement calls alone was sufficient to vocally suppress all exposed males (Tobias et al., 2010). Vocal suppression was achieved whether the playback recordings were derived from a vocally dominant or subordinate animal; low-intensity playbacks of either call were less effective in vocal suppression than high-intensity playbacks. Quantity or intensity of calling thus appears to be a determining factor in establishing dominance.

Whether the establishment or maintenance of vocal dominance is hormone-dependent has yet to be investigated. Because advertisement calling depends on circulating androgens (see below), differential levels of circulating androgens could be correlated with vocal dominance, as observed in social dominance in other species such as cichlids (Maruska, 2014), electric fish (Cuddy et al., 2012), and rodents (Shen et al., 2015). Another possible contributor is corticosterone, as the stress-related glucocorticoids are known to be correlated with social dominance in many species (Abbott et al., 2003; Goymann and Wingfield, 2004; Jeffrey et al., 2012; Ode et al., 2015). Because of its prolonged nature, advertisement calling could be energetically costly, perhaps contributing to the scarcity of calling in any given population during the breeding season. In a corticosterone-regulated pattern of vocal dominance, males in an optimal physiological state (i.e., well-fed, metabolically stable, low stress) would be most likely to gain vocal dominance, leading to greater reproductive success.

2.08.2.3 Hormone-Dependence of Vocal Behaviors

2.08.2.3.1 Advertisement Calls

Male advertisement calling depends on endocrine state. Most castrated males show a complete loss of advertisement calling after about 1 month (Wetzel and Kelley, 1983), although some animals do produce small amounts of abnormal calling as long as 1 year after castration (Zornik and Yamaguchi, 2011). Normal calling in castrated males resumes following treatment with either T or DHT (Wetzel and Kelley, 1983).

In the lab, male calling is greatly enhanced by injection of hCG (Wetzel and Kelley, 1983; Tobias et al., 2004; Yang et al., 2007). Very little calling is observed in uninjected male pairs. However, when one or both males in a pair is injected (100 IU, 24 h and 6–8 h before each trial), the amount of calling is greatly increased, with all six call types represented. Although some of this effect is likely due to an increase in circulating androgens, hCG also appears to affect calling by directly activating vocal nuclei in the brain. The original evidence for a central mechanism of hCG function came from experiments in castrated, hormone-replaced frogs (Wetzel and Kelley, 1983). As described above, calling was rapidly eliminated following castration, but could be reinstated by replacing T or DHT. Calling rates in hormone-replaced animals were significantly lower than intact frogs. However, when these animals were injected with hCG, time spent calling increased as it did during presurgery control recordings. Since the testes were no longer present, an extragonadal target was implicated.

Later experiments provided strong support for the hypothesis that gonadotropins enhance advertisement calling via direct action in the brain (Yang et al., 2007). Intracerebroventricular (ICV) injection of hCG was effective at enhancing calling at far lower doses than systemic injections. This effect appears to be transduced via luteinizing hormone receptors, which are expressed in the central amygdala (CeA), a known vocal control region (**Figure 3**; Brahic and Kelley, 2003; Hall et al., 2013). Exposure of the isolated brain to hCG induced upregulation of the immediate early gene egr-1 in the CeA, even when synaptic transmission was blocked. Endogenous gonadotropin signaling must not be sufficient to induce calling, however, because hCG injections in castrated males do not enhance male calling (Zornik and Yamaguchi, 2011). Thus, androgens and gonadotropins are both required in the brain to allow normal production of male advertisement calls.

As described above, linear vocal hierarchies between males can be stable for up to 2 weeks, but subordinate males only suppress their calling when in the presence of a dominant male. The mechanisms that underlie rapid suppression of calling is unknown, but must involve processing of auditory information, given that playbacks alone are sufficient to suppress calling. A likely candidate nucleus for regulating vocal suppression is the CeA. Because this region receives auditory information (**Figure 3(a)**; Hall et al., 2013) and is a target of gonadotropin (Yang et al., 2007), one intriguing possibility is that vocal suppression may be a result of changes in endogenous gonadotropin signaling in the CeA.

2.08.2.3.2 Female Calling

While females do not use vocalizations when paired with other females (Tobias et al., 2004), reproductive state is reflected in their vocalizations when paired with a male. Unreceptive females produce the slow call, ticking, while receptive females produce the faster call, rapping (**Figure 1**; Tobias et al., 1998b). Although hormones that promote vitellogenin synthesis (e.g., estradiol), oocyte maturation (e.g., androgens) and female receptivity (GnRH, progesterone, and estradiol) have been described, we do not have a complete picture of how endogenous hormones modulate female vocal behaviors. The time course of changes in female vocal behaviors induced by hCG in the presence of an advertising, clasping male has been followed in intact females (Wu et al., 2001). All uninjected females ticked when clasped; 6 h after injection, all females

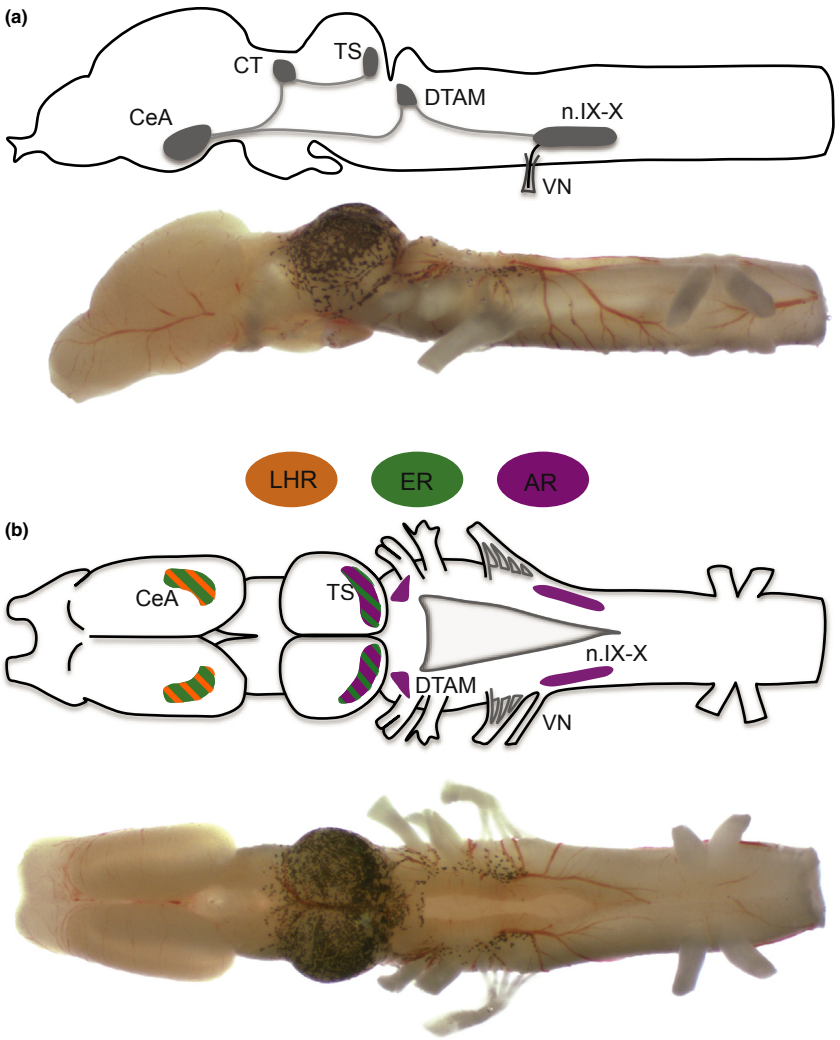

Figure 3 Auditory and vocal pathways in *Xenopus laevis*. (a) Diagram (top) and photograph (bottom) of a sagittal view of the *X. laevis* brain and anterior spinal cord (anterior is left, dorsal is up). Key auditory, sensorimotor and vocal central pattern generator (CPG) nuclei are shown with their known connections. The midbrain torus semicircularis (TS) processes auditory information and projects to the central thalamus (CT), which in turn projects to the central amygdala (CeA). CeA projections to the hindbrain are thought to regulate social context-dependent activation of the vocal CPG. CPG nuclei DTAM and n.IX-X are reciprocally connected. (b) Diagram (top) and photograph (bottom) of a dorsal view of the *X. laevis* brain and anterior spinal cord (anterior is left). Color coded nuclei indicate adult patterns of hormone receptor expression (LHR, luteinizing hormone receptor; ER, estrogen receptor; AR, androgen receptor). Laryngeal motor neuron axons exit the brain via the caudal root of cranial nerve IX-X, the vocal nerve (VN).

were silent when clasped. Rapping in response to male calling appeared in a smaller subset of females by 9 h postinjection and was recorded out to 18 h postinjection.

What are the mechanisms underlying suppression of ticking and promotion of rapping? A potential endocrine suppressor of ticking is prostaglandin; PGE2 rapidly suppresses ticking behaviors when given exogenously but does not elicit rapping (Weintraub et al., 1985). In cichlid fish, PGF2α activates female reproductive behaviors and appears to act directly on CNS targets via a gonadal hormone-induced PGF2 receptor (Juntti et al., 2016). A similar scenario might operate via PGE2 in *Xenopus*.

What is the trigger for rapping? hCG-induced serum steroid hormones in females are dominated by androgens, and their local action triggers the final reduction divisions of meiosis responsible for oocyte formation (Lutz et al., 2001). While T and DHT treatment masculinize female vocal behavior, the primary circulating androgen in females, androstenedione, might preferentially act on a subset of neural target cells that promote rapping. An acute increase in circulating androgen, associated with imminent oviposition, might activate neural targets responsible for producing rapping. Another possibility is that rapping arises in response to gonadotropin signaling in the CeA. Recent experiments in the *ex vivo* brain preparation (described below) showed that CeA electrical stimulation preferentially induce rappinglike vocal patterns, suggesting that increased activity in the CeA may promote receptive vocalizations in females (Ballagh, 2014).

2.08.2.3.3 Male Interaction Calls

As described above, Rhodes et al. (2014) observed that males clasping other males (male preference) were less likely to clasp females in a male–male–female triad, indicating that male–male clasping may represent an alternative (i.e., 'sneaker male') mating strategy rather than an agonistic interaction. Because they did not report vocal patterns in their study, however, it is not clear whether vocal dominance is associated with female-preference or male-preference clasping.

In an earlier study, Tobias et al. (2004) showed that vocally dominant males produce many chirps while clasping, while vocally subordinate males clasping another male produced few chirps. These results support the idea that chirping is an aggressive vocal behavior that functions in regulating reproductive dominance. Clasping by a dominant male may represent aggression, while clasping by a subordinate male may represent a 'sneaker' strategy. Males in the Rhodes et al. (2014) study were not injected with hCG, while both males in the Tobias et al. (2004, 2010) paired recordings were. The hCG treatments may have led to aggressive clasping absent from the Rhodes et al.'s study.

These laboratory results leave open the question of how, and under what circumstances, endogenous gonadotropins act to promote aggressive clasping and chirping. Field recordings in South Africa reveal that while advertisement calling is produced at high levels throughout the breeding season, chirping peaks at the end of the season (Tobias et al., 2004). Growling, produced by males clasped by other males, drops immediately prior to the surge in chirping and answer calling. This rapid change could reflect a population-wide surge in endogenous gonadotropin in the late South African winter, as has been observed in females (King and Millar, 1979). Testing this hypothesis will require replication of the vocal results together with hormone measurements.

2.08.2.3.4 Insights from Endocrine Disrupting Compounds

Most hormone manipulation studies in *Xenopus* have addressed long-term (i.e., weeks to months) effects. Recently, a series of studies investigating the effects of endocrine disrupting compounds have begun to address short-term effects of endocrine signaling on vocal behaviors. In one study, exposure of hCG-injected males to the antiandrogen flutamide (10^{-6} M) significantly reduced the amount of advertisement calling compared to hCG-injected controls 3 and 4 days after exposure, resulting in calling levels as low as non-hCG-injected animals (Behrends et al., 2010). Similar reductions in calling were observed during exposure to 10^{-6} M of the antiandrogen vinclozolin; advertisement calling and chirping were reduced while ticking and growling were increased (Hoffmann and Kloas, 2010). In contrast, 4-day exposure to low doses (30.5 ng l^{-1}, 3.05 μg l^{-1}, 30.5 μg l^{-1}) of the xenoandrogen methyldihydrotestosterone (MDHT) induced significant increases in the percentage of advertisement calling (Hoffmann and Kloas, 2012a). The increase in percentage of advertisement calls produced by males was paralleled by a significant decrease in a call the authors referred to as rasping. The spectrogram of rasping reveals a call that appears similar to an elongated (over 5 s) slow trill uninterrupted by fast trills (Hoffmann and Kloas, 2012b). Interestingly, a similar pattern of calling was observed in a study of the effects of long-term (5–18 months) castration (Zornik and Yamaguchi, 2011). These calls (referred to as 'isolated slow trill' in Zornik and Yamaguchi) therefore may be generated by males with relatively low androgen signaling, but can be rapidly eliminated by exogenous androgens. The behavioral relevance of 'isolated slow trills' is not understood.

2.08.3 Sex Differences and Hormone Targets in the Vocal System

The vocal behaviors of *X. laevis* are highly differentiated between the sexes. Nearly all neural and muscular components of the vocal circuits are regulated by one or more steroid hormones. The vocal organ, which contains the single muscle group needed to generate vocal sounds, differs markedly in males and females (Sassoon and Kelley, 1986). In the brain, many nuclei related to auditory processing and vocal motor production were first identified by their ability to concentrate steroid hormones (Kelley et al., 1975; Morrell et al., 1975; Kelley, 1980) and have been shown to express mRNA for the androgen receptor (Pérez et al., 1996). More recently, functional contributions of these vocal system components and the mechanisms whereby steroid hormones influence the vocal system have been investigated. In the following section we describe known components of the vocal system and briefly describe the hormones that regulate their development and function.

2.08.3.1 Sex Differences and Hormone Targets in the Larynx

The adult *X. laevis* vocal organ is markedly sexually dimorphic both in size and in tissue components and is functionally specialized for producing sex-specific vocal behaviors (described in detail below). Male larynges contain a greater number of muscle fibers than female larynges and express a number of structural and functional adaptations that support the production of fast rhythmic vocalizations. Female laryngeal features support the production of slower calls (Tobias and Kelley, 1987; Sassoon and Kelley, 1986; Sassoon et al., 1987; Marin et al., 1990; Tobias et al., 1995). Larynges of both sexes express androgen and estrogen receptors (Segil et al., 1987; Fischer et al., 1995; Wu et al., 2003). At metamorphosis, male and female larynges are structurally and functionally similar; sex-specific traits arise during juvenile development. All identified sexually distinct laryngeal traits are established and maintained by either androgens or estrogens. Although some traits gradually lose hormone sensitivity, most sex-specific traits remain at least partially steroid sensitive in adulthood (Zornik and Kelley, 2011).

2.08.3.2 Sex Differences and Hormone Targets in the Vocal Circuit

2.08.3.2.1 Motor Circuit

Laryngeal muscle fibers are innervated by motor neurons located in cranial nerve nucleus (n.) IX-X in the caudal hindbrain (Kelley, 1980; Wetzel et al., 1985). Molecular markers (transcription factors and neurotransmitter enzymes) support the homology of the *Xenopus* n.IX-X to nucleus ambiguus of mammals (Albersheim-Carter et al., 2016). As in the sexually

dimorphic larynx with its greater number of muscle fibers in males, the number of male vocal motor neurons in n.IX-X is greater than in females (Kay et al., 1999). Unlike the larynx, however, differences in laryngeal motor neuron number develop before metamorphosis (Kelley and Dennison, 1990), arising through an androgen-dependent reduction in cell death in males compared to females, a process that can be partially rescued in females by androgen exposure (Kay et al., 1999). In adulthood, motor neurons remain sensitive to androgens, and many, but not all, of the laryngeal motor neurons express androgen receptors (Perez et al., 1996). Following axotomy in adult males, the total number of n.IX-X motor neurons is greatly reduced, and cell loss can be partly prevented by DHT treatment in both males and females (Pérez and Kelley, 1996). Androgens upregulate expression of the calcium-binding protein calbindin in axotomized female vocal motor neurons, suggesting a possible mechanism for its neuroprotective effects (Perez and Kelley, 1996).

Vocal rhythms are generated by a hindbrain CPG (Rhodes et al., 2007). The two primary vocal CPG nuclei are n.IX-X in the caudal medulla, which contains both motor neurons and interneurons, and a nucleus, DTAM (used as a proper noun), located in the anterior brain stem (**Figure 3(a)**). These two CPG nuclei are reciprocally connected via n.IX-X projection neurons (Wetzel et al., 1985; Zornik and Kelley, 2007), and communication between them is critical for generating vocal rhythms (Rhodes et al., 2007; Zornik et al., 2010). In addition to motor neurons, some interneurons in n.IX-X also express androgen receptors, as do neurons in DTAM (Pérez et al., 1996). Thus the two primary CPG nuclei are both under direct control of androgens (**Figure 3(b)**).

2.08.3.2.2 Auditory Pathways

Androgen receptors are expressed in the auditory ganglion, indicating that peripheral coding of auditory information is regulated by gonadal steroids (Pérez et al., 1996). An important auditory processing nucleus, the laminar nucleus of the torus semicircularis (the homologue of the mammalian inferior colliculus) located in the midbrain, concentrates three classes of steroid hormones: androgens, estrogens, and progesterone (**Figure 3(b)**; Morrell et al., 1975; Kelley, 1980). The basic sensory–motor integration pathways have been identified anatomically. Auditory information appears to primarily travel from the torus to central thalamus (CT) and then on the central amygdala (CeA), which completes the vocal pathway circuit by projecting back to the CPG via DTAM (**Figure 3(b)**; Hall et al., 2013). Neurons in the CeA express estrogen receptors and luteinizing hormone receptors (**Figure 3(b)**; Morrell et al., 1975; Yang et al., 2007). Thus, nearly every component of the anatomically identified auditory circuit is sensitive to reproductive hormones, indicating that sensory processing of vocal signals and sensorimotor integration are likely to be as sexually distinct as the behaviors themselves.

2.08.4 Generating Sexually Differentiated Vocalizations

Vocal behaviors of males and females differ markedly in at least three ways.

1. *Rate*: Except for ticking and amplectant calls, male vocalizations consist of rapid sound pulses, between 30 and 80 Hz, whereas the fastest female call does not exceed ~12 Hz.
2. *Complexity*: Male advertisement and answer calls are temporally complex: trills with different durations and pulse repetition rate alternate. Both female calls are simpler trains of sound pulses.
3. *Spectral features*: Male advertisement and answer calls are spectrally complex: each sound pulse includes two well-defined frequency peaks. Sound pulses in both female calls contain a single, less sharply defined peak.

In this section, we discuss each class of sexually differentiated vocal features, focusing on the operation of multiple levels of mechanistic control and the role of hormonal regulation.

2.08.4.1 Generating Fast and Slow Calls

The most dramatic vocal difference between the sexes is pulse rate. The biphasic male advertisement call consists of two rate-specific phases, fast trill and slow trill. The typical fast trill pulse rate is ~60 Hz (interpulse interval: ~16 ms), while the slow trill pulse rate is ~30 Hz (interval, ~30 ms). In contrast, rates of sound pulses in the female receptive call, rapping, range from ~8 to 12 Hz (interval, ~80–125 ms), much slower than male advertisement calls. The female unreceptive call, ticking, is even slower, with pulse rates at ~4 Hz. How are these sex differences in sound pulse rhythms (biphasic vs monophasic) and rates (fast vs slow) produced?

Two reduced preparations – the isolated larynx and brain – have greatly advanced our understanding of the mechanisms underlying sex-specific vocal behaviors. We review our current understanding of how sex-specific vocalizations are generated and perceived and discuss experiments aimed at determining the precise role of hormones in shaping vocal output.

2.08.4.1.1 Larynx

Xenopus vocalizations are produced under water by the contraction of a single muscle group, the laryngeal dilators. Each sound pulse in a call is generated by a synchronous bilateral contraction of these muscles (Tobias and Kelley, 1987; Yager, 1992). Investigations of laryngeal mechanisms of sound production have been greatly facilitated by the fact that the isolated larynx can generate sounds *in vitro* when both laryngeal nerves are stimulated synchronously (Tobias and Kelley, 1987). This experimental preparation – the '*vox in vitro*' – has allowed the identification of numerous sex-specific traits of male and female vocal muscle.

Sound pulses are generated when laryngeal cartilages, the arytenoid discs, are rapidly pulled apart by a tendon into which the laryngeal muscle inserts; before the next pulse can be generated, muscles must relax completely to allow laryngeal cartilages to regain contact (Yager, 1992). Speed of contraction and relaxation are major functional differences between male and female larynges. Male laryngeal muscle fibers are fast twitch, and the muscles can fully contract and relax when stimulated at fast trill rates, 60 Hz (Tobias and Kelley, 1987). In contrast, female laryngeal muscle cannot generate sound pulses faster than ~30 Hz (associated with the upper limit for pulse

intervals in rapping; Tobias and Kelley, 1987; Tobias et al., 1998b). Above these sex-typical rate ceilings, the fused tension that can be recorded from the tendon prevents the arytenoid discs from regaining contact; subsequent sound pulses cannot be generated in response to nerve activity.

The sexually differentiated expression pattern of a larynx-specific myosin heavy chain is a major factor underlying sex-specific contraction rates. Adult male larynges contain a single fiber type – medium-sized, fast-twitch muscle fibers – whereas female larynges contain a mixture of fast-twitch and slow-twitch fibers (Sassoon and Kelley, 1986). The sex difference in fiber types reflects expression of a laryngeal-specific myosin heavy chain (LM) in all male muscle fibers, but only in a subset of female muscle fibers (Catz et al., 1992). The development of ubiquitous LM expression in males requires androgen, and DHT-exposure in juvenile females upregulates LM expression. In addition, androgen treatment in females leads to a dramatic increase in the number of histologically identified fast-twitch fiber types (Sassoon et al., 1987) and also leads to rapid increase in contraction and relaxation rate of the muscle (Potter et al., 2005). Although long-term castration of adult males does not affect the histological profiles of male muscle fibers (Sassoon et al., 1987), there is a dramatic slowing of muscle contraction and an accompanying increase in fused tension during nerve stimulation trains at fast trill rates (Zornik and Yamaguchi, 2011). Whether this castration-induced slowing of the male larynx is due to effects on LM (as opposed to other components of the muscle's contractile machinery) is not known. In summary, larynges of males and females support the sex-specific calls: male larynges are specialized for generating fast calls, while female vocal organs can generate only relatively slow calls. These laryngeal characteristics are androgen-dependent and many remain hormone-dependent in adulthood.

2.08.4.1.2 Central Pattern Generator

Laryngeal muscles provide constraints on the rates of sound pulse generation but do not shape most features of vocal patterning; these are generated within the brain. Yamaguchi and Kelley (2000) recorded from the laryngeal nerve of male and female frogs during vocalization and found that each sound pulse is preceded by a compound action potential (CAP). For both sexes and all call types, synchronous firing of laryngeal motor neurons in the brain generates a CAP in the nerve that, in turn, induces rapid contraction of the laryngeal dilators and a resulting sound pulse. Thus, the temporal features of each *Xenopus* call primarily reflect patterns of brain activity.

Because the temporal patterns of nerve activity and sound are matched, vocal 'intention' can be identified unambiguously by measuring nerve activity. This feature of the *Xenopus* vocal system has been put to effective use through the development of a 'singing brain in a dish' preparation (Rhodes et al., 2007). The CNS from forebrain to hindbrain is removed from the frog, pinned to a coated dish, and maintained in an oxygenated artificial cerebrospinal fluid while recording from the laryngeal nerve, the most caudal nerve rootlet of N.IX-X (Simpson et al., 1986). Bath-application of serotonin (5-HT) reliably induces 'fictive vocalizations' – nerve activity that matches the sound pulse patterns of singing frogs (Rhodes et al., 2007); some spontaneous vocal-like nerve activity patterns can also be recorded from the isolated brain (e.g., fictive amplectant calling; Zornik and Kelley, 2008). Though 5-HT initiates fictive calling in both sexes, the patterns are sex-specific as they are *in vivo*; 5-HT induces fictive advertisement calls in male brains and fictive ticking in female brains.

The isolated brain preparation facilitates localization of the vocal pattern generator to major brain regions. Transections of the isolated brain at the border between the midbrain and hindbrain do not eliminate 5-HT-induced fictive calls, indicating that the vocal CPG is located in the hindbrain (Yu and Yamaguchi, 2010). The vocal CPG includes two reciprocally connected nuclei: n.IX-X in the caudal hindbrain and a premotor nucleus, DTAM, in the rostral hindbrain (Brahic and Kelley, 2003; Rhodes et al., 2007). In *Xenopus*, both vocal CPG nuclei were first identified by their ability to concentrate androgen (Kelley, 1980), and both are sexually dimorphic (Wetzel et al., 1985; Kay et al., 1999; Brahic and Kelley, 2003). Homologues of these two nuclei are also believed to participate in generating vocal rhythms in a distantly related frog species, *Rana pipiens* (Schmidt, 1992), and expiration in lamprey ('paratrigeminal respiratory group' nucleus; Cinelli et al., 2013; Leininger and Kelley, 2015) and mammals (parabrachial complex; Dick et al., 1994; Song and Poon, 2009). It is likely that vocal CPGs in many, if not all, frog species comprise these two nuclei and that both contribute to coordinating vocalization with breathing in mammals.

2.08.4.1.2.1 Motor Neurons

As described above, many, but not all, laryngeal motor neurons express androgen receptors (Kelley, 1980; Pérez et al., 1996), which suggests an ongoing role for androgens in maintaining appropriate motor neuron function throughout adulthood. Motor neuron firing rates are significantly higher during fictive vocalizations in males than in females, and membrane properties and firing patterns of motor neurons express sexually distinct features for generating their sex-specific motor patterns. The majority of male motor neurons spike with short latency following current injection and are thus well suited to the demands of rapid firing with high temporal precision (Yamaguchi et al., 2003). In contrast, female motor neurons have longer spike onset times (Yamaguchi et al., 2003), which may account for the less precise firing patterns, and resulting longer nerve CAPs, than males during both *in vivo* and *ex vivo* vocalizations (Yamaguchi and Kelley, 2000; Rhodes et al., 2007). Patch-clamp recordings in slice preparations also revealed that male motor neurons have higher capacitance than female motor neurons (Yamaguchi et al., 2003). Larger motor neuron somata volumes in male motor neurons could contribute to these sex differences (Potter et al., 2005). The size difference is highly plastic, and female motor neurons increase to male size after only 1 week of testosterone treatment.

2.08.4.1.2.2 Premotor Neurons

The major source of input to laryngeal motor nucleus is the premotor nucleus, DTAM, located in the anterior hindbrain (Wetzel et al., 1985; Zornik and Kelley, 2007). Whole-cell patch-clamp recordings in isolated male brains during fictive advertisement calling have revealed a population of

premotor neurons that are active only during fictive fast trills (Zornik and Yamaguchi, 2012). These fast trill neurons produce long-lasting depolarizations that coincide with each fast trill. During these depolarizations, fast trill neurons produce action potentials that precede each fast trill CAP recorded from the nerve. Most fast trill neurons project directly to n.IX-X, suggesting that these neurons provide premotor signals that drive motor output. Earlier physiological studies showed that DTAM neurons provide monosynaptic glutamatergic excitatory input to laryngeal motor neurons (Zornik and Kelley, 2008), supporting the notion that synchronous firing of fast trill neurons is responsible for generating each fast trill CAP.

When depolarized by current steps, fast trill neurons preferentially generate spikes around 60 Hz, the approximate rate of fast trills (Zornik and Yamaguchi, 2012). Intrinsic fast trill neuron membrane properties may be tuned to fire at 60 Hz, and thus be critical for setting the fast trill rhythm. DTAM neurons express androgen receptors (Kelley, 1981; Pérez et al., 1996), and it is likely that fast trill neurons in particular are directly regulated by androgens. When androgens were ablated by castration, the rate of fictive fast trills was reduced over a period of months, indicating a role for androgens in maintaining normal fast trill rhythms (Zornik and Yamaguchi, 2011). We hypothesize that androgens act directly on fast trill neurons to ensure high levels of expression of androgen-regulated proteins that permit firing at 60 Hz for long periods and that the loss of these properties in the absence of androgen shifts vocal rhythms. Exactly how androgens act to regulate fast trill neurons and possible roles for an activity-dependent mechanism remain to be explored.

The sensitivity of sexually differentiated behavioral systems to reproductive hormones depends on developmental stage: early actions can produce permanent changes in the behavioral repertoire, while actions during adulthood can be transient (Phoenix et al., 1959). Sensitivity to androgens is often studied by manipulating hormone levels across development. For *X. laevis*, androgen promotion of clasping serves as an example of a transient action of a hormone: abolished in adult males by gonadectomy, reinstated by androgen replacement, and induced, in a form not distinguishable from that of males, in adult females by androgen treatment (Kelley, 1982). Similarly, male calling is abolished by castration and reinstated by androgen replacement (Wetzel and Kelley, 1983). However, masculinization of calling in females appears less malleable. Watson and Kelley (1992) provided testis implants or androgen pellets to juveniles at a number of postmetamorphic stages. They noted a decline in the ability to masculinize call temporal patterns with age in females but not in males. Long-term testis implants and androgen treatment (10–15 months) in adult females, however, did induce some biphasic advertisement calls. Potter et al. (2005) found that within 8 weeks, androgen-treated adult females produced biphasic, advertisement call-like vocalizations. Their fast trills approached, but did not quite equal, the 60 Hz rates typical of males. These results confirm that the adult female CPG can be masculinized to generate male-like vocal rhythms, albeit incompletely. While the processes underlying this androgen-induced masculinization of adult females is unclear, cells fated to become fast trill neurons are likely participants. Identifying androgen-induced functional, structural, and/or synaptic changes in these neurons that drive the ontogeny of fast trill rhythms is a challenge for the future. A goal is to identify both CPG components that remain androgen-sensitive, as well as those that lose androgen-sensitivity and thus constrain masculinization in adulthood.

2.08.4.2 Generating Complex and Simple Vocal Patterns

2.08.4.2.1 Biphasic versus Monophasic Calling

Fast trill neurons in DTAM produce long-lasting depolarizations that coincide with each fast trill (Zornik and Yamaguchi, 2012). These depolarizations require NMDA receptor signaling and are blocked by NMDA receptor antagonists. In the presence of TTX (to block spike-mediated synaptic transmission) and NMDA (to activate NMDA receptors), fast trill neurons generate membrane potential oscillations in which the duration and period of each depolarization is similar to the duration and period of fast trills. The constellation of ion currents in DTAM must support membrane potential oscillations with advertisement call patterns: that is, about one call per second, with each fast trill lasting about 300 ms. Slow trills only occur while fast trill neurons are hyperpolarized. Thus fast trill neurons may be largely responsible for controlling the temporal patterns of male advertisement calls.

Because fast trill neurons produce these membrane potential oscillations synchronously, population–level activity in DTAM can be readily recorded as local field potential (LFP) waves that correspond to fast trills. DTAM activity in females does not reveal the LFP waves seen in males (Ballagh, 2014). During androgen treatment, however, females gradually develop biphasic calls. Masculinization of existing neurons in female DTAM probably involves the rearrangement of ion currents to promote long-lasting depolarizations that lead to membrane oscillations with an ~1 Hz period.

During 5-HT evoked fictive ticking in the *ex vivo* female brain, CAP rates recorded from the laryngeal nerve occur at ~4 Hz (Rhodes et al., 2007). In the initial stages of testosterone-induced masculinization, single sound pulses transition to doublet, triplet, and quadruplet pulse bursts (Hannigan and Kelley, 1986; Potter et al., 2005). The appearance of pulse bursts could be due to prolonged depolarization in female premotor neurons.

Which neuronal properties are most strongly regulated by androgens and which neuronal properties are most closely associated with overall circuit activity? Elongating bursts may be correlated with increasing NMDA receptor current density. Alternatively, NMDA currents may change little during masculinization; instead, an upregulation of slower outward currents could underlie changes in temporal patterns of vocal output. Combining androgen-induced masculinization treatments in adult females with physiological recordings in the isolated brain will permit the identification of steroid-induced neuronal traits that contribute to sex-specific vocal characteristics.

2.08.4.2.2 Intensity Modulation of Vocal Trills

2.08.4.2.2.1 Synaptic Strength in the Larynx

In addition to rhythm and rate, the intensity modulation of male and female calls also differs. In male advertisement and answer calls, sound pulses early in fast trills are of low intensity and become gradually louder throughout each trill;

female ticking and rapping sound pulses, in contrast, do not vary systematically in intensity (**Figure 1**; Tobias et al., 1998b). The laryngeal neuromuscular synapse contributes to intensity modulation. When the male laryngeal nerve is stimulated at advertisement call rates *ex vivo*, amplitudes of the electromyogram, tension, and sound amplitudes all increase progressively throughout the stimulus train (Tobias and Kelley, 1987). This increase in amplitudes reflects the use-dependent facilitation of the weak male laryngeal synapse (Tobias et al., 1995; Ruel et al., 1998). In contrast, females have strong laryngeal synapses, well suited to ensuring that slower rates of nerve activity are faithfully converted to sound. The sex difference in synaptic strength arises presynaptically and is due to elevated quantal content at the synaptic terminals of female motor neurons (Tobias et al., 1995). Strong female laryngeal synapses arise during juvenile development and require prolonged elevated estrogen levels; ovariectomy leads to a decrease in synaptic strength (Tobias et al., 1998a). In contrast, androgen treatment weakens female synapses (Tobias and Kelley, 1995). In adult males, long-term castration appears to reduce male-typical EMG potentiation during nerve stimulation trains, reflecting castration-dependent synaptic strengthening (Zornik and Yamaguchi, 2011).

Laryngeal neuromuscular junctions are well suited to generating intensity-modulated male calls via weak facilitating synapses and slower female calls via strong synapses, both of which depend on appropriate androgen and estrogen levels during development and in adulthood. Thus, as is the case for clasping behavior, synaptic strength displays endocrine-regulated flexibility, even in adulthood.

2.08.4.2.2.2 Motor Neuron Recruitment

Laryngeal nerve recordings in singing frogs revealed that CAP amplitudes during fast trills also progressively increase throughout each trill (Yamaguchi and Kelley, 2000), and this pattern is preserved in fictive vocalizations (Rhodes et al., 2007). The most likely mechanism is sequential recruitment of motor neurons. The neural basis for this recruitment appears to have at least two components. First, the premotor fast trill neurons are gradually recruited across each trill. Early in a trill only a few fast trill neurons spike; the number of spiking neurons then increases, and nearly all fast trill neurons spike at the end of a trill (Zornik and Yamaguchi, 2012). Second, the DTAM-to-motor neuron synapses facilitate; when DTAM is electrically stimulated in trains from 20 to 60 Hz, nerve CAPs progressively increase in amplitude, as is also seen during *in vivo* calling (Zornik and Kelley, 2008). Thus, intensity modulation of fast trills is coded in three ways: premotor neuron activation, premotor-to-motor synaptic facilitation, and neuromuscular junction facilitation. We know that the weak, facilitating male neuromuscular synapse is regulated by hormones. Because both DTAM and motor neurons express androgen receptors, it is likely that some aspects of this trait are also androgen-dependent. Finally, because of the redundant mechanisms underlying intensity modulation, this aspect of fast trills could be a behaviorally critical component of the communication signal, a hypothesis supported by the even more dramatic intensity modulation of the answer call during male/female duets (Tobias et al., 1998b).

2.08.5 Processing Vocal Signals

2.08.5.1 Sexually Differentiated Auditory Processing

Auditory sensitivity also appears to differ in females and males. While no sex differences in spectral preference were identified in auditory nerve fibers using single-cell electrophysiological recordings (N.VIII; Elliott et al., 2007), auditory brain stem response (ABR) measurements have revealed sex-specific frequency preferences (Hall et al., 2016). *Xenopus laevis* male sound pulses contain two major DFs: ~1.9 and ~2.2 kHz (**Figure 2**). As would be predicted from the matched-filter hypothesis (Frishkopf et al., 1968; Capranica and Moffat, 1983; Moreno-Gomez et al., 2013; Simmons, 2013), in females, ABR thresholds for combinations of these two frequencies are lower than either frequency alone; small shifts in either abolish this enhanced sensitivity. This enhanced sensitivity is absent in males. Instead, ABR sensitivity peaks at lower frequencies, just under 1.5 kHz.

These results indicate that, at the level of the hindbrain, the female auditory system is more highly tuned to male call frequencies than is the male auditory system; this acoustic advantage may facilitate locating a mate. Males also communicate vocally, but most of their vocal interactions are close range, often while clasping, and enhanced sensitivity may be less important. However, males do establish vocal dominance hierarchies, which are sensitive to advertisement call intensity (Tobias et al., 2010). Relative insensitivity to advertisement calls could release males from a vocally subordinate condition at shorter distances from a dominant male. Field recordings do indicate that very few frogs advertise on any given night (Tobias et al., 2004); the number may depend on how well sound travels in particular bodies of water.

An alternative possibility is that slow trills, which contain a lower frequency band below 1.5 kHz not present in fast trill sound pulses (**Figure 2**; Yamaguchi et al., 2010), may be the more salient feature for establishing dominance. This hypothesis has some support from experimental evidence: a subset of males was preferentially vocally suppressed by artificial slow-trill-only stimuli, which were more effective than either fast-trill-only stimuli or natural advertisement call playbacks (Tobias et al., 2010). If true, these findings may indicate a sex-specific tuning to distinct features of the male advertisement call: females attending to fast trills, males attending to slow trills. This arrangement is analogous to sex-specific preferences in the tree frog, *Eleutherodactylus coqui*, in which the male auditory system preferentially responds to the first 'co' note of a male calls, while female auditory systems preferentially respond to the second 'qui' component (Narins and Capranica, 1976).

Male auditory sensitivity may also be tuned to female frequencies, which have a DF ~1.2 kHz (Vignal and Kelley, 2007). Although the unreceptive call, ticking, is used at close range, the female 'advertisement call,' rapping, is a high-intensity signal that should carry longer distances under water, allowing males to find distant receptive mates (Tobias et al., 1998b).

Sex-specific auditory sensitivities are steroid-dependent. Ovariectomy masculinized female sensitivity to DF1–DF2 dyads and to frequencies between 1 and 1.5 kHz, while androgen treatment with DHT prevented this masculinization

(Hall et al., 2016). These results are consistent with the finding that androgens are the primary circulating steroids in female *X. laevis* (Lutz et al., 2001). These results also suggest a possible functional role for the observation that androgen receptors are expressed in the auditory ganglion (Pérez et al., 1996), but one would then expect a similar change in males. It is possible that male and female auditory ganglia respond differently to androgen exposure, or that there is a dose–response difference between the sexes. Alternatively, sex-specific production of the DHT metabolite 5α-androstane-3β,17β-diol (3β-diol), a potent agonist of ERβ, could be a DHT-dependent source of estrogen (Oliveira et al., 2007). Although ERβ expression in the *Xenopus* auditory periphery has not been explored, if estrogen receptors are expressed in hair cells of the inner ear, as they are in vocal fish (Forlano et al., 2015), then selective production of 3β-diol only in female hair cells could account for DHT-dependent feminization of the auditory periphery.

2.08.5.2 Generating Socially Appropriate Vocal Responses

To facilitate effective social interactions, vocal responses of a receiver must be appropriately based on auditory information from the sender. Examples of socially appropriate vocal responses in *X. laevis* include the stimulation of male answer calling by female rapping, the transient suppression of male calling by female ticking, and the prolonged suppression of male calling by intense advertisement calling of another male (**Figure 1**; Tobias et al., 1998b, 2004; Elliott and Kelley, 2007). Activation of CNS auditory regions must be sufficient to promote appropriate vocal responses since broadcast calls are effective in eliciting these responses. The CeA plays a critical role in matching auditory signals to call-type responses (Hall et al., 2013). Neurons in the CeA receive synaptic input from the CT, which in turn receives inputs from the auditory midbrain (**Figure 3**). When the CeA is lesioned, males continue to generate spontaneous advertisement calls, and thus are not mute. Lesioned males also can still respond to intense advertisement call broadcasts with prolonged vocal suppression, and thus are not deaf. However, both ticking and rapping also elicit prolonged vocal suppression, a response that intact males do not normally make to either female call. In addition to socially inappropriate vocal suppression to female calling, CeA-lesioned males also produce a lower proportion of answer calls when paired with another male. These results indicate that the CeA may be a positive regulator of vocal responses, as such responses are diminished after lesions. Because the CeA is reciprocally connected with the CPG nucleus DTAM (**Figure 3**), there is a direct anatomical pathway by which CeA may activate socially appropriate vocal responses.

2.08.6 Evolution of Vocal Effectors in *Xenopus*

The *ex vivo* brain and larynx preparations have been used to probe the mechanisms underlying the evolutionary convergence of a rare vocal pattern in *Xenopus* (Leininger and Kelley, 2013; Leininger et al., 2015). Each *Xenopus* species has a unique advertisement call that can be distinguished by call temporal patterns and spectral properties of sound pulses (Tobias et al., 2011). Three classes of call pattern can be identified: fast and long-lasting (trill-type (as in *X. laevis*)), fast and brief (burst-type), and slow and ongoing (click-type). Each type occurs in multiple branches of the phylogeny, and parsimony suggests that the ancestral call type was burst-type. The click-type pattern occurs in two species – *Xenopus boumbaensis* and *Xenopus borealis* – which are found on distantly related branches of the *Xenopus* phylogeny, indicating that their click-type calls evolved independently. As in *X. laevis*, the *X. boumbaensis* larynx is strongly sexually dimorphic; male fibers are large and fast twitch, while female fibers are small and mixed twitch type. Because male *X. boumbaensis* laryngeal fibers are mostly fast twitch, they could support the production of fast trills as found in *X. laevis*. However, the CPG generates very short vocal bursts consisting of two CAPs in the nerve (Leininger and Kelley, 2013). These doublets are translated into single sound pulses due to the laryngeal synapse's requirement for facilitation (see Section 2.08.4.2.2.1). In *X. borealis* the larynges are less dramatically sexually dimorphic. Male and female muscle fibers are both a mixture of fast and slow twitch, though male fibers are larger and greater in number (Leininger et al., 2015). The *X. borealis* vocal CPG produces only single CAPS. Because the male *X. borealis* laryngeal synapse does not require facilitation, the single CAPs in the nerve faithfully produce single sound pulses. Thus, the vocal CPG rhythm and the laryngeal synapse in *X. borealis* are similar to features associated with ticking in female *X. laevis*. Since male laryngeal muscle fibers in the very distantly related species *Xenopus tropicalis* are entirely fast twitch (Baur et al., 2008), this feature is presumed to represent the ancestral state. The convergent evolution of click-type calls in *X. borealis* and *X. boumbaensis* are thus due to distinct mechanisms: in *X. boumbaensis*, the number of CAPs produced by the CPG is greatly reduced, while in *X. borealis*, the developmental program for the CPG, the laryngeal synapse, and muscle fiber types are all demasculinized (Leininger and Kelley, 2015).

2.08.7 Conclusion

Despite the conceptual value of the organization-activation dichotomy (Phoenix et al., 1959), this stark distinction is often an oversimplification with many exceptions (Arnold and Breedlove, 1985; Tobet and Fox, 1992; Arnold, 2009; McCarthy and Nugent, 2013). Hormonal regulation occurs throughout development and into adulthood. While some traits are irreversibly set during a narrow critical period, others retain full hormone sensitivity throughout life, while still others retain intermediate sensitivity. *Xenopus* vocalizations are complex, context-dependent and sexually differentiated, making them excellent subjects for identifying the role of reproductive hormones in regulating behaviors. The two *ex vivo* vocal preparations, the isolated larynx and brain, have permitted numerous advances in understanding the neural and muscular mechanisms of vocal production. Each can produce fictive vocalizations independent of the other, a feature that has enabled identification of separate hormonal effects on discrete vocal effectors that work together in the animal. For example, although the larynges of many androgen-treated females are not capable of generating sound pulses at fast trill rates, the isolated brain preparation permits an accurate assessment of the hormone sensitivity of the vocal CPG in adults. Similarly,

castration leads to nearly complete elimination of *in vivo* male calling, yet fictive vocalizations can still be generated by the isolated brain, allowing the identification of castration-induced functional changes in the CPG. Because the full acoustic communication pathway from ear to brain to vocal organ has been outlined, with nearly every component known to be hormone-sensitive, the *Xenopus* vocal system provides an experimentally powerful preparation for discovering novel roles for hormone-directed behaviors and should offer new insights about fundamental mechanisms for sexually differentiated vertebrate behaviors.

References

Abbott, D.H., Keverne, E.B., Bercovitch, F.B., Shively, C.A., Mendoza, S.P., Saltzman, W., Snowdon, C.T., Ziegler, T.E., Banjevic, M., Garland Jr., T., Sapolsky, R.M., 2003. Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Horm. Behav. 43, 67–82.

Albersheim-Carter, J., Blubaum, A., Ballagh, I.H., Missaghi, K., Siuda, E.R., McMurray, G., Bass, A.H., Dubuc, R., Kelley, D.B., Schmidt, M.F., Wilson, R.J., Gray, P.A., 2016. Testing the evolutionary conservation of vocal motoneurons in vertebrates. Respir. Physiol. Neurobiol. 224, 2–10.

Arnold, A.P., Breedlove, S.M., 1985. Organizational and activational effects of sex steroids on brain and behavior: a reanalysis. Horm. Behav. 19, 469–498.

Arnold, A.P., 2009. The organizational-activational hypothesis as the foundation for a unified theory of sexual differentiation of all mammalian tissues. Horm. Behav. 55, 570–578.

Ballagh, Irene H., 2014. Sex Differences in the Structure, Function and Regulation of Vocal Circuits in Xenopus (Ph.D. thesis). Columbia University.

Baur, L.A., Nasipak, B.T., Kelley, D.B., 2008. Sexually differentiated, androgen-regulated, larynx-specific myosin heavy-chain isoforms in *Xenopus tropicalis*; comparison to *Xenopus laevis*. Dev. Genes Evol. 218, 371–379.

Behrends, T., Urbatzka, R., Krackow, S., Elepfandt, A., Kloas, W., 2010. Mate calling behavior of male South African clawed frogs (*Xenopus laevis*) is suppressed by the antiandrogenic endocrine disrupting compound flutamide. Gen. Comp. Endocrinol. 168, 269–274.

Brahic, C.J., Kelley, D.B., 2003. Vocal circuitry in *Xenopus laevis*: telencephalon to laryngeal motor neurons. J. Comp. Neurol. 464, 115–130.

Capranica, R.R., Moffat, A.J.M., 1983. Neurobehavioral correlates of sound communication in anurans. In: Ewert, J.P., Capranica, R.R., Ingle, D. (Eds.), Advances in Vertebrate Neuroethology. Plenum Press, New York, pp. 701–730.

Catz, D.S., Fischer, L.M., Moschella, M.C., Tobias, M.L., Kelley, D.B., 1992. Sexually dimorphic expression of a laryngeal-specific, androgen-regulated myosin heavy chain gene during *Xenopus laevis* development. Dev. Biol. 154, 366–376.

Cinelli, E., Robertson, B., Mutolo, D., Grillner, S., Pantaleo, T., Bongianni, F., 2013. Neuronal mechanisms of respiratory pattern generation are evolutionary conserved. J. Neurosci. 33, 9104–9112.

Cuddy, M., Aubin-Horth, N., Krahe, R., 2012. Electrocommunication behaviour and non invasively-measured androgen changes following induced seasonal breeding in the weakly electric fish, *Apteronotus leptorhynchus*. Horm. Behav. 61, 4–11.

Dick, T.E., Bellingham, M.C., Richter, D.W., 1994. Pontine respiratory neurons in anesthetized cats. Brain Res. 636, 259–269.

Elkan, E.R., 1938. The Xenopus pregnancy test. Br. Med. J. 2, 1253–1256, 1274–2.

Elliott, T.M., Kelley, D.B., 2007. Male discrimination of receptive and unreceptive female calls by temporal features. J. Exp. Biol. 210, 2836–2842.

Elliott, T.M., Christensen-Dalsgaard, J., Kelley, D.B., 2007. Tone and call responses of units in the auditory nerve and dorsal medullary nucleus of *Xenopus laevis*. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 193, 1243–1257.

Elliott, T.M., Christensen-Dalsgaard, J., Kelley, D.B., 2011. Temporally selective processing of communication signals by auditory midbrain neurons. J. Neurophysiol. 105, 1620–1632.

Fischer, L.M., Catz, D., Kelley, D.B., 1995. Androgen-directed development of the *Xenopus laevis* larynx: control of androgen receptor expression and tissue differentiation. Dev. Biol. 170, 115–126.

Forlano, P.M., Sisneros, J.A., Rohmann, K.N., Bass, A.H., 2015. Neuroendocrine control of seasonal plasticity in the auditory and vocal systems of fish. Front. Neuroendocrinol. 37, 129–145.

Frishkopf, L.S., Capranica, R.R., Goldstein Jr., M.H., 1968. Neural coding in the bullfrog's auditory system—a teleological approach. Proc. IEEE 56, 969–980.

Goymann, W., Wingfield, J.C., 2004. Allostatic load, social status and stress hormones: the costs of social status matter. Anim. Behav. 67, 591–602.

Hall, I.C., Ballagh, I.H., Kelley, D.B., 2013. The Xenopus amygdala mediates socially appropriate vocal communication signals. J. Neurosci. 33, 14534–14548.

Hall, I.C., Woolley, S.M., Kwong-Brown, U., Kelley, D.B., 2016. Sex differences and endocrine regulation of auditory-evoked, neural responses in African clawed frogs (Xenopus). J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 202, 17–34.

Hannigan, P., Kelley, D.B., 1986. Androgen-induced alterations in vocalizations of female *Xenopus laevis*: modifiability and constraints. J. Comp. Physiol. A 158, 517–527.

Hoffmann, F., Kloas, W., 2010. An environmentally relevant endocrine-disrupting antiandrogen, vinclozolin, affects calling behavior of male *Xenopus laevis*. Horm. Behav. 58, 653–659.

Hoffmann, F., Kloas, W., 2012a. Effects of environmentally relevant concentrations of the xeno-androgen, methyldihydrotestosterone, on male and female mating behavior in *Xenopus laevis*. Chemosphere 87, 1246–1253.

Hoffmann, F., Kloas, W., 2012b. Estrogens can disrupt amphibian mating behavior. PLoS One 7, e32097.

Hogben, L., Charles, E., Slome, D., 1931. Studies on the pituitary VIII. J. Exp. Biol. 8 (4), 345–354.

Jeffrey, J.D., Esbaugh, A.J., Vijayan, M.M., Gilmour, K.M., 2012. Modulation of hypothalamic-pituitary-interrenal axis function by social status in rainbow trout. Gen. Comp. Endocrinol. 176, 201–210.

Juntti, S.A., Hilliard, A.T., Kent, K.R., Kumar, A., Nguyen, A., Jimenez, M.A., Loveland, J.L., Mourrain, P., Fernald, R.D., 2016. A neural basis for control of cichlid female reproductive behavior by prostaglandin F2alpha. Curr. Biol. 26, 943–949.

Kay, J.N., Hannigan, P., Kelley, D.B., 1999. Trophic effects of androgen: development and hormonal regulation of neuron number in a sexually dimorphic vocal motor nucleus. J. Neurobiol. 40, 375–385.

Kelley, D.B., Dennison, J., 1990. The vocal motor neurons of *Xenopus laevis*: development of sex differences in axon number. J. Neurobiol. 21, 869–882.

Kelley, D.B., Pfaff, D.W., 1976. Hormone effects on male sex behavior in adult South African clawed frogs, *Xenopus laevis*. Horm. Behav. 7, 159–182.

Kelley, D.B., Morrell, J.I., Pfaff, D.W., 1975. Autoradiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. I. Testosterone. J. Comp. Neurol. 164, 47–59.

Kelley, D.B., 1980. Auditory and vocal nuclei in the frog brain concentrate sex hormones. Science 207, 553–555.

Kelley, D.B., 1981. Locations of androgen-concentrating cells in the brain of *Xenopus laevis*: autoradiography with 3H-dihydrotestosterone. J. Comp. Neurol. 199, 221–231.

Kelley, D.B., 1982. Female sex behaviors in the South African clawed frog, *Xenopus laevis*: gonadotropin-releasing, gonadotropic, and steroid hormones. Horm. Behav. 16, 158–174.

King, J.A., Millar, R.P., 1979. Hypothalamic luteinizing hormone-releasing hormone content in relation to the seasonal reproductive cycle of *Xenopus laevis*. Gen. Comp. Endocrinol. 39, 309–312.

Leininger, E.C., Kelley, D.B., 2013. Distinct neural and neuromuscular strategies underlie independent evolution of simplified advertisement calls. Proc. Biol. Sci. 280, 20122639.

Leininger, E.C., Kelley, D.B., 2015. Evolution of courtship songs in Xenopus : vocal pattern generation and sound production. Cytogenet Genome Res. 145, 302–314.

Leininger, E.C., Kitayama, K., Kelley, D.B., 2015. Species-specific loss of sexual dimorphism in vocal effectors accompanies vocal simplification in African clawed frogs (Xenopus). J. Exp. Biol. 218, 849–857.

Lutz, L.B., Cole, L.M., Gupta, M.K., Kwist, K.W., Auchus, R.J., Hammes, S.R., 2001. Evidence that androgens are the primary steroids produced by *Xenopus laevis* ovaries and may signal through the classical androgen receptor to promote oocyte maturation. Proc. Natl. Acad. Sci. U.S.A. 98, 13728–13733.

Marin, M.L., Tobias, M.L., Kelley, D.B., 1990. Hormone-sensitive stages in the sexual differentiation of laryngeal muscle fiber number in *Xenopus laevis*. Development 110, 703–711.

Maruska, K.P., 2014. Social regulation of reproduction in male cichlid fishes. Gen. Comp. Endocrinol. 207, 2–12.

McCarthy, M.M., Nugent, B.M., 2013. Epigenetic contributions to hormonally-mediated sexual differentiation of the brain. J. Neuroendocrinol. 25, 1133–1140.

McLean, J., Bricault, S., Schmidt, M.F., 2013. Characterization of respiratory neurons in the rostral ventrolateral medulla, an area critical for vocal production in songbirds. J. Neurophysiol. 109, 948–957.

Moreno-Gomez, F.N., Sueur, J., Soto-Gamboa, M., Penna, M., 2013. Female frog auditory sensitivity, male calls, and background noise: potential influences on the evolution of a peculiar matched filter. Biol. J. Linn. Soc. 110, 814–827.

Morrell, J.I., Kelley, D.B., Pfaff, D.W., 1975. Autoradiographic localization of hormone-concentrating cells in the brain of an amphibian, *Xenopus laevis*. II. Estradiol. J. Comp. Neurol. 164, 63–77.

Narins, P.M., Capranica, R.R., 1976. Sexual differences in the auditory system of the tree frog *Eleutherodactylus coqui*. Science 192, 378–380.

Ode, M., Asaba, A., Miyazawa, E., Mogi, K., Kikusui, T., Izawa, E., 2015. Sex-reversed correlation between stress levels and dominance rank in a captive non-breeder flock of crows. Horm. Behav. 73, 131–134.

Oliveira, A.G., Coelho, P.H., Guedes, F.D., Mahecha, G.A., Hess, R.A., Oliveira, C.A., 2007. 5alpha-Androstane-3beta,17beta-diol (3beta-diol), an estrogenic metabolite of 5alpha-dihydrotestosterone, is a potent modulator of estrogen receptor ERbeta expression in the ventral prostrate of adult rats. Steroids 72, 914–922.

Perez, J., Kelley, D.B., 1996. Trophic effects of androgen: receptor expression and the survival of laryngeal motor neurons after axotomy. J. Neurosci. 16, 6625–6633.

Pérez, J., Cohen, M.A., Kelley, D.B., 1996. Androgen receptor mRNA expression in *Xenopus laevis* CNS: sexual dimorphism and regulation in laryngeal motor nucleus. J. Neurobiol. 30, 556–568.

Phoenix, C.H., Goy, R.W., Gerall, A.A., Young, W.C., 1959. Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65, 369–382.

Picker, M., 1980. *Xenopus laevis* (Anura: Pipidae) mating systems-a preliminary synthesis with some data on the female phonoresponse. South Afr. J. Zool. 15 (3), 150–158.

Picker, M.D., 1983. Hormonal induction of the aquatic phonotactic response of Xenopus. Behavior 84, 74–90.

Potter, K.A., Bose, T., Yamaguchi, A., 2005. Androgen-induced vocal transformation in adult female African clawed frogs. J. Neurophysiol. 94, 415–428.

Rhodes, H.J., Yu, H.J., Yamaguchi, A., 2007. Xenopus vocalizations are controlled by a sexually differentiated hindbrain central pattern generator. J. Neurosci. 27, 1485–1497.

Rhodes, H.J., Stevenson, R.J., Ego, C.L., 2014. Male-male clasping may be part of an alternative reproductive tactic in *Xenopus laevis*. PLoS One 9, e97761.

Ruel, T.D., Kelley, D.B., Tobias, M.L., 1998. Facilitation at the sexually differentiated laryngeal synapse of *Xenopus laevis*. J. Comp. Physiol. A 182, 35–42.

Sassoon, D., Kelley, D.B., 1986. The sexually dimorphic larynx of *Xenopus laevis*: development and androgen regulation. Am. J. Anat. 177, 457–472.

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

Schmidt, R.S., 1992. Neural correlates of frog calling: production by two semi-independent generators. Behav. Brain Res. 50, 17–30.

Segil, N., Silverman, L., Kelley, D.B., 1987. Androgen-binding levels in a sexually dimorphic muscle of *Xenopus laevis*. Gen. Comp. Endocrinol. 66, 95–101.

Shen, W., Zhang, X.Y., Liu, D.Z., Wang, D.H., 2015. Hormones orchestrated pre- and post-copulatory sexual traits in male Mongolian gerbils. Physiol. Behav. 143, 90–96.

Simmons, A.M., 2013. "To ear is human, to frogive is divine": Bob Capranica's legacy to auditory neuroethology. J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol. 199, 169–182.

Simpson, H.B., Tobias, M.L., Kelley, D.B., 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.

Song, G., Poon, C.S., 2009. Lateral parabrachial nucleus mediates shortening of expiration and increase of inspiratory drive during hypercapnia. Respir. Physiol. Neurobiol. 165, 9–12.

Stacey, N.E., Goetz, F.W., 1982. Role of prostaglandins in fish reproduction. Can. J. Fish. Aquat. Sci. 39, 92–98.

Tobet, S.A., Fox, T.O., 1992. Sex differences in neuronal morphology influenced hormonally throughout life. In: Gerall, A.A., Moltz, H., Ward, I.L. (Eds.), Sexual Differentiation. Springer, USA, pp. 41–83.

Tobias, M.L., Kelley, D.B., 1987. Vocalizations by a sexually dimorphic isolated larynx: peripheral constraints on behavioral expression. J. Neurosci. 7, 3191–3197.

Tobias, M.L., Kelley, D.B., 1995. Sexual differentiation and hormonal regulation of the laryngeal synapse in *Xenopus laevis*. J. Neurobiol. 28, 515–526.

Tobias, M.L., Kelley, D.B., Ellisman, M., 1995. A sex difference in synaptic efficacy at the laryngeal neuromuscular junction of *Xenopus laevis*. J. Neurosci. 15, 1660–1668.

Tobias, M.L., Tomasson, J., Kelley, D.B., 1998a. Attaining and maintaining strong vocal synapses in female *Xenopus laevis*. J. Neurobiol. 37, 441–448.

Tobias, M.L., Viswanathan, S.S., Kelley, D.B., 1998b. Rapping, a female receptive call, initiates male-female duets in the South African clawed frog. Proc. Natl. Acad. Sci. U.S.A. 95, 1870–1875.

Tobias, M.L., Barnard, C., O'Hagan, R., Horng, S.H., Rand, M., Kelley, D.B., 2004. Vocal communication between male. Anim. Behav. 67, 353–365.

Tobias, M.L., Corke, A., Korsh, J., Yin, D., Kelley, D.B., 2010. Vocal competition in male *Xenopus laevis* frogs. Behav. Ecol. Sociobiol. 64, 1791–1803.

Tobias, M.L., Evans, B.J., Kelley, D.B., 2011. Evolution of advertisement calls in African clawed frogs. Behaviour 148, 519–549.

Tupal, S., Rieger, M.A., Ling, G.Y., Park, T.J., Dougherty, J.D., Goodchild, A.K., Gray, P.A., 2014. Testing the role of preBötzinger Complex somatostatin neurons in respiratory and vocal behaviors. Eur. J. Neurosci. 40, 3067–3077.

Vignal, C., Kelley, D., 2007. Significance of temporal and spectral acoustic cues for sexual recognition in *Xenopus laevis*. Proc. Biol. Sci. 274, 479–488.

Watson, J.T., Kelley, D.B., 1992. Testicular masculinization of vocal behavior in juvenile female *Xenopus laevis* reveals sensitive periods for song duration, rate, and frequency spectra. J. Comp. Physiol. A 171, 343–350.

Weintraub, A.S., Kelley, D.B., Bockman, R.S., 1985. Prostaglandin E2 induces receptive behaviors in female *Xenopus laevis*. Horm. Behav. 19, 386–399.

Wetzel, D.M., Kelley, D.B., 1983. Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. Horm. Behav. 17, 388–404.

Wetzel, D.M., Haerter, U.L., Kelley, D.B., 1985. A proposed neural pathway for vocalization in South African clawed frogs, *Xenopus laevis*. J. Comp. Physiol. A 157, 749–761.

Wu, K.H., Tobias, M.L., Kelley, D.B., 2001. Estrogen and laryngeal synaptic strength in *Xenopus laevis*: opposite effects of acute and chronic exposure. Neuroendocrinology 74, 22–32.

Wu, K.H., Tobias, M.L., Thornton, J.W., Kelley, D.B., 2003. Estrogen receptors in Xenopus: duplicate genes, splice variants, and tissue-specific expression. Gen. Comp. Endocrinol. 133, 38–49.

Yager, D.D., 1992. A unique sound production mechanism in the pipid anuran *Xenopus borealis*. Zool. J. Linn. Soc. Lond. 104, 351–375.

Yamaguchi, A., Kelley, D.B., 2000. Generating sexually differentiated vocal patterns: laryngeal nerve and EMG recordings from vocalizing male and female African clawed frogs (*Xenopus laevis*). J. Neurosci. 20, 1559–1567.

Yamaguchi, A., Kaczmarek, L.K., Kelley, D.B., 2003. Functional specialization of male and female vocal motoneurons. J. Neurosci. 23, 11568–11576.

Yamaguchi, A., Munoz, M.M., Bose, T.O., Oberlander, J.G., Smith, S., 2010. Sexually distinct development of vocal pathways in *Xenopus laevis*. Dev. Neurobiol. 70, 862–874.

Yang, E.J., Nasipak, B.T., Kelley, D.B., 2007. Direct action of gonadotropin in brain integrates behavioral and reproductive functions. Proc. Natl. Acad. Sci. U.S.A 104, 2477–2482.

Yu, H.J., Yamaguchi, A., 2009. 5-HT2C-like receptors in the brain of *Xenopus laevis* initiate sex-typical fictive vocalizations. J. Neurophysiol. 102, 752–765.

Yu, H.J., Yamaguchi, A., 2010. Endogenous serotonin acts on 5-HT2C-like receptors in key vocal areas of the brain stem to initiate vocalizations in *Xenopus laevis*. J. Neurophysiol. 103, 648–658.

Zornik, E., Kelley, D.B., 2007. Breathing and calling: neuronal networks in the *Xenopus laevis* hindbrain. J. Comp. Neurol. 501, 303–315.

Zornik, E., Kelley, D.B., 2008. Regulation of respiratory and vocal motor pools in the isolated brain of *Xenopus laevis*. J. Neurosci. 28, 612–621.

Zornik, E., Kelley, D.B., 2011. A neuroendocrine basis for the hierarchical control of frog courtship vocalizations. Front. Neuroendocrinol. 32, 353–366.

Zornik, E., Yamaguchi, A., 2011. Vocal pathway degradation in gonadectomized *Xenopus laevis* adults. J. Neurophysiol. 105, 601–614.

Zornik, E., Yamaguchi, A., 2012. Coding rate and duration of vocalizations of the frog, *Xenopus laevis*. J. Neurosci. 32, 12102–12114.

Zornik, E., Katzen, A.W., Rhodes, H.J., Yamaguchi, A., 2010. NMDAR-dependent control of call duration in *Xenopus laevis*. J. Neurophysiol. 103, 3501–3515.