# NEUROENDOCRINE REGULATION OF FEMALE MATE RECOGNITION BEHAVIOR IN TÚNGARA FROGS

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## ABSTRACT

MUKTA CHAKRABORTY: Neuroendocrine Regulation of Female Mate Recognition Behavior in Túngara Frogs
(Under the direction of Sabrina S. Burmeister)

An enduring question in sexual selection is how females choose mates to increase their reproductive success. Therefore, understanding the proximate mechanisms underpinning female choice is essential to understanding speciation and evolution. An important mechanism underlying female mate choice is modulation of neural pathways by sex steroid hormones. My dissertation explores how steroid hormones influence female sexual behavior and auditory processing of species-specific signals in túngara frogs.

To determine which hormonal conditions promote sexual behavior in female túngara frogs, I assessed the effect of hormone manipulations on phonotaxis behavior toward conspecific calls in post-reproductive females. I found that estradiol is sufficient for the expression of phonotaxis behavior.

Steroid hormones exert their effects by acting through steroid receptors in the brain. I found expression of androgen receptor (AR), estrogen receptors alpha and beta (*ER*$\alpha$ and *ER*$\beta$) mRNA in parts of the auditory system and the forebrain auditory targets. I identified new putative sites of steroid action within the pallium, posterior tuberculum, locus coeruleus, and the principal nucleus of the torus semicircularis. Females had higher *ER*$\alpha$ and *ER*$\beta$ expression than males in the auditory midbrain, whereas males had higher *AR* expression than females, indicating that sex steroid hormones are likely to have sexually dimorphic effects on auditory processing.

Neural representation of species-specific signals is thought to emerge at higher levels of processing. I measured expression of the immediate early gene *egr-1* in response to conspecific, heterospecific, or no sound stimuli in parts of the ascending auditory system and the primary forebrain targets. With three exceptions, all auditory nuclei showed greater responses to the conspecific call than the heterospecific call, suggesting that the auditory system responds preferentially to conspecific stimuli.

Finally, I measured expression of *egr-1* after estradiol injections in parts of the ascending auditory system and its forebrain targets and the nucleus accumbens. Both estradiol and conspecific calls together induced greater neural responses than either alone in most auditory nuclei and the nucleus accumbens, suggesting an additive effect on *egr-1* induction. I conclude that estradiol is an important neuromodulator and may influence mate recognition systems that are critical for mate choice.

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## ABBREVIATIONS

| | |
|---|---|
| Acc | Nucleus accumbens |
| AP | Amphibian papilla |
| AR | Androgen receptor |
| ASIH | American Society of Ichthyologists and Herpetologists |
| Athal | Anterior thalamus |
| BP | Basilar papilla |
| Cthal | Central thalamus |
| DMN | Dorsal medullary nucleus |
| dMP | Dorsal medial pallium |
| DP | Dorsal pallium |
| dSt | Dorsal striatum |
| DTAM | Dorsal tegmental area of medulla |
| ER | Estrogen receptor |
| HCG | Human chorionic gonadotropin |
| IACUC | Institutional Animal Care and Use Committee |
| La | Lateral thalamus |
| LH | Lateral hypothalamus |
| LP | Lateral pallium |
| LSD | Least significant difference |
| Ltor | Laminar nucleus of torus semicircularis |
| MINAE | Ministerio del Ambiente Y Energia |
| MP | Medial pallium |
| MCtor | Magnocellular nucleus of torus semicircularis |
| OT | Optic tectum |
| POA | Preoptic area |

| | |
|---|---|
| Pthal | Posterior thalamus |
| Ptor | Principal nucleus of torus semicircularis |
| SCN | Suprachiasmatic nucleus |
| Sep | Septum |
| Sd | Dorsal septal nucleus |
| SINAC | Sistema Nacional de Áreas de Conservación |
| Sl | Lateral septum |
| Sld | Dorsal part of lateral septum |
| Slv | Ventral part of lateral septum |
| Sm | Medial septal nucleus |
| SON | Superior olivary nucleus |
| SSAR | Society for the Study of Amphibians and Reptiles |
| St | Striatum |
| STRI | Smithsonian Tropical Research Institute |
| SVL | Snout vent length |
| Teg | Tegmentum |
| Tel | Telencephalon |
| TH | Tyrosine hydroxylase |
| VH | Ventral hypothalamus |
| VL | Ventrolateral thalamus |
| VM | Ventromedial thalamus |
| vMP | Ventral medial pallium |
| VP | Ventral pallium |
| vSt | Ventral striatum |

# CHAPTER 1

## INTRODUCTION

Choosing a mate is one of the most important decisions a sexually reproducing individual makes in its lifetime. An enduring question in sexual selection is how females choose mates to increase their reproductive success (Andersson, 1994). Male courtship signals may encode information important for both species recognition and mate quality assessment (Gerhardt, 1992). Therefore, female preference for male traits constitutes an important mechanism for sexual selection and speciation (Andersson, 1994; Doebeli, 2005; Panhuis et al., 2001; Ryan et al., 2009; van Doorn et al., 2004), where females are known to display strong preferences for elaborate male secondary sexual traits, and for conspecifics over heterospecifics (but see Pfennig, 2007). Since females invest a large proportion of energy in reproduction and incur greater costs for mating with heterospecifics they are generally the more discriminating sex. Consequently, the timing of expression of sexual behavior is highly regulated in females to ensure reproductive success. Therefore, understanding the proximate mechanisms underlying expression of female sexual behavior is essential to understanding variation in female mate choice and speciation.

Mate choice requires detection and perception of information through sensory processing mechanisms and then evaluating this information using a set of decision-making rules (Ryan et al., 2009). Information transmitted by the signaler must traverse a noisy environment to reach the receiver where it is transduced into a neural code that can then be evaluated by the receiver. Therefore, receivers must possess physiological adaptations that

enhance detection and perception of sexual stimuli. Although we know why females vary their mating preferences (Andersson, 1994), we know less about the physiological and neural mechanisms underpinning mate choice decisions. What physiological and cognitive conditions are necessary for females to be sexually receptive and be able to discriminate among sexual signals? What neural pathways are involved in recognizing, processing, and categorizing male sexual signals to ensure mate detection and species recognition? These are some of the critical unanswered questions in sexual selection studies. To fully comprehend the biology of mate choice behavior, it is important that we apply an integrative approach since data and interpretations that are available from one level of analysis can inform data and interpretations from another level (Ryan, 2005). While an understanding of behavioral ecology helps to identify the selection pressures that led to the evolution of a trait, examining the neural substrate for decision-making helps to identify the mechanistic basis of female preference for that trait. Females must first be sexually receptive in order to respond to male courtship signals, which require a suite of physiological (e.g. hormonal) changes that enhance detection and perception of sexual stimuli. Therefore, an insight into the neuroendocrine mechanisms underlying expression of sexual receptivity is important for understanding how the brain evolved to process species-specific signals, important in mate choice.

In the following studies, I use the túngara frog (*Physalaemus pustulosus*) as a model system to investigate the neuroendocrine mechanisms of female sexual behavior important for mate recognition. Specifically, I ask four questions: (1) Which hormone(s) influence sexual receptivity to species-specific calls? (2) What are the target areas of hormone action in the brain of túngara frogs? (3) What are the brain regions involved in processing species-specific signals in females? (4) What is the role of estradiol in neural processing of species-specific signals in female túngara frogs? In this chapter, I first provide a brief review of the role of steroid hormones in expression of sexual behavior in anurans. Second, I describe the

central auditory pathways in anurans, and third, I describe the túngara frogs (*Physalaemus pustulosus*) as a model system to investigate the neuroendocrine mechanisms underpinning female sexual behavior.

## A. Neuroendocrinology of sexual behavior

### General introduction

There is considerable interest in the role of sex steroid hormones in regulating expression of mating behavior. For example, estradiol is a potent regulator of both male and female sexual behaviors in birds and mammals (Ball and Balthazart, 2004; Meisel and Sachs, 1994). Lordosis behavior in rodents, a classic example of steroidal regulation of female sexual behavior is dependent on the presence of estradiol and progesterone. In reptiles, testosterone is known to facilitate female sexual behavior, which is in part due to aromatization of the hormone to estradiol (Noble and Greenberg, 1940; Winkler and Wade, 1998). Unlike tetrapods, estrogen does not appear to be essential for the expression of female sexual behavior for fish with external fertilization (see reviews by Munakata and Kobayashi, 2010; Stacey, 1983). Thus, it appears that there is considerable diversity in steroidal regulation of sexual behavior in mammals, reptiles, and fish. The role of steroid hormones in modulating female neural pathways that process male mating signals is unclear.

Animal communication systems generally involve the production of species-typical signals produced by males that evoke a response in the receiver, the female (Ryan, 1980; Searcy, 1992). In many taxa, it is not clear which aspects of the species-typical signal evokes behavioral responses from females. Anurans serve as excellent model systems to assess female responses to mating signals since females base their mate choice decisions almost entirely on the acoustic properties of the mating signal produced (Gerhardt and

Huber, 2002; Ryan, 1985; Wells, 1977). In addition, the central auditory pathways are well characterized (Wilczynski and Capranica, 1984; Wilczynski and Endepols, 2007), which allows us to investigate the effects of hormones on sensory processing of mating signals.

Reproductive behavior in anurans: a brief review

Anurans have been of longstanding interest as model organisms to study behavioral neuroendocrinology for a long time and therefore have a distinguished history. Seminal studies by researchers such as Dodd (1960) demonstrated causal relationships between elevations of androgen levels and the expression of sexual behaviors in amphibians and led to numerous later studies of how steroid hormones regulate vocal communication in anurans. Like most other vertebrate species, reproduction occurs seasonally and is regulated by steroid hormones and a variety of peptide neuromodulators which implies that there is considerable diversity in hormone-behavior relationships among species (see review by Yamaguchi and Kelley, 2002). Given the importance of acoustic signaling for expression of social and sexual behaviors, it is not surprising that the vocal and auditory systems are strongly influenced by steroid hormones and contain steroid concentrating cells (Kelley, 1980; Kelley et al., 1975; Morrell et al., 1975), although little is known about the distribution of sex steroid hormone receptors.

Male anurans typically use a few stereotyped vocalizations to communicate with conspecifics (see reviews by Arch and Narins, 2009; Moore et al., 2005). The most widely studied vocal behaviors include “advertisement calls” that are used to attract females and defend territories, and the “release calls” that are typically produced by unreceptive females to prevent unwanted clasping by males for reproduction. While castration will usually result in the cessation of advertisement calling behavior, the cause and effect relationships between the display of advertisement calls and plasma androgen levels in natural populations have not been resolved. While several studies have demonstrated a positive

correlation between calling and plasma testosterone levels (Harvey and Propper, 1997; Marler and Ryan, 1996; Solis and Penna, 1997; Wada and Gorbman, 1977), others have found an inverse relationship (Mendonca et al., 1985). Furthermore, the neuropeptide arginine vasotocin has been shown to facilitate advertisement calling in several amphibian species. Treatment with vasotocin facilitates the display of advertisement calling in *Rana catesbeiana*, *Hyla cinerea*, *Hyla versicolor*, *Acris crepitans*, and *Bufo cognatus* (Boyd, 1994; Chu et al., 1998; Marler et al., 1995; Penna et al., 1992b; Propper and Dixon, 1997; Tito et al., 1999). It appears that there is considerable variation in hormone-behavior relationships in male anurans.

Studies that have investigated hormone-behavior relationships in female anurans typically focused on hormonal induction of receptivity to advertisement signals of males. Sexual behavior in females in most anuran species includes approaching an advertising male (phonotaxis) during the time of ovulation (Gerhardt and Huber, 2002), emitting a vocalization in some species (Shen et al., 2008; Tobias et al., 1998), or inhibiting release calls and leg extensions (Boyd, 1992; Diakow and Nemiroff, 1981; Kelley, 1982). Early hormone studies in females demonstrated that female American toads (*Bufo americanus*) will approach a conspecific mate signal when injected with a variety of peptide or steroid hormones such as human chorionic gonadotropin (HCG) or prostaglandin (Schmidt, 1984a; Schmidt, 1985b; Weintraub et al., 1985). In *Xenopus laevis*, females vocalize when they have mature eggs (Tobias et al., 1998) and display sexual receptivity when their hormone levels are high (Kelley, 1982). Females also vary in their degree of receptivity to advertisement calls depending on reproductive state. For example, female túngara frogs exhibit their highest level of receptivity when they near ovulation, but also become less choosy while accepting less attractive calls (Lynch et al., 2005). Female gonadal hormones are seasonally modulated (Licht and McCreery, 1983), but in species with long breeding seasons, steroid concentrations may fluctuate and females may cycle through breeding

stages multiple times (Harvey et al., 1997; Lynch and Wilczynski, 2005; Medina et al., 2004). Earlier studies have shown that receptivity can be induced in ovariectomized *X. laevis* with estrogen and progesterone injections, but requires an additional injection of HCG for expression of maximal receptivity (Kelley, 1982). Additionally, studies indicate that testosterone levels are higher in reproductive females than in males and higher than estrogen levels in many anuran species (Harvey and Propper, 1997; Itoh and Ishii, 1990; Medina et al., 2004). Taken together, these studies indicate that there is significant diversity among anurans in the hormonal mechanisms underlying female sexual behavior.

Male advertisement calling may induce female sexual receptivity by eliciting increase in plasma hormone levels (Lynch and Wilczynski, 2006) which suggests that hormones may directly influence auditory processing in females. Female Majorcan midwife toads (*Alytes mulentensis*) exposed to mate choruses continued to ripen and mature eggs whereas females that were exposed to heterospecific calls or random tones reabsorbed resources from their eggs (Lea et al., 2001). In a recent study, Lynch and Wilczynski (2008) found that injections of HCG and exposure to conspecific mating choruses induced expression of the activity-dependent immediate early gene (IEG) *egr-1* (early growth response 1), within the auditory midbrain of female túngara frogs. The most familiar IEGs are c-fos and *egr-1* (also known as *zif268,* and *ZENK*), which are often used as a means of measuring neural activity (Burmeister et al., 2008; Hoke et al., 2004; Jarvis, 2004b; Mello et al., 1992). These results indicate that hormones such as HCG can enhance the stimulatory effect of the conspecific calls. At present, it is unknown if the enhanced *egr-1* responses to conspecific calls is due to the direct binding of HCG to Luteinizing hormone receptors, or due to the downstream effects of HCG administration (i.e., the induced release of gonadal hormones which influence auditory neurons through steroid receptors).

To date, only a handful of studies have addressed the effects of hormonal modulation of acoustic processing in female frogs. For example, Yovanof and Feng (1983)

demonstrated that auditory evoked potentials recorded from the midbrain torus semicircularis of female leopard frogs (*Lithobates pipiens*) in response to tones that matched frequencies contained in conspecific advertisement calls increased in amplitude after injections with estradiol. Other studies have shown that gonadectomy influences multiunit audiograms in the torus semicircularis of male *Hyla cinerea* (Penna et al., 1992), and that single-unit and multiunit neural responses in the torus semicircularis vary seasonally (Goense and Feng, 2005; Hillery, 1984; Walkowiak, 1980). Furthermore, in female green treefrogs (*Hyla cinerea*), implantation with testosterone increased midbrain auditory thresholds for frequencies corresponding to the male advertisement call, but not for frequencies outside these spectral bands (Miranda, 2007). Recently, Miranda and Wilczynski (2009) showed that testosterone may influence the filtering properties of the auditory system in a sex-specific manner. The midbrain torus semicircularis is known to contain androgen concentrating cells in *Xenopus laevis* (Kelley, 1980), indicating that the auditory system is a target of steroid action. However, since a detailed description of the neuroanatomical distribution of sex steroid receptors in anurans is still unknown, we do not have a clear idea about neural pathways that are potential targets of hormone action.

**B. The central auditory system in anurans**

The auditory system is more closely tied physiologically and behaviorally to social communication in anuran amphibians than in any other vertebrate species and is therefore well characterized. The anuran central auditory system has been characterized in some species belonging to the genus *Rana*, *Xenopus*, and *Hyla*, which share common anatomical features. Although there is some variation among species we can assume that anatomical connections are generally similar across most genus including *Pustulosus*. The largest component of the auditory system, the midbrain torus semicircularis (homologous to

mammalian inferior colliculus), serves as a key point in the central auditory pathways integrating ascending auditory and descending forebrain auditory inputs. However, anurans do not possess a telencephalic auditory area such as the mammalian auditory cortex or the avian Field L. There are three levels of the central auditory system: lower brainstem, midbrain, and forebrain (Fig. 1.1). Anurans also have two inner ear auditory papillae that are sensitive to acoustic stimuli of different, but overlapping frequencies, the amphibian papilla (AP), and the basilar papilla (BP).

Lower brainstem auditory nuclei

Anurans have a single primary auditory nucleus in the dorsal lateral medulla at the entrance of the eighth cranial nerve, which is known as the dorsal medullary nucleus (DMN). Afferents from the AP (low and mid frequencies) and BP (high frequencies) enter with AP fibers more dorsal than BP fibers in the nerve (Fuzessery and Feng, 1981). Two efferent pathways ascend from the DMN (homologous to the mammalian cochlear nucleus), similar to the dual ascending auditory pathways from the mammalian cochlear nucleus. The first connection (Fig. 1.1) extends to the midbrain torus semicircularis (Edwards and Kelley, 2001; Pettigrew, 1981), and the second is a bilateral connection to the superior olivary nucleus (SON) (Feng, 1986a; Will et al., 1985). The SON receives bilateral projections from the DMN (Feng, 1986b; Wilczynski, 1988; Will et al., 1985) and sends an ascending connection to the torus semicircularis (Edwards and Kelley, 2001; Feng, 1986b; Wilczynski, 1981) and the caudal thalamus (Feng, 1986b). The SON and DMN exhibit tonotopy but do not demonstrate complex feature detection.

The midbrain torus semicircularis

The torus semicircularis is a major integrative center which receives ascending auditory projections from all hindbrain auditory nuclei, and descending inputs from the

forebrain (Endepols and Walkowiak, 2001; Wilczynski, 1981). The torus sends efferents to thalamic nuclei and parts of the subpallial telencephalon (Fig. 1.1). The torus contains several nuclei, which are organized differently from the mammalian inferior colliculus with which it shares homology. The three primary nuclei include the laminar, principal, and magnocellular nuclei (Potter, 1965). The principal nucleus is the primary target of ascending auditory fibers (Feng and Lin, 1991; Matesz and Kulik, 1996; Walkowiak and Luksch, 1994), whereas the laminar and magnocellular nuclei receive weaker input. Projections from the hypothalamus (Wilczynski, 1981) and anterior preoptic area (Edwards and Kelley, 2001) converge in the laminar nucleus providing an endocrine input. The laminar nucleus of the torus semicircularis sends projections to the ventral part of the caudal striatum/dorsal pallium via the ventrolateral pathway, and some neurons project to the lateral septal complex (Endepols and Walkowiak, 2001; Neary, 1988).

Thalamus and forebrain auditory targets

Forebrain auditory pathways extend from the midbrain and spread extensively to the diencephalon and telencephalon. Most of the thalamic nuclei receive some form of toral connections (Fig. 1.1). The anterior, anterior lateral, and ventrolateral thalamic nuclei receive connections from the laminar nucleus of the torus semicircularis (Endepols and Walkowiak, 2001; Luksch and Walkowiak, 1998). The lateral anterior and central thalamic nuclei send connections to the striatum/dorsal pallium (Endepols et al., 2004; Marín et al., 1997a, 1997b; Neary, 1988), an area that is considered to be a motivational/associative pathway modulating motor output (Walkowiak et al., 1999). Furthermore, the anterior thalamic nucleus projects to the septal complex and the medial pallium (Neary, 1984; Northcutt and Ronan, 1992; Roden et al., 2005) which constitutes a limbic pathway, although its function remains obscure (Wilczynski and Endepols, 2007).

Neurophysiology

Feature detectors emerge in the auditory midbrain and the caudal thalamus (Fuzessery and Feng, 1983; Mudry et al., 1977). Electrophysiological studies have suggested that toral neurons with complex feature detection properties presumably contribute to representation of mating signals (Edwards et al., 2002; Fuzessery, 1988; Penna et al., 1997; Rose and Capranica, 1984). Furthermore, there is significant diversity in response properties of toral neurons suggesting that they possess spectral and temporal selectivity (Fuzessery, 1988; Penna et al., 1997). Some neurons in the torus respond only to two-tone combinations (Fuzessery and Feng, 1983), while others are sensitive to the number of pulses in a call (Edwards et al., 2002). Feng and Lin (1991) proposed that the principal nucleus is dedicated to spectral processing whereas the laminar nucleus is dedicated to temporal processing. Other authors speculate that the laminar and magnocellular nuclei are sites of integration of auditory, motor, and motivational systems (Endepols and Walkowiak, 2001) because inputs from the forebrain nuclei converge here and they project to the spinal cord (Endepols and Walkowiak, 1999).

**C. Functional mapping of neural activity using immediate early genes (IEG)**

The expression of immediate early genes (Fig. 1.2) has been used to investigate neural correlates of mate choice decisions. Operationally, immediate early genes are those genes that are inducible in the presence of protein synthesis inhibitors and therefore must not require the preceding activation of any other responsive genes (Clayton, 2000). Therefore, they represent the earliest genomic response to a particular inducing stimulus. The IEG proteins are divided into two categories, the transcription factors and the direct effectors. Direct effector IEGs act directly to modify synaptic structure and function, whereas the transcription factor IEGs act by altering the transcription of other target genes encoding

downstream effector proteins. The most familiar IEGs are c-fos and *egr-1* (also known as *zif268,* and *ZENK*), which are often used as a means of detecting increased neural activity. Expression of *egr-1* is linked to the activity of postsynaptic receptors by second messenger cascades and their expression can be brought about by enhancing the firing rate of presynaptic neurons onto target cells (Jarvis, 2004a). However, *egr-1* expression can be uncoupled from production of action potentials (Keefe and Gerfen, 1999) since *egr-1* induction relies on the suite of activators and repressors present in the cell that may vary spatially with cell type and temporally with context (Jarvis, 2004b). A single neuron can express multiple immediate early genes in response to a stimulus and therefore if a brain area lacks expression of a specific IEG, it does not necessarily represent a lack of neuronal activation (Jarvis, 2004a). Nonetheless, IEG mapping offers several advantages, the foremost being that it allows simultaneous functional mapping of the entire brain in freely moving animals, which makes it an attractive molecular tool in studies of avian (Jarvis, 2004b; Maney et al., 2006; Mello et al., 1992; Sockman et al., 2002) and anuran acoustic communication (Burmeister et al., 2008; Hoke et al., 2005; Hoke et al., 2004). In chapter 4, I use the immediate early gene *egr-1* to investigate neural responses in the central auditory system and its primary forebrain targets in response to mating signals. In chapter 5, I combine the *egr-1* mapping techniques with estradiol manipulations to assess the effects of estradiol in modulating neural response patterns in the central auditory system and its forebrain auditory targets.

**D. The túngara frogs (*Physalaemus pustulosus*) as a model system**

Túngara frogs (*Physalaemus pustulosus*) has been a focus of sexual selection studies for decades and thus we know a great deal about their behavioral responses to communication signals (Ryan, 1985). In this series of investigations, I examine the

neuroendocrine mechanisms of female mate recognition behavior in *P. pustulosus*, which is allopatric with other species in the genus *Physalaemus* throughout most of its range, with the exception of the Llanos region in Venezuela, where it is sympatric with a heterospecific species, *Physalaemus enesefae* (La Marca, 1992). Similar to other anurans, male túngara frogs aggregate at night in choruses producing advertisement calls to attract females, and compete with rival males. Males produce a simple advertisement call that is a frequency-modulated "whine", and can increase the attractiveness of the whine by adding up to 7 suffixes called "chucks" to produce a complex "whine-chucks" call that is strongly preferred by females over the simple whine-only call (Rand and Ryan, 1981). Females express mating preferences by differential phonotaxis toward the call of choice, preferring higher amplitude calls over low amplitude calls (Rand et al., 1997). However, females in this species do not produce advertisement calls (Ryan, 1980; Ryan, 1985).

Female behavioral responses for species-specific and heterospecific calls are well documented in *P. pustulosus* (Ryan, 1985; Ryan and Rand, 2003; Ryan et al., 1990). Female túngara frogs make mate choice decisions in acoustically complex environments, and their recognition processes have presumably been shaped by the costs associated with incorrect responses to biologically relevant signals. Female recognition and discrimination of mating stimuli is usually demonstrated using one-choice and two-choice phonotaxis tests, where females approach a specific mating stimulus (Ryan, 1985; Ryan and Rand, 1993). Females appear to use categorical discrimination of calls by perceiving whines as "simple" calls and whines appended with chucks as "complex calls", both of which elicit species recognition (Rand and Ryan, 1981; Ryan, 1985). Chucks alone are not sufficient to evoke species recognition in females (Rand and Ryan, 1981). Although females prefer whines with chucks, the number of chucks does not influence female mate preferences (Bernal et al., 2009). Moreover, females vary in choosiness depending on body condition (Baugh and Ryan, 2009), actively assess multiple signalers simultaneously, and are sensitive to the

location of preferred call types using an open-ended mate choice process that was previously unknown in anurans (Baugh and Ryan, 2010).

*P. pustulosus* and *P. enesefae* advertisement calls

The advertisement call of *P. pustulosus* and *P. enesefae* is a descending frequency sweep called a whine (Fig. 1.3). The *P. pustulosus* whine is sufficient for species recognition, beginning at approximately 1000 Hz and sweeping to 400 Hz in 350 ms (Ryan, 1985). Male *P. pustulosus* can adorn the simple call by appending up to 7 chucks (40 ms bursts of sound) to produce the complex whine-chucks call (Fig. 1.3B). Although females strongly prefer the whine-chucks calls, predatory bats and parasitic flies localize males based on their calls and prefer males that produce chucks, thereby imposing a negative selection on males that produce the attractive complex call (Rand and Ryan, 1981; Tuttle and Ryan, 1981). The whine of *P. enesefae* shares many features with those of its congeners and has the longest call duration (720 ms) compared to other species of its genus (Tárano, 2001). Furthermore, the *P. enesefae* whine is frequency modulated, beginning with 1060 Hz and descending to 590 Hz (Fig. 1.3A). It also contains a rich harmonic structure, with the dominant call frequency in the second harmonic (Tárano, 2001). Past studies have shown that the whine stimulates the amphibian papilla (AP), whereas the chuck stimulates the basilar papilla (Ryan and Rand, 1990).

Functional mapping of the túngara auditory system in response to mating signals

Functional mapping studies using *egr-1* in túngara frogs have offered important insights into the neural representation of complex stimuli within the anuran brain (Burmeister et al., 2008; Hoke et al., 2005; Hoke et al., 2007; Hoke et al., 2008; Hoke et al., 2004). A recent study has demonstrated that the laminar nucleus of the torus semicircularis might be a key region that elicits behavioral selectivity to mating signals (Hoke et al., 2008). In light of

the known connections of the laminar nucleus, it is possible that mating call representation emerges first within the torus. However, without a detailed functional mapping of the central auditory system in response to conspecific calls, it is difficult to assess the roles of the hindbrain, midbrain, and forebrain auditory nuclei, and how they each contribute to mate call recognition. In order to address this limitation, I examine system-wide neural responses to species-specific mating calls in females in Chapter 4 to explore where response biases to species-specific calls may emerge.

**E. Steroid-dependent plasticity in neural processing of social and mating signals**

Steroid hormones may influence female receptivity to male courtship signals. One possible mechanism by which steroid hormones affect behavioral responses in the signal receiver is by modifying processing of the signal. Effects of steroid hormones on sensory systems originates from clinical studies in humans which report that females experience shifts in olfactory, auditory, and visual systems during natural fluctuations in the menstrual cycle (Avitabile et al., 2007; Pause et al., 1996; Walpurger et al., 2004). Neuroendocrine modulation of sensory processing has also been reported in other mammals (Moffat, 2003), birds (Hinde and Steele, 1964; Maney et al., 2006; Maney et al., 2008), reptiles (Rose and Moore, 2002), fish (see review by Sisneros, 2009; Zakon and Smith, 2002), and amphibians (Lynch and Wilczynski, 2008; Penna et al., 1992). For example, in female white-throated sparrows, the expression of the immediate early gene, *egr-1* in the auditory system is selective for song only when plasma estradiol levels exceed non-breeding levels (Maney et al., 2006). A recent study has shown that estradiol influences auditory processing through rapid changes in neuronal excitability and modulation of plasticity-associated genes in songbirds, indicating mechanisms through which estradiol may influence sensory processing (Tremere et al., 2009). It appears that steroid-dependent plasticity in sensory

processing may influence how females respond to species-specific signals. However, the mechanisms underlying such effects are largely unknown, as are the target sites in the brain in which potential interactions between sensory and endocrine systems occur. In Chapter 5, I investigate the target sites in the brain that are sensitive to hormonal modulation and how estradiol may influence auditory processing of species-specific signals which is important in mate recognition.

**F. Summary**

This dissertation topic is composed of a series of related empirical studies that aim to understand the neuroendocrine mechanisms underpinning female sexual behavior, which is important in female mate recognition. In Chapter 2, I first investigate which hormonal conditions promote sexual behavior in female túngara frogs. My results show that estradiol is sufficient to induce female sexual behavior and also induces the same mate call preferences as observed in naturally breeding females. In Chapter 3, I investigate the distribution of androgen and estrogen receptors in the brain of reproductive, adult túngara frogs to assess the target sites of hormone action. This chapter also represents the first study in addressing sexual dimorphism in steroid receptor expression in amphibians and provides the first description of the neuroanatomical distribution of estrogen receptors in an amphibian brain. In Chapter 4, I examine system-wide neural responses to species-specific mating calls in females to understand where neural biases to species-specific calls may emerge. Finally, in Chapter 5, I examine if estradiol modulates responses to species-specific stimuli within the auditory system and its primary forebrain auditory targets as a possible mechanism for steroid-dependent auditory plasticity regulating behaviors. Each chapter in this dissertation has been written to stand alone as a separate study to address a specific aim of this dissertation project.

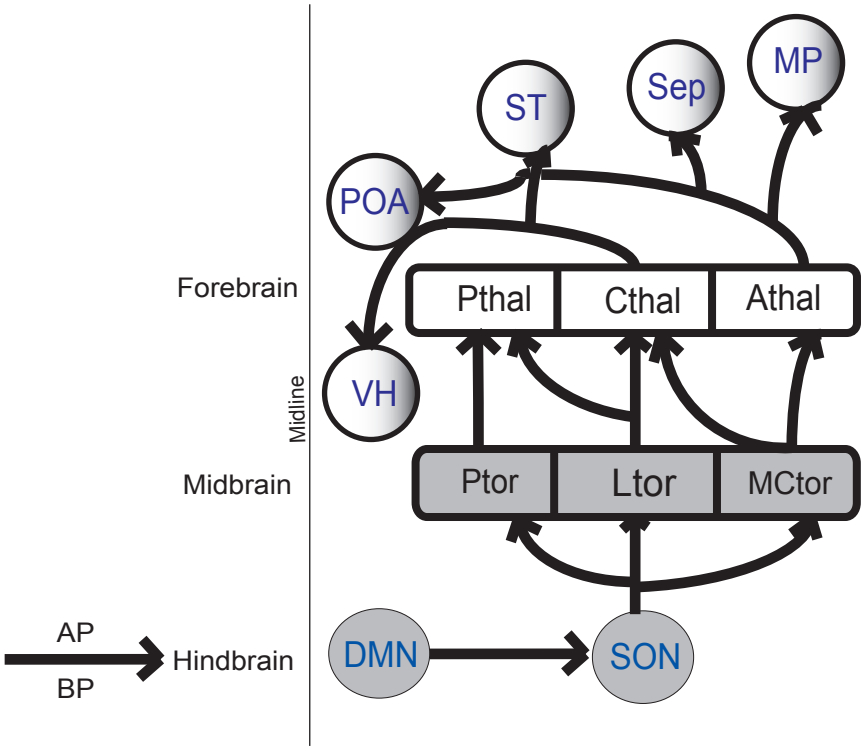
Figure Legends

Figure 1.1. Ascending pathways of the anuran central auditory system.

Figure 1.2. Model showing intracellular cascades leading to *egr-1* transcription.

Figure 1.3. Sonograms of calls. A. *Physalaemus enesefae* whine. B. *Physalaemus pustulosus* whine with one chuck.

Figure 1.1




See page xiv for abbreviations

Figure 1.2

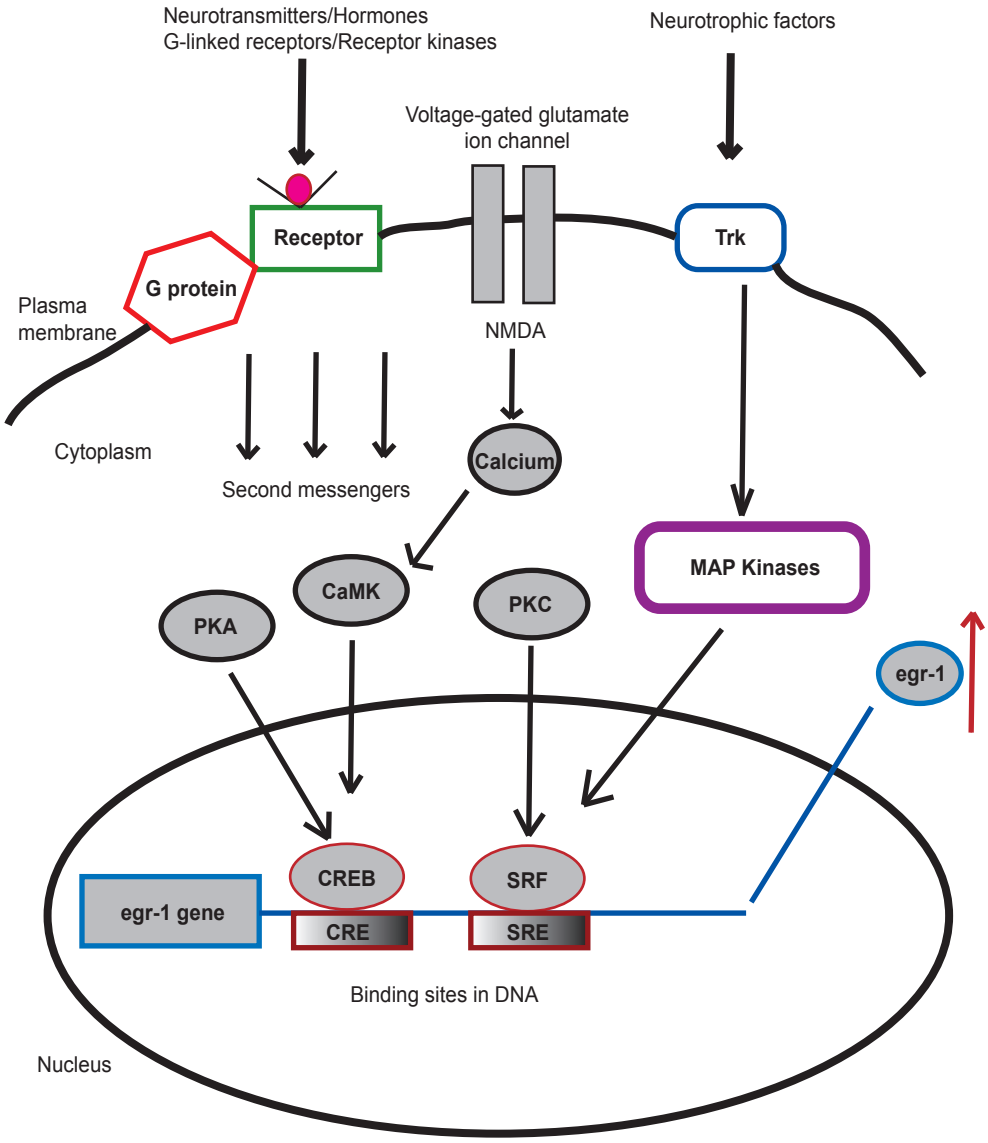
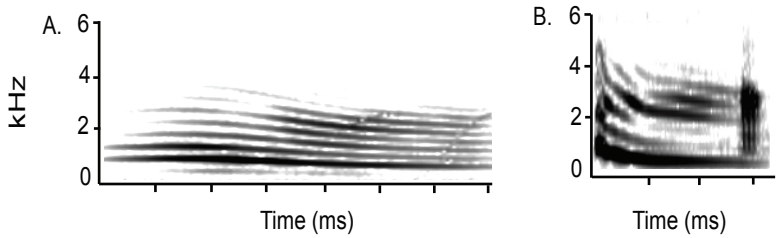

Neurotransmitters/Hormones
G-linked receptors/Receptor kinases
Neurotrophic factors
Voltage-gated glutamate
ion channel
Receptor
Trk
G protein
Plasma
membrane
NMDA
Cytoplasm
Second messengers
Calcium
MAP Kinases
CaMK
PKA
PKC
egr-1
CREB
SRF
egr-1 gene
CRE
SRE
Binding sites in DNA
Nucleus

Figure 1.3



## CHAPTER 2

# ESTRADIOL INDUCES SEXUAL BEHAVIOR IN FEMALE TÚNGARA FROGS


**Summary**

Steroid hormones play an important role in regulating vertebrate sexual behavior. In frogs and toads, injections of exogenous gonadotropins, which stimulate steroid h[1]ormone production, are often used to induce reproductive behavior, but steroid hormones alone are not always sufficient. To determine which hormonal conditions promote sexual behavior in female túngara frogs, we assessed the effect of hormone manipulation on the probability of phonotaxis behavior toward conspecific calls in post-reproductive females. We injected females with human chorionic gonadotropin (HCG), estradiol, estradiol plus progesterone, saline, or HCG plus fadrozole (an aromatase blocker) and tested their responses to mating calls. We found that injections of HCG, estradiol, and estradiol plus progesterone all increased phonotaxis behavior, whereas injections of saline or HCG plus fadrozole did not. Since injections of estradiol alone were effective at increasing phonotaxis behavior, we concluded that estradiol is sufficient for the expression of phonotaxis behavior. Next, to determine if estradiol-injected females display the same behavioral preferences as naturally breeding females, we compared mating call preferences of naturally breeding females to


[1] This chapter is reprinted with permission from Hormones and Behavior; Chakraborty, M. and Burmeister, S. S. 2009. Hormones and Behavior 55, 106-112.

those of post-reproductive females injected with estradiol. We found that, when injected with estradiol, females show similar call preferences as naturally breeding females, although they were less likely to respond across multiple phonotaxis tests. Overall, our results suggest that estradiol is sufficient for the expression of sexual responses to mating calls in túngara frogs. To our knowledge, ours is the only study to find that estradiol alone is capable of promoting phonotaxis behavior in a frog.

**Introduction**

Steroid hormones are important regulators of sexual behavior in vertebrates. In females, studies conducted on a variety of vertebrates have shown that estrogen plays an important role in facilitating sexual behavior (Ball and Balthazart, 2004; Moore et al., 2005). For example, both estrogen and progesterone are required for expression of estrous behavior and mating in rodents (Luttge et al., 1977). In reptiles, testosterone is known to facilitate female sexual behavior, which is in part due to aromatization of the hormone to estradiol (Noble and Greenberg, 1940; Winkler and Wade, 1998). In anurans (frogs and toads), however, there appears to be diversity in hormone-behavior relationships among species, with a variety of hormones implicated as being important.

In anurans, female sexual behavior can be expressed as movement towards conspecific calling males ("phonotaxis") (Gerhardt and Huber, 2002), as producing vocalizations to attract males (Shen et al., 2008; Tobias et al., 1998), or as the inhibition of behaviors typical of unreceptive females, such as release calls and leg extensions (Boyd, 1992; Diakow and Nemiroff, 1981; Kelley, 1982). As in many other vertebrates, female anurans exhibit sexual behavior when they near oviposition (Lynch et al., 2005), a time when sex steroid hormones also tend to be high (Lynch and Wilczynski, 2005). A number of studies have found that injections of human chorionic gonadotropins (HCG) effectively

increases sexual behavior in female frogs (Kelley, 1982; Lynch et al., 2006; Schmidt, 1984a). HCG mimics the effects of endogenous gonadotropins and can stimulate the gonads to produce sex steroid hormones. Thus, these studies raise the possibility that, like other vertebrates, ovarian steroids regulate female sexual behavior in anurans. However, some studies suggest that sex steroids, alone, are insufficient to induce sexual behavior. For example, although receptivity to male clasping can be induced in ovariectomized *Xenopus laevis* with a combination of estradiol and progesterone, an additional injection of luteinizing hormone-releasing hormone caused females to be more sexually responsive compared to estradiol and progesterone injections alone (Kelley, 1982). Arginine vasotocin and/or prostaglandins are effective at inhibiting unreceptive calling behavior in the Northern leopard frog (Diakow and Nemiroff, 1981) and *X. laevis* (Kelley, 1982; Weintraub et al., 1985). In the American toad, HCG induces phonotaxis, but its action is blocked by inhibition of prostaglandin synthesis (Schmidt, 1984a). However, prostaglandin-induced phonotaxis appears to require progesterone (Schmidt, 1985a). In summary, it appears that there is significant diversity among anurans in the hormonal mechanisms underlying female sexual behavior.

Túngara frogs (*Physalaemus pustulosus*) have been a focus of sexual selection research. As a result, we know a great deal about their behavioral responses to mating calls (Ryan, 1985), and this makes them an excellent model for testing the effects of steroid hormones on female sexual behavior. Male túngara frogs produce a simple advertisement call that is a frequency-modulated “whine” (Rand and Ryan, 1981). Males can increase the attractiveness of the whine by adding up to 7 “chucks” to produce a complex “whine-chucks” call that is strongly preferred by females over the simple whine-only call (Rand and Ryan, 1981). Females express mating preferences by differential phonotaxis toward the call of choice, but females in this species do not produce advertisement calls.

Female túngara frogs go to ponds only on the night they are ready to mate (Ryan, 1985), and when unmated females are present at ponds, they have high concentrations of plasma estradiol and androgens (Lynch and Wilczynski, 2005). After a female chooses a mate and allows the male to clasp her in amplexus, she has high plasma estradiol and progesterone concentrations and low androgen levels (Lynch and Wilczynski, 2005). The high levels of estradiol and progesterone disappear within 7-10 days after the female has oviposited (Lynch and Wilczynski, 2005). In addition, injections of HCG, which increase plasma estradiol concentrations, raise the probability that a female will approach conspecific calls (Lynch et al., 2006). Together, these data suggest that estradiol and/or progesterone may be mediators of changes in female sexual behavior in this species. Therefore, we tested the effects of estradiol and progesterone on sexual motivation and female preferences for conspecific calls. Because HCG increases estradiol, as well as phonotaxis behavior, we first asked whether the HCG-induced increase in phonotaxis could be replicated by steroid hormone manipulation (Experiment 1). Our results suggest that estradiol is sufficient to increase phonotaxis. Therefore, we next asked whether estradiol injections elevate phonotaxis behavior to levels seen in naturally breeding females, and whether estradiol-injected females show the same call preferences as naturally breeding females (Experiment 2).

## Experiment 1: Which hormonal conditions promote phonotaxis behavior?

### Materials and Methods

To determine which hormonal conditions promote phonotaxis behavior, we assessed the effects of hormone manipulation on the probability of phonotaxis behavior toward conspecific calls in post-reproductive females. To do so, we collected pairs during the

breeding season, brought them back to the laboratory, and allowed them to make nests. Ten days after females had oviposited we injected all females with saline and tested them in phonotaxis behavior tests. Following the first set of phonotaxis tests, we injected females with one of five hormone treatments and tested them again with the same set of phonotaxis tests. Finally, to validate the hormone manipulations we bled the females to collect plasma to measure their hormone concentrations at the end of phonotaxis tests.

Frog collection

We collected adult females (n = 76) individually or paired with males from breeding ponds between 19:00 – 23:00 hours near Gamboa, Panamá in 2006. After capture, we placed amplexed pairs or individual females in plastic bags and brought them back to the Smithsonian Tropical Research Institute (STRI) laboratory. We paired females that were caught individually with males that were calling in the same pond. We allowed the pairs to make foam nests after which we returned the foam nests and males to their original site of capture. We toe-clipped females for permanent identification following the recommended toe-clipping Guidelines for Live Amphibians and Reptiles in Field Research compiled by the American Society of Ichthyologists and Herpetologists (ASIH) and the Society for the Study of Amphibians and Reptiles (SSAR). We measured the snout vent length (SVL) to the nearest 0.01 mm using digital slide calipers (Mitutoyo Corporation, Aurora, IL), and body mass to the nearest 0.1 g using a Pesola spring scale (Pesola, Baar, Switzerland). The mean SVL of females was 28.54 mm and the mean body mass at capture was 1.92 g. After oviposition, we kept the females at the STRI laboratory in Gamboa for ten days before hormone manipulations because plasma hormone concentrations decline to non-breeding levels within 7 - 10 days after oviposition (Lynch and Wilczynski, 2005). During this time, we housed the females in 10-liter terrariums with substrate containing a mix of damp soil, leaf litter, and small twigs, and maintained them under ambient conditions (light: approximately

12 hours 35 min from sunrise to sunset; temperature: approximately 28° C). We provided the females with water, and fed them termites every other day. This work was approved by the University of North Carolina Institutional Animal Care and Use Committee (UNC IACUC) and was permitted by the National Authority for the Environment of Panamá (Autoridad Nacional del Ambiente).

Hormone manipulations

We followed one of two timelines for injections and phonotaxis testing for females in different treatment groups. Females from the HCG (n = 16), estradiol (E; n = 16), estradiol plus progesterone (E+P; n = 16), and saline (n = 12) groups were first injected with saline only followed 24 hours later by phonotaxis testing. Females were then injected with either HCG (500 IU per g of body mass), E (0.07 μg per g of body mass), E+P (0.07 μg of E and 0.7 μg of P per g of body mass), or saline, and tested again 24 hours later in the same phonotaxis tests. Females from the HCG plus fadrozole group (HCG+fad; n = 16) followed the second timeline which was based on a previous study that demonstrated that fadrozole blocks HCG-induced estradiol production in túngara frogs (Lynch, 2005). We first injected females with saline followed by phonotaxis tests 24 hours later. Females were then injected with a single dose of fadrozole (50 μg per frog), followed 24 hours later by injections of fadrozole and HCG. Finally, another 24 hours later we tested the females again in the phonotaxis tests. At the end of phonotaxis testing, all females were returned to their original site of capture. Each injection was 50-μl in volume and all substances were dissolved in saline (0.9% sodium chloride in water), although estradiol and progesterone were first dissolved in a small amount of ethanol. All substances were purchased from Sigma-Aldrich (St. Louis, MO) except fadrozole (4 - (5, 6, 7, 8 –tetrahydrimidazo [1, 5a] pyridine-5 - yl) benzonitrile monohydrochloride), which was acquired from Novartis (Basel, Switzerland).

Phonotaxis tests

We conducted phonotaxis tests between 19:00 and 06:00 hours. We tested each subject in four consecutive phonotaxis tests, each up to 15 minutes duration. In each test, the female heard two calls from opposing speakers. In the first and fourth tests we gave the females a choice between a conspecific whine (W) and a whine with 1 chuck (W1C) (see Stimuli, below). We separated tests one and four by up to 40 minutes during which we conducted two intervening tests to assess the ability of the females to choose between a conspecific and a heterospecific call, and between an artificial hybrid call and noise. We did not analyze the data from tests two and three due to low response from females. Instead, we used responses from tests 1 and 4 to determine a female's willingness to approach conspecific calls. Specifically, females who approached either one of the conspecific calls in both tests were defined as showing "Persistent Phonotaxis." Our definition of Persistent Phonotaxis is identical to the definition of receptivity used in prior studies (Lynch et al., 2005; Lynch et al., 2006). We chose a different moniker so as to not confound our specific definition of behavior with the more general concept of sexual receptivity.

The phonotaxis chamber (1.5 m W x 1.5 m L x 1 m H) was made of mattress foam (Allegro Medical, Tempe, AZ) suspended by PVC pipes. We placed two audio speakers (Cambridge Soundworks, North Andover, MA) at equal distances from the center of the chamber. We set the peak intensity of the acoustic stimuli at 82 dB SPL measured from the center of the chamber where we released the female. We conducted the behavioral observations in a semi-dark room and from outside the chamber. We also ensured that the observer stayed still during testing to avoid any sudden movements that could have disturbed the female. The observer was not blind to the treatment groups. At the beginning of the phonotaxis tests we placed each subject in the center of the chamber under an inverted funnel for 3 minutes. During these 3 minutes, acoustic stimuli were broadcast antiphonally from the two opposite speakers with a 1 second delay between presentations.

To control for side bias, we alternated the side on which each stimulus was presented in tests one and four for each individual female. We lifted the funnel 3 minutes after the start of the broadcasts and allowed the female up to 15 minutes to respond during which time the stimuli continued to be broadcast. Females had to approach within 10 cm of a speaker to have made a choice. We regarded the female as non-responsive if she remained stationary for more than 5 minutes after the funnel was lifted, or if she did not approach within 10 cm of a speaker. For the females that showed Persistent Phonotaxis, we calculated the mean latency to respond (time to approach within 10 cm of a speaker after the funnel was lifted) in the first and fourth phonotaxis tests.

Stimuli

We used natural túngara calls recorded from the Gamboa population, and we assembled all stimuli on a Macintosh computer using Raven Version 1.2.1 (Cornell Laboratory of Ornithology, BioAcoustics Research Program) and Garageband (Apple, Cupertino, CA). To maximize the generalizability of our conclusions (Kroodsma, 1989; Wiley, 2003), we used multiple call exemplars as follows. We used 7 pairs of mating calls recorded from 7 different males. Each pair of mating calls consisted of a W and a W1C call from the same male. In each phonotaxis test, the female was presented with a pair of calls recorded from an individual male. No individual female heard the same pair of calls twice during the course of the experiment. All stimuli were adjusted to the same peak amplitude.

Hormone assays

To validate the endocrine manipulations, total estradiol and progesterone concentrations were determined using enzyme immunoassay kits (Cayman Chemicals, Ann Arbor, Michigan). To collect plasma, we bled frogs from the retro-orbital sinus using a heparinized microcapillary tube, centrifuged the blood samples at 6000 rpm for 4 minutes,

and stored the plasma supernatant at – 20° C until later analysis. Plasma volumes ranged from 5 - 40 µl for individual frogs. If we had less than 20 µl of plasma, we could not conduct both hormone assays on the same sample. Therefore, sample sizes vary. Plasma samples were extracted twice with 2 ml of ether, evaporated, and then resuspended in enzyme immunoassay buffer. Recovery estimations were performed according to the Cayman kit instructions. These kits had previously been validated in this species (Lynch and Wilczynski, 2005; Lynch et al., 2006). However, we also validated the kits by adding known and unknown amounts of hormones to samples and measuring them repeatedly in different assays. The mean recovery after extraction was 52% for estrogen and 56% for progesterone, respectively. Recovery values were used to correct the concentration of hormone estimated in each sample. Each sample was assayed at two dilutions and each dilution was assayed in duplicates. The dilution value that fell within the most sensitive part of the standard curve generated from each assay was subsequently used for calculation of plasma samples. In total, five separate estradiol and five progesterone assays were conducted to analyze all the samples. Inter-assay variation was 18.4% and 9.65% for estrogen and progesterone, respectively. Cross reactivity in the estrogen kit was 0.1% for testosterone and 5-$\alpha$-DHT, 0.07% for 17$\alpha$-estradiol, and 0.03% for progesterone with a detection limit of 8 pg/mL. Cross reactivity in the progesterone kit was 7.2 % and 0.01% for 17$\beta$-estradiol and 17$\alpha$-estradiol respectively, with a detection limit of 10 pg/mL. Samples that were measured at the lowest dilution but were outside the sensitive area of the standard curve (i.e. very low amounts of hormone present in the plasma) were assigned the lowest detectable amount for the assay.

Statistical analyses

We analyzed plasma estradiol and progesterone concentrations for all treatment groups using a one-way ANOVA and we conducted least significant difference (LSD) post-

hoc analyses to examine pair-wise differences in hormone concentrations among the treatment groups. We used McNemar's 'test of significant change' (Zar, 1999) to determine whether, for each group, hormone treatment changed the probability of showing Persistent Phonotaxis compared to the initial saline injection. McNemar's test takes into account the within-subject nature of this comparison. We used Fisher's exact chi square to compare the effects of hormone treatment on the probability of Persistent Phonotaxis directly to one another. In addition, among females that showed Persistent Phonotaxis in the E, E+P, and HCG groups, we used ANOVA to test for the effect of hormone treatment on the latency to respond to calls. We did not include females from the saline or HCG+fad groups in this analysis since the number of females that showed Persistent Phonotaxis in these groups was 2 and 3, respectively. Throughout, instead of using a threshold alpha level to interpret our results, we describe the pattern of results and use p values to support our statements as recommended by Hurlbert and Lombardi (2003) and Stewart-Oaten (1995). We consider p to be a continuous variable and we consider lower p values to represent a lower probability of incorrectly rejecting the null hypothesis of no difference.

**Results**

Estradiol injections successfully elevated plasma estradiol concentrations and generated substantial variation in estradiol concentrations among groups ($F_{4,45} = 5.872$, $p < 0.001$; Fig. 2.1). Estradiol injections increased estradiol concentrations by approximately three-fold compared to saline-treated females. The magnitude of the change in estradiol concentrations is comparable to that observed in amplexed females compared to post-reproductive females (Lynch and Wilczynski, 2005), although absolute levels of estradiol of all groups were lower in that earlier study. Unlike previous studies (Lynch et al., 2006), HCG injections did not increase estradiol concentrations significantly above females injected with

saline or HCG+fad. In contrast to estradiol levels, we did not detect a substantial difference in progesterone concentrations among the treatment groups ($F_{4,47}$ = 1.60, p = 0.190; Fig. 2.1). Although we were surprised that our hormone manipulation did not elevate progesterone concentrations, a prior study similarly failed to elevate progesterone concentrations using HCG in túngara frogs (Lynch and Wilczynski, 2008).

Females injected with estradiol showed the highest rates of Persistent Phonotaxis (75%), followed by those injected with E+P (56%) and HCG (44%). Compared to when the same females were injected with saline, the increase in Persistent Phonotaxis was strong for estradiol ($\chi^2$ = 11.0, p < 0.001) and HCG ($\chi^2$ = 6.0, p = 0.014), but was more modest for E+P ($\chi^2$ = 2.8, p = 0.096) because of higher rates of Persistent Phonotaxis after initial saline injections in this group (Fig. 2.2). In contrast, females injected with HCG+fad did not change their probability of Persistent Phonotaxis compared to when they were injected with saline ($\chi^2$ = 0.0, p = 1.0; Fig. 2.2), nor did females who received a second injection of saline ($\chi^2$ = 1.0, p = 0.32; Fig. 2.2). These results suggest that injections of HCG, E, and E+P all increase the probability of phonotaxis. In order to determine if the hormone injections increased Persistent Phonotaxis to different levels, we compared the effect of hormone treatments directly to one another. We found that females injected with E alone had similar rates of Persistent Phonotaxis as females injected with E+P ($\chi^2$ = 1.3, p = 0.46). Compared to HCG-injected females, both E-injected females ($\chi^2$ = 3.3, p = 0.15), and E+P-injected females ($\chi^2$ = 0.5, p = 0.72) showed similar rates of Persistent Phonotaxis. Furthermore, among females showing Persistent Phonotaxis in the E, E+P, and HCG treated females, hormone treatment had no effect on latency to respond to conspecific calls ($F_{3,27}$ = 0.256, p = 0.86). The mean ± SE latency to respond in the E, E+P, and HCG-treated females were 333.83 ± 40.01, 358.2 ± 42.9, and 321.5 ± 76.1 seconds, respectively. Thus, injections of HCG, E, and E+P had similar effects on the motivation to approach conspecific calls.

**Experiment 2: Does estradiol elicit natural responses to mating calls?**

**Materials and Methods**

Results of Experiment 1 show that estradiol was sufficient to increase phonotaxis to levels observed in HCG-injected females. However, that experiment did not test whether estradiol-injected females show a similar degree of sexual motivation as observed in naturally breeding females, or whether they display the same call preferences as naturally breeding females. Therefore, we next compared phonotaxis responses of females tested right after capture to when they were post-reproductive and injected with either estradiol or saline. In this experiment, we assessed sexual motivation as the probability of Persistent Phonotaxis and as the probability of approaching a speaker during any given test. As a reminder, females who approached either one of the conspecific calls in the first and last tests were defined as showing Persistent Phonotaxis.

Frog collection and hormone manipulation

Experimental procedures were identical to Experiment 1, except where noted. In 2007, we collected 48 amplexed females from breeding ponds between 20:00 – 24:00 hours near Rio Píro on the Osa Peninsula in Costa Rica. The mean SVL of females was 29.63 mm and the mean body mass at capture was 1.84 g. After capture, we removed the male and tested the female's behavior in a series of two-choice phonotaxis tests within 10 hours of capture at the Osa Biodiversity Research Station. We then returned the females to their mate to allow the pairs to complete nesting, and we housed females in terrariums under ambient conditions (approximately 12 hours 20 min from sunrise to sunset and 28° C). Ten days following oviposition, we injected females with estradiol (n = 33), or saline (n = 15), and 24 hours after injection tested their behavior in the same series of two-choice tests. This

work was approved by the UNC IACUC and permitted by Costa Rica's Ministerio del Ambiente Y Energia (MINAE) and Sistema Nacional de Áreas de Conservación (SINAC).

Phonotaxis tests

We tested each subject in five consecutive phonotaxis choice tests between 19:00 and 05:00 hours. Basic phonotaxis procedures were identical to those in Experiment 1, except we used Tivoli Portable Audio Laboratory speakers (Tivoli Audio, Cambridge MA). In order to test a range of responses, we included phonotaxis tests where clear and strong preferences have been well documented, as well as tests for which we expected no strong call preferences, as follows. Tests 1 and 5 assessed the preference for the complex whine-chuck call over the simple whine, and test 2 assessed the preference for a conspecific whine-chuck call over a heterospecific whine. In these cases, a strong preference for the whine-chuck call over the alternative is well documented (Griddi-Papp et al., 2006; Ryan, 1980). Tests 3 and 4 compared responses to conspecific whine-chuck calls that varied in the number of chucks. In tests 3 and 4, females were assessed for their preference for a whine with 1 chuck over a whine with three chucks, and a whine with six chucks, respectively. Prior studies have shown that, at the amplitudes used in our experiment, females do not discriminate among whine-chuck calls based on the number of chucks (M. J. Ryan, personal communication).

Stimuli

We used natural túngara calls recorded near Puerto Jiménez on the Osa Peninsula; the heterospecific whines were recorded from *Physalaemus enesefae (fischeri)* in Venezuela. We assembled all stimuli on a Macintosh computer using the software programs Raven and Audacity (audacity.sourceforge.net). We used call exemplars from 4 different male túngaras and 4 different *P. enesefae* males. For the túngara calls, the W and W1C

calls were unmanipulated calls. To create the calls with multiple chucks, we added two (W3C) or five (W6C) chucks to the end of the W1C calls with 50 msec of intervening silence. For the túngara call stimuli, we presented each female with stimuli from the same male, and females were presented with the same set of stimuli when they were tested under both hormonal conditions. Call exemplars were distributed among the different treatment groups.

Statistical analyses

We assessed sexual motivation as the probability of Persistent Phonotaxis (approaching either conspecific call in the first and last tests), and the probability of responding during a test (approaching any speaker during a given test). As in Experiment 1, we used McNemar's test of significant change to assess the effect of hormonal condition on the probability of showing Persistent Phonotaxis. We used Fisher's exact chi square to compare the probability of responding in each phonotaxis test when tested after amplexus versus after estradiol injection. Because chi square assumes independence of each observation, we assigned each female to one of two groups as follows. To represent amplexed females (n = 15), we included the responses generated following amplexus of females in the saline group. To represent the estradiol group, we included the responses generated following estradiol injection of the estradiol-treated group (n = 33). Thus, each female was only included once in these analyses.

Finally, we used Fisher's exact chi square to assess the effect of estradiol injection on call preferences in comparison to amplexus. To do so, for each phonotaxis test, it was necessary to only consider a female's response once in order to satisfy the assumption of independence. Our strategy for sorting the data was designed to maximize the sample sizes representing each group. Females were included in the amplexed group if they were originally assigned to the saline treatment group or if they were originally assigned to the estradiol treatment group but failed to respond after estradiol injection. Females were

included in the estradiol treatment group if they were injected with estradiol and responded. Since we considered each preference test separately, sample sizes varied for each analysis. In order to facilitate direct comparisons between amplexed females and estadiol-injected females, we expressed the data as the number of females who chose the W1C, since this call was common to all phonotaxis tests. We conducted analyses of preferences for the W1C call in test 1 (W1C vs. W), test 2 (W1C vs. Het), and test 3 (W1C vs. W3C). We excluded analyses of test 4 because the number of estradiol-injected females that responded during that test was prohibitively low (n = 8). We did not include an analysis of test 5 because it was redundant with test 1.

**Results**

Overall, a high percentage of amplexed females showed Persistent Phonotaxis. Compared to when they were amplexed, saline-injected females were less likely to show Persistent Phonotaxis ($\chi^2 = 6.0$, $p = 0.014$; Fig. 2.3). Females that were injected with E had similar probability of Persistent Phonotaxis compared to when they were tested after amplexus ($\chi^2 = 1.6$, $p = 0.21$; Fig. 2.3), suggesting that E-injected females exhibit similar levels of motivation to respond to calls as naturally breeding females. In addition, we found that amplexed females were more responsive across tests compared to E-injected females (Fig. 2.4A). Specifically, E-injected females were less likely to respond in tests 2-5 compared to amplexed females (test 1: $\chi^2 = 0.18$, $p = 1.0$; test 2: $\chi^2 = 6.4$, $p = 0.037$; test 3: $\chi^2 = 4.1$, $p = 0.065$; test 4: $\chi^2 = 7.9$, $p = 0.009$; test 5: $\chi^2 = 7.7$, $p = 0.041$). Qualitatively, saline-injected females showed a similar decline in responses during tests 2-5 (data not shown), but we could not test this statistically due to low sample sizes. Nonetheless, the similar response of saline- and E-injected females suggests that this decline in responsiveness is not a result of

estradiol treatment, *per se*, but is more likely due to some aspect of housing, passage of time, or being injected.

We also compared call preference of amplexed females to E-treated females. We found that amplexed females chose the W1C about 83% of the time and E-injected females chose the W1C 74% of the time (test 1: $\chi^2 = 0.55$, $p = 0.72$; Fig. 2.4B), demonstrating that the preference for the complex whine-chuck call is intact in E-injected females. In test 2, all females showed a strong preference for the conspecific W1C call over the heterospecific whine regardless of reproductive condition ($\chi^2 = 3.5$, $p = 0.18$; Fig. 2.4B). In addition, we found that females chose the W1C over W3C about 63% of the time, regardless of whether they were tested after amplexus or after E injection ($\chi^2 = 0.007$, $p = 1.0$; Fig. 2.4B). In summary, E-injected females show similar call preferences as amplexed females.

**Discussion**

We found that injections of human chorionic gonadotropins (HCG), estradiol (E), and estradiol plus progesterone (E+P) all increased phonotaxis behavior, whereas injections of saline or HCG plus fadrozole (HCG+fad) did not. Since injections of estradiol alone were effective at increasing phonotaxis behavior, we conclude that estradiol is sufficient for the expression of phonotaxis behavior, a critical feature of sexual behavior in female túngara frogs. We also found that estradiol-injected females were just as likely to show phonotaxis, and expressed similar call preferences, as females in natural breeding condition. Prior evidence from HCG manipulations and hormonal studies of naturally breeding females have shown that the expression of sexual behavior in female túngara frogs is accompanied by elevated estrogen and progesterone concentrations (Lynch and Wilczynski, 2005; Lynch et al., 2005; Lynch et al., 2006). Taken together, these data suggest that the natural changes

in female sexual behavior that occurs over the reproductive cycle is controlled primarily by fluctuations in estradiol concentrations.

Our hormonal manipulations show that injections of estradiol alone can increase Persistent Phonotaxis (approaching either conspecific call in both the first and last phonotaxis tests) leading to our conclusion that estradiol is sufficient for sexual responses to mating calls. However, whether estradiol is necessary for phonotaxis remains unclear. Although we found that HCG injections effectively increased phonotaxis behavior, they failed to substantially elevate estradiol concentrations, suggesting that HCG could modulate phonotaxis behavior in an estradiol-independent manner. Nonetheless, combining HCG with the aromotase inhibitor fadrozole blocked HCG-induced phonotaxis. Although estradiol levels in the HCG+fad group were similar to saline-injected females, we were unable to conclude that fadrozole blocked HCG-induced phonotaxis by inhibiting estradiol since HCG alone failed to substantially elevate estradiol. Thus, it is possible that fadrozole inhibited phonotaxis through some estradiol-independent pathway. Since an earlier study using the same injection protocol demonstrated that fadrozole blocks HCG-induced production of estradiol in túngara frogs (Lynch, 2005) we suspect that the ambiguity in our data stems from our inability to demonstrate elevated levels of estradiol in our HCG-injected females. Regardless, future studies will be necessary to determine whether estradiol is necessary for phonotaxis behavior in female túngara frogs. In addition, we cannot draw strong conclusions about the role of progesterone from our data, since we were unable to demonstrate that our injections increased progesterone concentrations to breeding levels (~20 ng/ml; Lynch and Wilczynski, 2005). It is possible that the progesterone dose that we used was not sufficiently high, that we failed to detect an increase in progesterone with the timing of our sampling, or that our progesterone assay failed. Nonetheless, since we did not observe any significant difference in the expression of sexual behavior among E-, E+P-, and the HCG-injected females, it appears that progesterone is not necessary for phonotaxis. However, it remains

to be conclusively determined whether progesterone modulates sexual behavior in the túngara frog.

Estradiol-injected females were similar to amplexed females in the probability of showing Persistent Phonotaxis and in their call preferences. Females injected with estradiol displayed strong preferences for the complex whine-chuck call over the simple whine, and for a conspecific call over a heterospecific call. They also failed to discriminate among calls based on the number of chucks in a manner similar to amplexed females. These data suggest that estradiol-induced phonotaxis behavior is indistinguishable from that of amplexed females. However, estradiol-injected females showed a decline in the probability of responding across sequential phonotaxis tests. Because saline-injected females seemed to show a similar decline, the waning of phonotaxis responses may be a consequence of housing or injection, and not a consequence of estradiol treatment *per se*. Nonetheless, estradiol-injected females were less reliable in their phonotaxis behavior than amplexed females, suggesting that, under these conditions, estradiol was unable to induce sexual motivation to levels as seen in amplexed females tested on the night of capture. Thus, estradiol injections are highly effective at inducing sexual behavior that is similar to naturally breeding females, but some differences in sexual motivation appear to exist.

Prior work suggests diversity in hormone-behavior relationships among anurans, although studies of different species do not always manipulate the same combination of hormones, making direct comparisons difficult. HCG has commonly been used to induce sexual behavior in frogs, including female phonotaxis (Lynch et al., 2006; Schmidt, 1984a). Presumably, HCG acts by mimicking endogenous gonadotropins to stimulate the production of ovarian hormones. HCG could also directly bind to luteinizing hormone receptors to affect behavior (Yang et al., 2007). To our knowledge, ours is the only study to demonstrate that estradiol alone is effective at inducing phonotaxis behavior in an anuran. In *X. laevis*, steroid hormones are effective at promoting receptivity to amplexus, but a combination of estradiol

and progesterone is necessary (Kelley, 1982). In addition, in the American toad, HCG-induced phonotaxis depends on the production of prostaglandins (Schmidt, 1984a) but prostaglandin-induced phonotaxis may require progesterone (Schmidt, 1985b); the priming effects of estradiol alone were not tested. It is worth noting, however, that the primary goal of these prior studies was to develop a pharmacological method for inducing phonotaxis, and they were not designed to discover the natural hormonal mechanisms of phonotaxis (Schmidt, 1984a; Schmidt, 1985a; Schmidt, 1985b). Nonetheless, the effects of prostaglandins on phonotaxis appear to be potent (Schmidt, 1985b).

Prostaglandins are non-steroid fatty acid hormones produced in many tissues, including the ovaries, and are associated with ovulation, oviposition, parturition, and sexual receptivity in widespread taxa (Gobbetti and Zerani, 1992; Gobbetti and Zerani, 1999; Guillette et al., 1991). Several studies have demonstrated reciprocal relationships between estradiol and prostaglandins, including the stimulation of aromatase activity by prostaglandins (Gobbetti and Zerani, 1992) and the stimulation of prostaglandin synthase expression by estradiol (Wu et al., 2005). Thus, it is possible that our estradiol manipulations were effective at inducing phonotaxis in túngara frogs, in part, through stimulation of prostaglandin production, or that prostaglandin injections in prior studies were effective because they also increased estradiol concentrations. If so, it would suggest that our results are not inconsistent with prior studies. Future studies of the interactions between steroid hormones and prostaglandins are necessary for a more complete understanding of the hormonal mechanisms of female sexual behavior in anurans.

Theoretical models suggest that both intrinsic and extrinsic factors may serve as constraints that can influence mate choice decisions (Jennions and Petrie, 1997). Intrinsic factors, such as hormonal state, can modulate female sexual behavior by allowing the female to be plastic in her mate choice behavior (Lynch et al., 2005). Our study demonstrates that estradiol can induce sexual behavior in female túngara frogs, which

suggests that steroid hormones are capable of inducing female mate choice behavior via modulation of neural pathways. Clearly more studies are needed to investigate the precise neuroendocrine mechanisms by which estradiol modulates sexual motivation and mate choice behavior in the túngara frog. Because female frogs base mate choice decisions largely on acoustic signals produced by males, anurans are an attractive model for investigating the effect of steroid hormones on the neural pathways that modulate sexual behavior. Our results illustrate an important proximate mechanism that could have an essential function in influencing female mate choice behavior in anurans within the context of sexual selection.

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Figure Legends

Figure 2.1. Plasma estradiol and progesterone concentrations (mean ± SE) 24 hours after the final injections in Experiment 1. Final injections were human chorionic gonadotropins (HCG), estradiol (E), estradiol plus progesterone (E+P), saline, or a combination of HCG and the aromatase inhibitor fadrozole (HCG+fad). Sample sizes are shown in parentheses and common letters indicate groups that are statistically indistinguishable at $p < 0.05$.

Figure 2.2. Effects of hormonal manipulation on the probability of showing Persistent Phonotaxis to conspecific mating calls in Experiment 1. Females were categorized as showing Persistent Phonotaxis if they approached one of two conspecific mating calls in two different phonotaxis tests. Persistent Phonotaxis was first assessed after injection with saline and then after one of 5 different hormone injections. Hormone treatments were human chorionic gonadotropins (HCG), estradiol (E), estradiol plus progesterone (E+P), saline, or a combination of HCG and the aromatase inhibitor fadrozole (HCG+fad).

Figure 2.3. Effects of hormonal condition on the probability of showing Persistent Phonotaxis to conspecific mating calls in Experiment 2. Females were categorized as showing Persistent Phonotaxis if they approached one of two conspecific mating calls in two different phonotaxis tests. Persistent Phonotaxis was first assessed within 10 hours of amplexus (Amp), and then 11 days later after an injection of saline (n = 15), or estradiol (E; n = 33).

Figure 2.4. **A**. Effects of hormonal condition on the probability of responding during 5 sequential phonotaxis tests in Experiment 2. Females were considered responsive if they approached any speaker during a test. Females were first tested within 10 hours of amplexus (n = 15), and then 11 days later after an injection of estradiol (n = 33). **B**. Effect of hormonal condition on the preference for the whine + 1 chuck call (W1C) in 3 sequential phonotaxis tests in Experiment 2. Females were either tested within 10 hours of amplexus or 11 days later after injection with estradiol. Sample sizes (indicated in parenthesis) vary depending on the proportion of females that responded in each test.

Figure 2.1

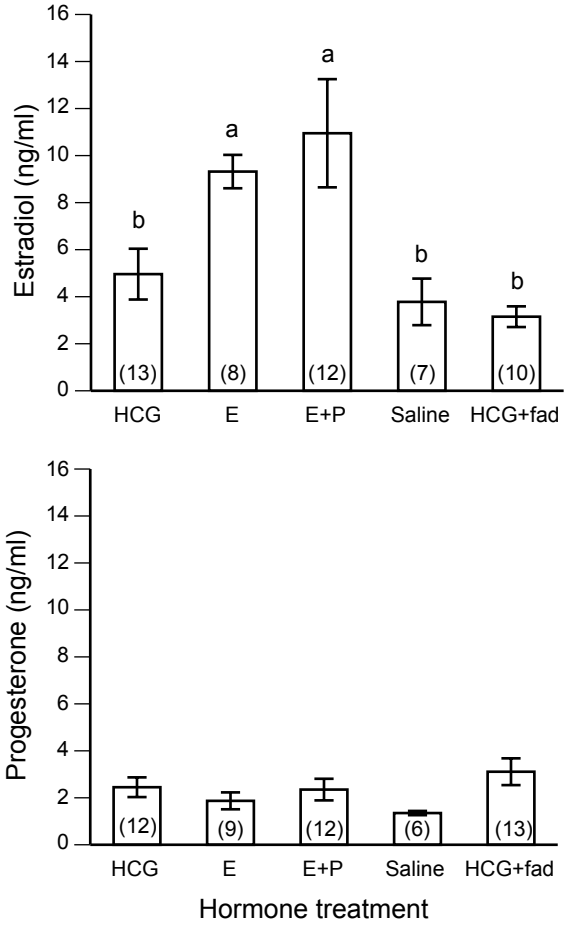

Estradiol (ng/ml)
16
14
12
10
8
6
4
2
0
b
a
a
b
b
(13)
(8)
(12)
(7)
(10)
HCG
E
E+P
Saline
HCG+fad
Progesterone (ng/ml)
16
14
12
10
8
6
4
2
0
(12)
(9)
(12)
(6)
(13)
HCG
E
E+P
Saline
HCG+fad
Hormone treatment

Figure 2.2

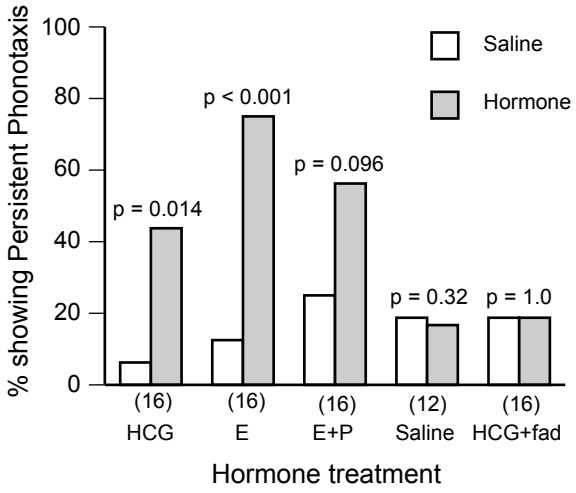

% showing Persistent Phonotaxis
100
80
60
40
20
0
Saline
Hormone
p = 0.014
p < 0.001
p = 0.096
p = 0.32
p = 1.0
(16)
HCG
(16)
E
(16)
E+P
(12)
Saline
(16)
HCG+fad
Hormone treatment

Figure 2.3

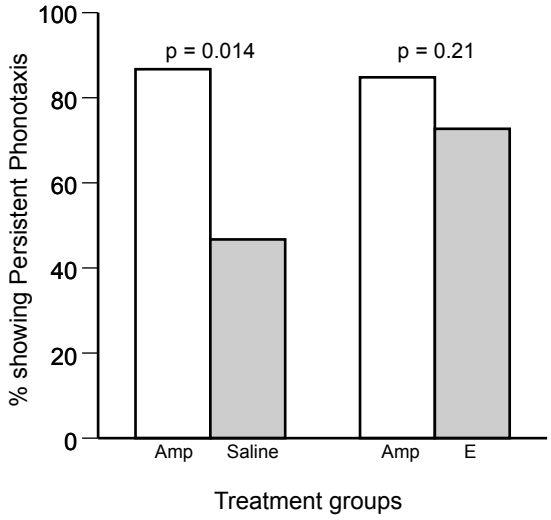
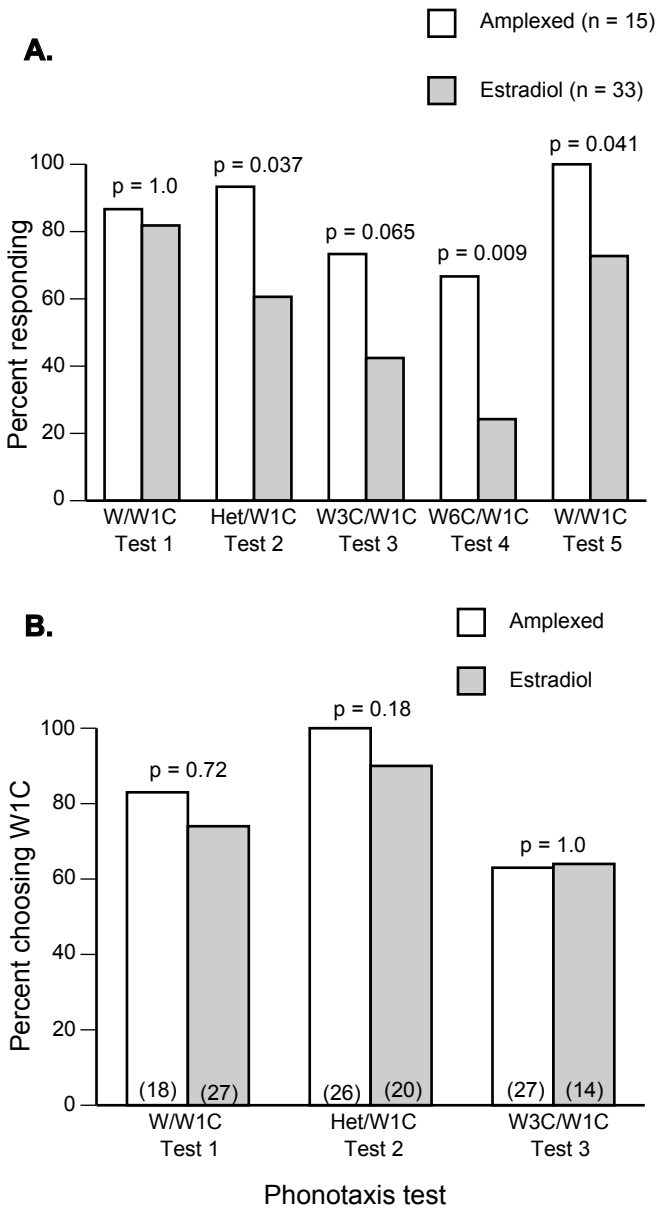

100
80
60
40
20
0
% showing Persistent Phonotaxis
p = 0.014
p = 0.21
Amp
Saline
Amp
E
Treatment groups

Figure 2.4


A.
Amplexed (n = 15)
Estradiol (n = 33)
Percent responding
100
80
60
40
20
0
p = 1.0
p = 0.037
p = 0.065
p = 0.009
p = 0.041
W/W1C
Test 1
Het/W1C
Test 2
W3C/W1C
Test 3
W6C/W1C
Test 4
W/W1C
Test 5
B.
Amplexed
Estradiol
Percent choosing W1C
100
80
60
40
20
0
p = 0.72
p = 0.18
p = 1.0
(18)
(27)
(26)
(20)
(27)
(14)
W/W1C
Test 1
Het/W1C
Test 2
W3C/W1C
Test 3
Phonotaxis test

## CHAPTER 3

# SEXUALLY DIMORPHIC ANDROGEN AND ESTROGEN RECEPTOR mRNA EXPRESSION IN THE BRAIN OF TÚNGARA FROGS


### Summary

Sex steroid hormones are potent regulators of behavior and exert their effects through influences on sensory, motor, and motivational systems. To elucidate where androgens and estrogens can act to regulate sex-specific behaviors in the túngara frog (*Physalaemus pustulosus*), we quantified expression of androgen receptor (AR), estrogen receptor alpha (ER$\alpha$), and estrogen receptor beta (ER$\beta$) in the brains of male and females. To do so, we cloned túngara-specific sequences for *AR, ER$\alpha$,* and *ER$\beta$*, determined their distribution in the brain, and then quantified their mRNA expression in sensory, motor, and motivational systems that are important in sexual communication. We observed expression of *AR*, *ER$\alpha$*, and *ER$\beta$* mRNA within the pallium, limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, amygdala, septum, striatum), parts of the thalamus, and the midbrain torus semicircularis. We found that males and females had similar distribution of *AR* and *ER* expression, but expression levels differed in some brain regions. In the auditory midbrain, females had higher *ER$\alpha$* and *ER$\beta$* expression than males, whereas males had higher *AR* expression than females. In the forebrain, females had higher *AR* expression than males within the ventral hypothalamus and medial pallium, whereas males had higher *ER$\alpha$* expression in the medial pallium. In the preoptic area, striatum, and septum, males and females had similar levels of *AR* and *ER* expression. The results of our study

indicate that sex steroid hormones are likely to have sexually dimorphic effects on auditory processing, and thus important implications for sexual communication in this system. To our knowledge, this is the first study to provide a detailed description of the neuroanatomical distribution of *AR*s and *ER*s in an anuran, and the first to report a sexual dimorphism in steroid receptor expression in the brain of amphibians.

**Introduction**

Steroid hormones regulate a wide variety of physiological functions, including reproduction. For example, androgens and estrogens exert profound cellular effects within sensory systems including cell proliferation, cognition, and neurogenesis, and may regulate a suite of behaviors such as aggression, spatial learning and memory (Dechering et al., 2000; DonCarlos et al., 2006; Liu et al., 2008; Lösel et al., 2003; McEwen, 2002; Nadal et al., 2001; Patchev et al., 2004). Steroid hormones influence the expression of sex-typical behaviors by modulating sensory, motor, and motivational systems. The relationship between gonadal steroid hormones and expression of male- and female-typical sexual behaviors is well established in vertebrates. In general, gonadectomy abolishes sexual behavior, which can then be reinstated by administration of androgens and/or estrogens (Adkins et al., 1980; Wallis and Luttge, 1975). Androgens and estrogens exert their effects by acting through steroid receptors in the brain. The classical mechanism of androgen and estrogen action is mediated by nuclear receptors that function as ligand-dependent transcription factors regulating transcription of target genes, although gonadal steroids can also exert effects through membrane-bound receptors (see review by Björnström and Sjöberg, 2005). In some vertebrates, the neuroanatomical distribution of nuclear sex steroid receptors in the brain is conserved between the sexes (e.g. Balthazart et al., 1989; Rhen and Crews, 2001; Rosen et al., 2002) but there is variation in steroid receptor expression

among brain regions, between sexes, between seasons, and across species, all of which can influence the expression of sex-typical behaviors (Young and Crews, 1995). Therefore, a detailed examination of the neuroanatomical distribution of steroid receptors is important for understanding sexually dimorphic, hormone-behavior relationships.

As in other vertebrates, anurans display sex-typical behaviors when plasma steroid hormone levels are high (see reviews by Arch and Narins, 2009; Moore et al., 2005; Wilczynski et al., 2005). Typically, male anurans produce mating calls to attract females and females, who do not typically produce advertisement calls, express mating preferences by differential phonotaxis toward the male of choice. Steroid hormones regulate advertisement calling in males (Burmeister and Wilczynski, 2001; Wetzel and Kelley, 1983) and phonotaxis in females (Chakraborty and Burmeister, 2009; Kelley, 1982; Schmidt, 1984a). Furthermore, parts of the neural pathways controlling communication concentrate androgens and estrogens (Kelley, 1980; Kelley et al., 1975; Morrell et al., 1975), although little is known about the distribution of sex steroid hormone receptors. Only androgen receptors have thus far been localized in the brain of anurans (Guerriero et al., 2005) and nothing is known about the distribution of estrogen receptors. Thus, our understanding of the neural targets of sex steroid hormones in anurans is incomplete.

To elucidate the neural targets of sex steroid hormones that may contribute to sexually dimorphic behaviors in anurans, we localized and quantified expression of androgen and estrogen receptors in the brains of male and female túngara frogs, an important model species in sexual selection studies (Endler and Basolo, 1998; Ryan, 1991; Ryan and Rand, 2003). Communication in túngara frogs is typical of many anurans: males produce mating calls to attract females while females, who do not vocalize, initiate mating by approaching a calling male (Ryan, 1985). We cloned túngara-specific sequences for *AR,* *ER*$\alpha$, and *ER*$\beta$, determined their distribution in the brain, and then quantified their mRNA expression in sensory, motor, and motivational systems that are important in sexual

communication. We found *AR*, *ER*$\alpha$, and *ER*$\beta$ expression in the limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, amygdala, septum, striatum), parts of the thalamus, and in the laminar nucleus of the torus semicircularis, areas that have been reported to contain steroid-concentrating cells in other anurans. In addition, we found new putative sites of steroid action within the pallium, posterior tuberculum, locus coeruleus, and the principal nucleus of the torus semicircularis. We found that, although males and females had similar distributions of *AR* and *ER* expression, expression levels differed in some brain regions. In the auditory midbrain, females had higher *ER*$\alpha$ and *ER*$\beta$ expression than males, whereas males had higher *AR* expression than females. In the forebrain, females had higher *AR* expression than males within the ventral hypothalamus and medial pallium, whereas males had higher *ER*$\alpha$ expression in the medial pallium. In the preoptic area, striatum, and septum, males and females had similar levels of *AR* and *ER* expression. The results of our study indicate that sex steroid hormones are likely to have sexually dimorphic effects on auditory processing, which may have important implications for sexual communication in this system. This is the first study to provide a detailed description of the neuroanatomical distribution of *AR*s and *ER*s in an anuran, and the first to report a sexual dimorphism in steroid receptor expression in the brain of amphibians.

## Materials and Methods

### Identification of túngara-specific receptor sequences

We first identified the túngara specific sequences for the *AR*, *ER*$\alpha$, and *ER*$\beta$ genes. To do so, we used degenerate PCR to clone partial cDNA sequences for each gene that we then used to generate probes for *in situ* hybridization (see Table 3.1 for primers). We extracted total RNA from ovaries (*AR* and *ER*$\beta$) or liver (*ER*$\alpha$) of adult females from a

laboratory stock maintained at the University of Texas at Austin that was originally derived from natural populations in Gamboa, Panama, and synthesized cDNA from 10 μg of RNA using an anchored poly-dT primer and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). For *AR*, we amplified a 641-base pair fragment using the following PCR conditions: denaturation at 95° C for 2 min followed by 35 cycles of denaturation at 93° C for 30 s, annealing at 55° C for 30 s, and elongation at 65 C for 1 min. For *ERβ*, we amplified a 877-base pair fragment using the following PCR conditions: denaturation at 94° C for 1 min followed by 35 cycles of denaturation at 94° C for 30 s, annealing at 55° C for 30 s, and elongation at 65° C for 1 min. For *ERα*, we amplified two overlapping fragments of one ~919-base pairs (primer pair 1; Table 3.1) and a second of 414-base pairs (primer pair 2; Table 3.1). To generate the ~919-base pair fragment, we used the following PCR conditions: denaturation at 94° C for 2 min followed by 20 cycles of denaturation at 94°C for 30 s, annealing at 60–51°C (decreasing 2 degrees per cycle) for 30 s, and elongation at 65°C for 90 s, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 65°C for 90 s, and a final elongation at 65°C for 7 min. To generate the 414-base pair fragment, we used the following PCR conditions: denaturation at 94° C for 2 min, followed by 35 cycles of denaturation at 94° C for 30 s, annealing at 58° C for 30 s, elongation at 65° C for 1 min, and a final elongation at 65° C for 7 min. We subcloned the PCR products into a TOPO TA cloning Vector TOP 10 (Invitrogen), sequenced the inserts, and confirmed our results by aligning the predicted amino acid sequences with that of other reported receptors using BLAST.

Neuroanatomical distribution of *AR*, *ERα*, and *ERβ* expression

Because we were interested in dimorphisms underlying sex-typical behaviors, we examined expression of *AR* and *ERs* in the brains of reproductively active males and

females. We collected 5 mating pairs at breeding ponds on the Osa Peninsula, Costa Rica in July 2007. We captured pairs in a mating clasp (amplexus) between 20:00 and 24:00 hours and brought them back to the laboratory at the Osa Biodiversity Center where we rapidly decapitated them. After decapitation, we opened the skull in order to fix the brains (10 min in 4% paraformaldehyde) before removing them. We then rinsed the brains in phosphate buffered saline for 10 min before freezing them in liquid nitrogen in 2 ml tubes containing Tissue-Tek OCT Compound (Sakura, Finetek, Torrance, CA). We kept the brains on dry ice during transportation to University of North Carolina where we stored them at –80° C until further processing. The University of North Carolina Institutional Animal Care and Use Committee (IACUC) approved our experimental procedures and Costa Rica's Ministerio del Ambiente Y Energia (MINAE) and Sistema Nacional de Áreas de Conservación (SINAC) permitted tissue collection and export.

We sectioned brains in the transverse plane at 16 µm in 3 series on a cryostat. To localize *AR, ER$\alpha$,* and *ER$\beta$* mRNA, we used radioactive *in situ* procedures previously described Burmeister et al. (2008) with some modifications. Briefly, we generated radioactively labeled sense and antisense probes from reverse transcription of 641-, 414-, and 877-base pair subclones for *AR, ER$\alpha$* and *ER$\beta$,* respectively. We linearized the plasmids with EcoRV or Hind III (New England BioLabs Inc., Ipswich, MA). We prepared the $S^{35}$-labeled RNA by *in vitro* transcription with Sp6 or T7 polymerase using a MAXISCRIPT kit (Ambion, Austin, TX), and we removed the unincorporated nucleotides by using NucAway spin columns (Ambion). Before hybridization, we fixed the tissue for 10 sec in 4% paraformaldehyde before washing in phosphate-buffered saline, triethanolamine, acetic anhydride, 2× SSC, and a series of ethanols. We hybridized the tissue with 90 µl of $3.0 \times 10^5$ cpm/ml of hybridization buffer at 65° C overnight and removed unbound probe with a series of 65° C washes, first in 50% formamide and 2× SSC (1.25 h) followed by two washes in

0.1× SSC (30 min each). We visualized the bound riboprobe as silver grains by exposing the slides to NTB emulsion diluted 1:1 in distilled water for 30 days at 4° C, and we visualized the cell bodies by staining the tissue with thionin. Tissue incubated with the sense probe showed no significant binding above background. We used darkfield and brightfield illumination on a compound microscope to qualitatively examine the neuroanatomical distribution of *AR, ER*$\alpha$*,* and *ER*$\beta$ mRNA expression. We paid particular attention to brain areas involved in sexual communication or areas previously reported to bind sex steroids or express their receptors.

Sex differences in receptor expression levels

Since we did not observe any obvious sex differences in *AR* or *ER* distribution, we quantified levels of mRNA expression focusing on a subset of brain regions involved in sexual communication. We quantified receptor expression in the principal and laminar nuclei of the torus semicircularis, central nucleus of the thalamus, preoptic area, ventral hypothalamus, medial pallium, medial septum, and ventral striatum. The torus semicircularis and central thalamus are important in processing acoustic communication signals. The striatum, preoptic area, and ventral hypothalamus all receive auditory input and play a role in the expression of sexual behavior. The medial pallium receives significant auditory input, although its role in sexual communication is unclear. Finally, the septum receives auditory input and septal lesions can disrupt phonotaxis (Walkowiak et al., 1999).

For each brain region, we calculated an individual's mean from between two to five consecutive photomicrographs captured at a magnification of 630× from one hemisphere of the brain that best represented the respective brain region morphologically. For the torus semicircularis, we calculated an individual's mean from between three to five photomicrographs whereas for all other brain regions we obtained the mean from between two to three photomicrographs. The quality of the brain sections influenced the sample sizes

reported for each brain region, as we were unable to obtain data from all individuals in the study for all brain regions sampled. We assessed relative levels of mRNA expression by quantifying the number of silver grains per cell body above background using methods described in Burmeister et al. (2008). Briefly, we used ImageJ (http:// rsbweb.nih.gov/ij/) to quantify silver grain number in the region of interest and in a nearby area of the slide that represented the background silver grain density. We manually counted the number of cell bodies in the region of interest from separate photomicrographs.

We conducted linear mixed models using the "lme" function in R (R Foundation for Statistical Computing, Vienna, Austria). For each receptor and brain region, we tested for a main effect of sex (fixed effect) with subject as a random effect.

**Results**

Identification of túngara-specific receptor sequences

We identified 641-, 414-, and 887-base pair fragments of *P. pustulosus AR*, *ER*$\alpha$, and *ER*$\beta$ mRNA, respectively. The *AR*, *ER*$\alpha$, and *ER*$\beta$ nucleotide sequences code for predicted protein sequences of 214, 138, and 292 amino acids, respectively. The *P. pustulosus* AR protein sequence shared over 90% similarity and 84% identity with AR of other tetrapods (Table 3.2, Fig. 3.2A). The *P. pustulosus* ER$\alpha$ protein sequence shared over 90% similarity and 78% identity (Fig. 3.2B), whereas the ER$\beta$ protein sequence shared over 89% similarity and 77% identity with the ERs of other tetrapods (Table 3.2, Fig. 3.2C). This sequence similarity supports our conclusion that our subclones correspond to *P. pustulosus* AR, ER$\alpha$, and ER$\beta$ gene sequences.

Neuroanatomical distribution of *AR*, *ER*$\alpha$, and *ER*$\beta$ expression

Males and females had similar anatomical distributions of *AR*, *ER*$\alpha$, and *ER*$\beta$ in the brain (Table 3.3). The steroid receptor distribution in the túngara frog brain parallels previous ligand autoradiography studies and extends those results by identifying new putative sites of steroid action. As in other anurans, *AR* was expressed in the limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, striatum, septum, amygdala), dorsal tegmental area of the medulla (DTAM), thalamus, cerebellum, optic tectum, tegmentum, reticular formation, and in the laminar nucleus of the torus semicircularis (Table 3.3). In addition to previously reported distributions, *AR* was expressed in the posterior tuberculum, locus coeruleus, and the principal nucleus of the torus semicircularis. We also found medium to high *AR* expression in the medial pallium, and medium to low expression in the dorsal, lateral, and ventral pallium. We found *ER*$\alpha$ and *ER*$\beta$ expression in the limbic areas (e.g. preoptic area, hypothalamus, nucleus accumbens, striatum, septum, amygdala), parts of the thalamus, and in the laminar nucleus of the torus semicircularis (Table 3.3). In addition, we found new sites of estrogen receptor expression including the optic tectum, posterior tuberculum, and principal nucleus of the torus semicircularis. We found high to medium levels of *ER*$\beta$ expression in the medial and dorsal pallia, and low levels of *ER*$\beta$ expression in the lateral and ventral pallia. In contrast, *ER*$\alpha$ was absent from the lateral and ventral pallia, and present in low to medium levels in the dorsal and medial pallia.

Not surprisingly, there was variation among steroid receptors in a given brain region (Table 3.3). For example, *AR* was expressed in the cerebellum, locus coeruleus, reticular formation and DTAM, whereas *ER*$\alpha$ and *ER*$\beta$ expression was undetectable in these areas. Interestingly, *ER*$\beta$ expression was ~ five-fold higher than *ER*$\alpha$ and *AR* in the preoptic area, suggesting that *ER*$\beta$ plays an important role in modulating sexual behavior in anurans. Finally, there was significant variation among brain regions for a given steroid receptor (Table 3.3). For example, *AR* and *ER*$\beta$ were expressed at higher levels in the medial pallium

than in the dorsal, ventral or lateral pallium. However, expression of the steroid receptors appeared to be similar among the subdivisions of the amygdala.

Sex differences in receptor expression levels

Although males and females had similar distributions of androgen and estrogen receptors, receptor expression levels differed in some brain regions (Figs. 3.1, 3.3). Females had higher *ER*$\beta$ and *ER*$\alpha$ expression than males in the laminar and principal nuclei of the torus semicircularis, respectively, and males had greater *AR* expression than females within the principal nucleus, suggesting that the túngara auditory system is sensitive to modulation by sex-typical steroid hormones. Although there was a trend for greater *AR* and *ER*$\beta$ expression in the central thalamus of females than males, this differences was not statistically robust (Fig. 3). We found no sex differences in levels of receptor expression within the preoptic area, whereas in the ventral hypothalamus females had slightly higher *AR* expression than males. We also observed a trend of greater *ER*$\beta$ expression in the ventral hypothalamus of females compared to males. In the medial pallium, *AR* was expressed at higher levels in females than males whereas males had higher *ER*$\alpha$ expression than females. Finally, we did not observe any sex differences in the medial septum or ventral striatum.

## Discussion

To identify sites of androgen and estrogen action where sex-specific behaviors may be regulated, we cloned sequences for *AR, ER*$\alpha$*,* and *ER*$\beta$ cDNA in the túngara frog and determined their distribution in the brain. The predicted proteins of our subclones had over 89% similarity to receptor sequences of other vertebrates, confirming that our subclones

represented túngara-specific steroid receptor genes. We found *AR*, *ER*$\alpha$, and *ER*$\beta$ expression in the limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, amygdala, striatum, septum), parts of the thalamus, optic tectum, and in the laminar nucleus of the torus semicircularis that parallels previous reports from other anurans (for example see Di Meglio et al., 1987; Guerriero et al., 2005; Kelley et al., 1975; Morrell et al., 1975). In addition, we found new putative sites of *AR* and *ER* action including the pallium, posterior tuberculum, locus coeruleus (*AR* only), optic tectum (*ER*$\alpha$ and *ER*$\beta$ only), and the principal nucleus of the torus semicircularis. However, *AR, ER*$\alpha$*,* and *ER*$\beta$ mRNA was undetectable in areas such as the olfactory bulb that are known to contain steroid concentrating cells in other anuran species. Although the receptors had similar neuroanatomical distributions in males and females, their expression levels varied in some brain regions. In the torus semicircularis, females had higher *ER*$\alpha$ and *ER*$\beta$ expression than males, whereas males had higher *AR* expression than females, suggesting that auditory processing in túngara frogs is subject to hormonal regulation in a sex-specific manner. In the forebrain, we found that females had higher *AR* expression than males within the ventral hypothalamus and medial pallium (homolog of the hippocampus), whereas males had higher *ER*$\alpha$ expression in the medial pallium. In contrast, we did not observe any sex differences in steroid receptor mRNA expression within limbic areas such as the preoptic area, striatum, and septum.

We found steroid receptor expression in many of the same brain regions identified by ligand autoradiography (Di Meglio et al., 1987; Kelley et al., 1975; Morrell et al., 1975) and immunocytochemistry (Guerriero et al., 2005) in other anurans and also identified additional putative sites of steroid action. We found that *AR* and *ERs* were expressed in some brain regions not previously identified in anurans, such as the pallium. The anuran pallium is not functionally differentiated to process sensory information as in amniotes (Butler and Hodos, 1996). Although the exact functions still remain obscure, the dorsal and medial pallia are

generally considered to be centers for multimodal sensory processing and integration (Laberge and Roth, 2007; Northcutt and Ronan, 1992), whereas the lateral pallium is thought to process olfactory input (Northcutt and Royce, 1975). Studies have shown that the medial pallium is acoustically sensitive (Mangiamele and Burmeister, 2008; Mudry and Capranica, 1980), and it has been hypothesized that it may direct sexual motivation and reward seeking behaviors through its connections to the amygdala and nucleus accumbens (Northcutt and Ronan, 1992; Westhoff and Roth, 2002), areas that also express *AR* and *ERs*. Additionally, since the anuran medial pallium is thought to be homologous to the mammalian hippocampus (Kicliter and Ebbesson, 1976), it is possible that auditory responses in the medial pallium may contribute to memory formation during mate choice when females assess multiple males before choosing a mate (Ryan, 1985). Androgen and estrogen receptor immunoreactive cells have been identified in the pallium in one other amphibian species, the male roughskin newt (Davis and Moore, 1996). Androgen receptors and estradiol concentrating neurons are known to be present in the pallium in lizards (Morrell et al., 1979; Tang et al., 2001), whereas *AR* and *ER* have been localized in the hippocampus of rats (Simerly et al., 1990), and birds (Gahr and Metzdorf, 1999; Hodgson et al., 2008; Soma et al., 1999). The mammalian hippocampus is known to express estrogen receptors (Register et al., 1998) and recent studies have shown that *ER*$\beta$ may play a significant role in hippocampal synaptic plasticity and in improving memory in rodents (Liu et al., 2008). Likewise, a recent study in songbirds has shown that steroid hormones improve spatial memory in songbirds (Hodgson et al., 2008). At present, the exact function of the medial pallium in anurans is obscure. Future studies investigating the role of steroid hormones on synaptic plasticity in anurans may be useful in understanding pallial function and contribution to memory formation.

We also found *AR* and *ER*$\beta$ (but not *ER*$\alpha$) expression in the posterior tuberculum, *AR*, *ER*$\alpha$, and *ER*$\beta$ expression in the principal nucleus of the torus semicircularis, and *AR*,

*ERα*, and *ERβ* expression in the optic tectum, where estrogen containing cells have hitherto been unidentified. The posterior tuberculum is a diencephalic region comparable to the mammalian substantia nigra pars compacta containing dopamine concentrating cells (González and Smeets, 1991), that sends projections to the striatum, an area thought to be involved in motor functions (Marín et al., 1997a). The posterior tuberculum is also acoustically responsive in túngara frogs (Hoke et al., 2005), which may indicate that steroid hormones may be involved in influencing motor output through connections of the posterior tuberculum to the striatum. The principal nucleus of the torus semicircularis is the primary target of ascending auditory fibers (Feng and Lin, 1991; Matesz and Kulik, 1996; Walkowiak and Luksch, 1994), and thought to be dedicated to spectral processing of calls in anurans (Feng and Lin, 1991). To date, steroid concentrating cells were observed in the laminar nucleus of the torus semicircularis, but whether the principal nucleus was also a target of steroid action was unclear. Electrophysiological studies have suggested that toral neurons with complex feature detection properties presumably contributes to representation of mating signals (Edwards et al., 2002; Fuzessery, 1988; Penna et al., 1997; Rose and Capranica, 1984). The presence of *AR*s and *ER*s in the principal nucleus indicates that androgens and estrogens may influence auditory processing during mate recognition in anurans. Furthermore, the presence of estrogen receptors within the optic tectum in túngara frogs suggests that the visual system may be regulated by estrogen. Two elegant studies by Taylor et al. (2008) and Rosenthal et al. (2004) have reported that female túngara frogs use both auditory (courtship calls) and visual (inflation of vocal sacs) cues when they are discriminating among potential males. The presence of *AR*s and *ER*s in both the auditory and visual systems indicate that steroid hormones may modulate multimodal signal processing in anurans with important implications for mate choice decisions.

Our results demonstrate that auditory processing in túngara frogs may be subject to hormonal regulation in a sex-specific manner. We found that, in the auditory torus

semicircularis, female túngara frogs express higher levels of *ERα* and *ERβ* expression than males, whereas males have higher *AR* expression than females. Hormone-behavior relationships in túngara frogs are well established (Chakraborty and Burmeister, 2009; Kime et al., 2007; Lynch, 2005; Marler and Ryan, 1996). Estradiol is sufficient to induce phonotaxis (acoustically-guided approach) to species-specific calls (Chakraborty and Burmeister, 2009) and females approaching oviposition have higher estradiol concentrations than when they are in the non-breeding condition (Lynch and Wilczynski, 2005). Gonadotropins (which stimulate release of estradiol) may modulate neural responses to mating signals (Lynch and Wilczynski, 2008; Lynch et al., 2006). In addition, testosterone influences the filtering properties of the auditory system in a sex-specific manner (Miranda and Wilczynski, 2009), and gonadectomy influences multiunit audiograms in the torus semicircularis of male *Hyla cinerea* (Penna et al., 1992). Taken together, it appears that steroid hormones influence auditory processing in anurans in a sex-specific manner thereby modulating behavioral responses to species-specific signals.

Females had higher *AR* expression than males within the ventral hypothalamus and medial pallium, whereas males had higher *ERα* expression in the medial pallium. At present the function of this higher *AR* expression within the ventral hypothalamus in females is unclear. It is possible that both androgens and estrogens are involved to some degree in influencing female receptivity. If so, enhanced expression of *AR* may be required in females to compensate for the low circulating androgens and to increase the sensitivity of the hormone. The ventral hypothalamus is important for expression of female sexual behavior in most vertebrates and facilitates female-specific receptivity in a variety of species (reviewed in Blaustein and Erskine, 2002; Flanagan-Cato, 2000). The findings from our study parallel studies from reptiles that have demonstrated enhanced *AR* expression in females than males within the hypothalamus (Rosen and Wade, 2000; Scott et al., 2004). The distribution pattern observed within the medial pallium in túngara frogs suggests that steroid hormones

may modulate pallial functions during reproduction. Since very little is currently known about pallial functions in anurans, the implications for the enhanced expression of *AR* in females, and *ER*$\alpha$ in males are unclear. Alternatively, since the pallium serves as a major multimodal center for sensory processing and integration (Laberge and Roth, 2007; Northcutt and Ronan, 1992), sex dimorphisms in receptor expression may represent underlying mechanisms that are associated with functions that are unrelated to reproduction.

In summary, we found widespread distribution of *AR, ER*$\alpha$*,* and *ER*$\beta$ mRNA within many brain regions, including sensory, motor, and motivational areas that are important for sexual communication in anurans. Although males and females showed similar distribution of *AR* and *ER* expression, expression levels varied in some brain regions that may possibly explain sex-specific, hormone-behavior relationships. Our results showed sex differences in receptor expression in the midbrain torus semicircularis, suggesting that auditory processing is regulated in a sex-specific manner. Furthermore, we found new putative sites of androgen and estrogen action including the pallium, principal nucleus of the torus semicircularis, locus coeruleus, and the posterior tuberculum. Since steroid hormones exert widespread cellular effects it is likely that sex differences within brain regions indicate aspects of social behaviors and physiological processes that are unrelated to reproduction. Clearly, further work will be necessary to investigate how and where steroid receptors exert physiological effects in the amphibian brain. To our knowledge, this is the first study to demonstrate sex differences in steroid receptor expression in amphibians, and the first to provide a detailed description of the neuroanatomical distribution of *AR*s and *ER*s in an anuran.

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Table 3.1

Primers (5' to 3') used to generate cDNA sequences.

| | | Forward | Reverse | Genbank No. of receptor |
|---|---|---|---|---|
| AR[1] | | GCS AGC AGR AAY GAY TGY AC | GCY TTC ATG CAS AGG AAY TC | DQ320626 |
| ERβ[2] | | ATI TGY CCI GCI ACI AAY CA | ARR TGY TCC ATI CCY TTR TT | pending |
| ERα | Pair 1[3] | GGD CAY AAY GAY TAY ATG TG | TCC ATK CCY TTR TTR CTC AT | pending |
| | Pair 2 | GTA TCA GGA ARG AYC GSA GRG | CGC CAA ATT AAD CCR ACC ATW | |

[1]Chattopadhayay et al. (2003)

[2]Wu et al. (2003)

[3]Ko et al. (2008)

Table 3.2

Percent sequence similarity and identity of predicted protein sequences of AR, ERα, and ERβ receptors among vertebrates.

| | AR | ERα | ERβ |
|---|---|---|---|
| Chicken | 90, 84 [1] | 90, 80 [6] | 89, 80 [11] |
| Zebra finch | 93, 85 [2] | 90, 80 [7] | 89, 78 [12] |
| Rat | 91, 86 [3] | 90, 78 [8] | 89, 78 [13] |
| Human | 91, 87 [4] | 91, 78 [9] | 90, 77 [14] |
| South African clawed frog | 97, 93 [5] | 98, 97 [10] | 93, 86 [15] |

Genbank numbers: [1]NP_001035179; [2]NP_ 001070156; [3]NP_036634; [4]AAA51772; [5]CAA41726; [6]NP_990514; [7]NP_ 001070169; [8]NP_ 036821; [9]AAD52984; [10]NP_001083086; [11]NP_990125; [12]XP_002200631; [13]NP_036886; [14]AAC05985; [15]NP_001124426.

Table 3.3

Relative pattern of expression of *AR*, *ER*$\alpha$, and *ER*$\beta$ mRNA in túngara brain.

| Brain region | AR | ERα | ERβ |
|---|---|---|---|
| *Hindbrain* | | | |
| Cerebellum | L | -- | -- |
| Reticular formation | M | -- | -- |
| Locus coeruleus | L | -- | -- |
| Dorsal tegmental area of medulla | L | -- | -- |
| *Midbrain* | | | |
| Tegmentum | L | -- | L |
| Magnocellular nucleus of torus semicircularis | -- | -- | -- |
| Principal nucleus of torus semicircularis | M-H | M-H | M |
| Laminar nucleus of torus semicircularis | H | M-H | L-H |
| Optic tectum | M | L | L |
| *Forebrain (Diencephalon)* | | | |
| Posterior tuberculum | M | -- | M |
| Lateral hypothalamus | M | M | M |
| Dorsal hypothalamus | M | M | M |
| Ventral hypothalamus | M-H | M | L |
| Ventrolateral thalamus | L | -- | L |
| Ventromedial thalamus | M | L | L |
| Posterior thalamus | -- | -- | -- |
| Central thalamus | L | L-M | M-H |

| | | | |
|---|---|---|---|
| Anterior thalamus | L | L | L |
| Habenula | -- | -- | -- |
| Preoptic area | H | H | H* |
| *Forebrain (Telencephalon)* | | | |
| Caudal amygdala | M | M | M |
| Lateral amygdala | M | M | M |
| Medial amygdala | L | M | M |
| Lateral septum | L | M | M |
| Medial septal nucleus | L | L | L |
| Dorsal pallium | M | L | M |
| Lateral pallium | L | -- | L |
| Medial pallium | M-H | L-M | H |
| Ventral pallium | L | -- | L |
| Nucleus accumbens | H | H | H |
| Ventral striatum | M-H | M | M |
| Dorsal striatum | L | L | L |
| Olfactory bulb | -- | -- | -- |

H*, Very high; H, High; M, Medium; L, Low; --, Undetectable

Figure Legends

Figure 3.1 Expression of *AR*, *ER*$\alpha$, and *ER*$\beta$ mRNA within the auditory torus semicircularis, thalamus, and forebrain auditory targets in males and females. Data are shown as mean (± SE) silver grains/cell. Sample sizes are indicated for each treatment group and p values are significant at an alpha level of 0.05.

Figure 3.2. Amino acid alignments of AR (A), ER$\alpha$ (B), and ER$\beta$ (C) protein sequences. The shading indicates parts of sequences that share > 80% (darkest gray), > 60% (mid-gray), > 40% (light gray), and < 40% (not colored) percent similarity with the consensus sequences of the respective receptor protein sequences.

Figure 3.3. Schematic diagrams (left column) and photomicrographs showing sex differences in AR, *ER*$\alpha$, and *ER*$\beta$ mRNA expression within sampling windows (boxes) in the laminar and principal nuclei of the torus semicircularis, ventral hypothalamus, and medial pallium. Scale bar represents 400 $\mu$m (brightfield images) and 100 $\mu$m (photomicrographs). Abbreviations: OT, optic tectum; Ltor, laminar nucleus of torus semicircularis; Ptor, principal nucleus of torus semicircularis; Teg, tegmentum; Cthal, central thalamus; La, lateral thalamus; VM, ventromedial thalamus; LH, lateral hypothalamus; VH, ventral hypothalamus; MP, medial pallium; LP, lateral pallium; DP, dorsal pallium; VP, ventral pallium; St, striatum; Sl, lateral septum; Acc, nucleus accumbens; Sm, medial septum.

Figure 3.1

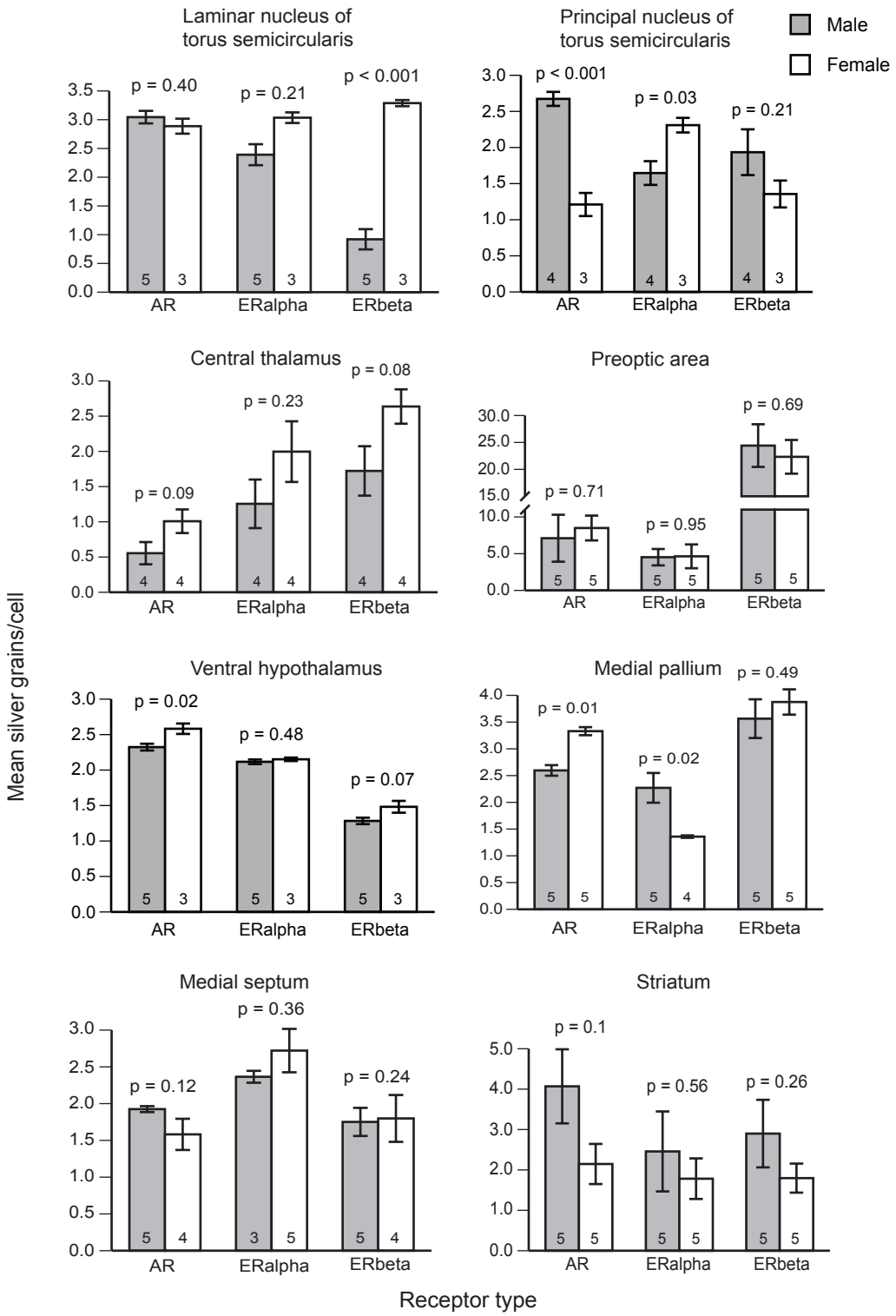
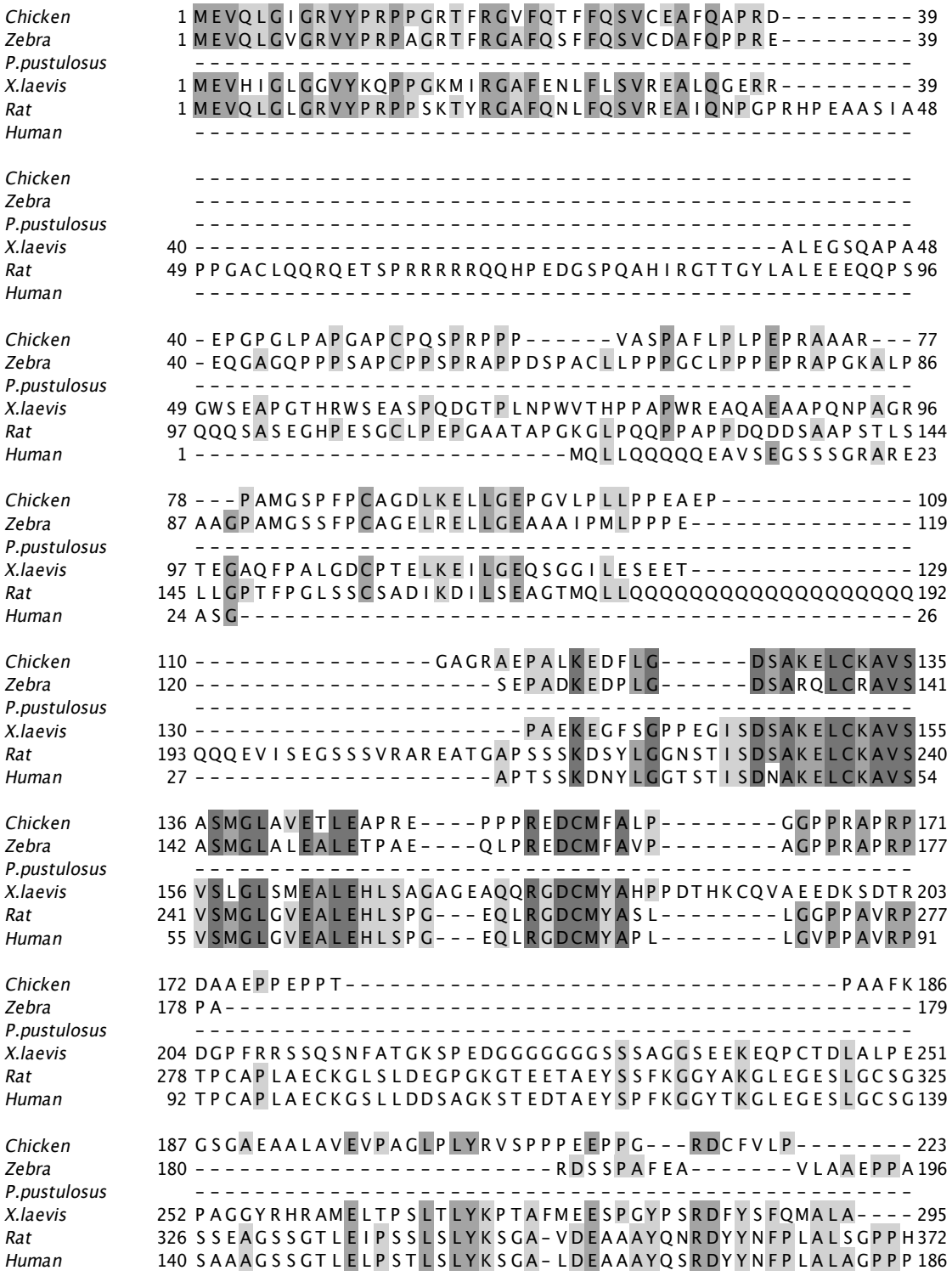
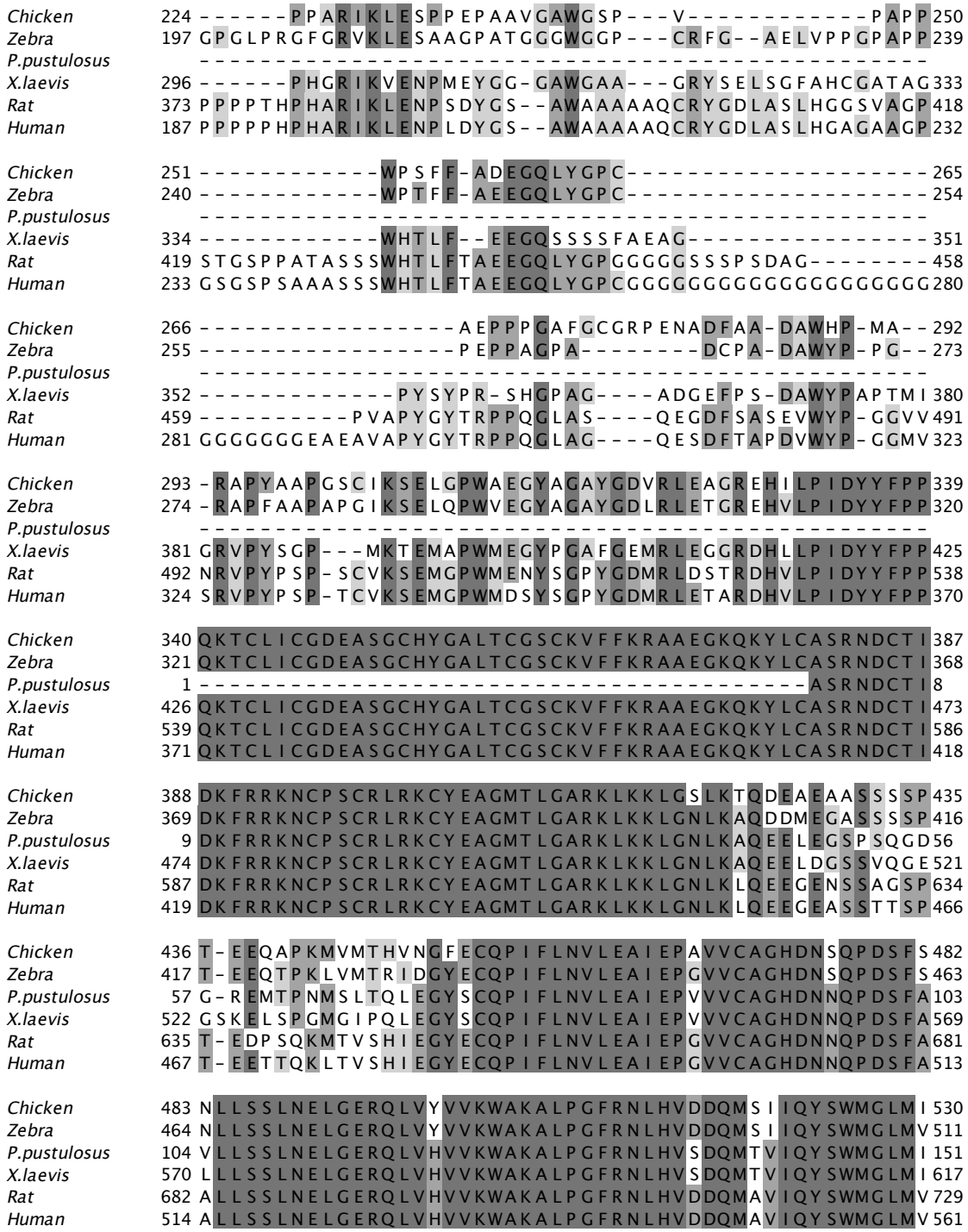

Laminar nucleus of torus semicircularis
Principal nucleus of torus semicircularis
Male
Female
p = 0.40
p = 0.21
p < 0.001
p < 0.001
p = 0.03
p = 0.21
Central thalamus
Preoptic area
p = 0.08
p = 0.23
p = 0.09
p = 0.69
p = 0.71
p = 0.95
Ventral hypothalamus
Medial pallium
p = 0.02
p = 0.48
p = 0.07
p = 0.49
p = 0.01
p = 0.02
Medial septum
Striatum
p = 0.36
p = 0.12
p = 0.24
p = 0.1
p = 0.56
p = 0.26
AR
ERalpha
ERbeta
Mean silver grains/cell
Receptor type

Figure 3.2A


Chicken 1 MEVQLGIGRVYPRPPGRTFRGVFQTFFQSVCEAFQAPRD---------39
Zebra 1 MEVQLGVGRVYPRPAGRTFRGAFQSFFQSVCDAFQPPRE---------39
P.pustulosus ----------
X.laevis 1 MEVHIGLGGVYKQPPGKMIRGAFENLFLSVREALQGERR---------39
Rat 1 MEVQLGLGRVYPRPPSKTYRGAFQNLFQSVREAIQNPGPRHPEAASIA48
Human ----------
Chicken ----------
Zebra ----------
P.pustulosus ----------
X.laevis 40 ----------ALEGSQAPA48
Rat 49 PPGACLQQRQETSPRRRRRQQHPEDGSPQAHIRGTTGYLALEEEQQPS96
Human ----------
Chicken 40 -EPGPGLPAPGAPCPQSPRPPP------VASPAFLPLPEPRAAAR---77
Zebra 40 -EQGAGQPPPSAPCPPSPRAPPDSPACLLPPPGCLPPPEPRAPGKALP86
P.pustulosus ----------
X.laevis 49 GWSEAPGTHRWSEASPQDGTPLNPWVTHPPAPWREAQAEAAPQNPAGR96
Rat 97 QQQSASEGHPESGCLPEPGAATAPGKGLPQQPPAPPDQDDSAAPSTLS144
Human 1 ----------MQLLQQQQQEAVSEGSSSGRARE23
Chicken 78 ---PAMGSPFPCAGDLKELLGEPGVLPLLPPEAEP----------109
Zebra 87 AAGPAMGSSFPCAGELRELLGEAAAIPMLPPPE----------119
P.pustulosus ----------
X.laevis 97 TEGAQFPALGDCPTELKEILGEQSGGILESEET----------129
Rat 145 LLGPTFPGLSSCSADIKDILSEAGTMQLLQQQQQQQQQQQQQQQQQQ192
Human 24 ASG----------26
Chicken 110 ----------GAGRAEPALKEDFLG------DSAKELCKAVS135
Zebra 120 ----------SEPADKEDPLG------DSARQLCRAVS141
P.pustulosus ----------
X.laevis 130 ----------PAEKEGFSGPPEGISDSAKELCKAVS155
Rat 193 QQQEVISEGSSSVRAREATGAPSSSKDSYLGGNSTISDSAKELCKAVS240
Human 27 ----------APTSSKDNYLGGTSTISDNAKELCKAVS54
Chicken 136 ASMGLAVETLEAPRE----PPPREDCMFALP--------GGPPRAPRP171
Zebra 142 ASMGLALEALETPAE----QLPREDCMFAVP--------AGPPRAPRP177
P.pustulosus ----------
X.laevis 156 VSLGLSMEALEHLSAGAGEAQQRGDCMYAHPPDTHKCQVAEEDKSDTR203
Rat 241 VSMGLGVEALEHLSPG---EQLRGDCMYASL---------LGGPPAVRP277
Human 55 VSMGLGVEALEHLSPG---EQLRGDCMYAPL---------LGVPPAVRP91
Chicken 172 DAAEPPEPPT----------PAAFK186
Zebra 178 PA----------179
P.pustulosus ----------
X.laevis 204 DGPFRRSSQSNFATGKSPEDGGGGGGSSSAGGSEEKEQPCTDLALPE251
Rat 278 TPCAPLAECKGLSLDEGPGKGTEETAEYSSFKGGYAKGLEGESLGCSG325
Human 92 TPCAPLAECKGSLLDDSAGKSTEDTAEYSPFKGGYTKGLEGESLGCSG139
Chicken 187 GSGAEAALAVEVPAGLPLYRVSPPPEEPPG---RDCFVLP--------223
Zebra 180 ----------RDSSPAFEA-------VLAAEPPA196
P.pustulosus ----------
X.laevis 252 PAGGYRHRAMELTPSLTLYKPTAFMEESPGYPSRDFYSFQMALA----295
Rat 326 SSEAGSSGTLEIPSSLSLYKSGA-VDEAAAYQNRDYYNFPLALSGPPH372
Human 140 SAAAGSSGTLELPSTLSLYKSGA-LDEAAAYQSRDYYNFPLALAGPPP186


Chicken 224 ------PPARIKLESPPEPAAVGAWGSP---V----------PAPP 250
Zebra 197 GPGLPRGFGRVKLESAAGPATGGGWGGP---CRFG--AELVPPGPAPP 239
P.pustulosus ----------
X.laevis 296 ------PHGRIKVENPMEYGG-GAWGAA---GRYSELSGFAHCGATAG 333
Rat 373 PPPPTHPHARIKLENPSDYGS--AWAAAAAQCRYGDLASLHGGSVAGP 418
Human 187 PPPPPHPHARIKLENPLDYGS--AWAAAAAQCRYGDLASLHGAGAAGP 232
Chicken 251 ----------WPSFF-ADEGQLYGPC---------- 265
Zebra 240 ----------WPTFF-AEEGQLYGPC---------- 254
P.pustulosus ----------
X.laevis 334 ----------WHTLF--EEGQSSSSFAEAG---------- 351
Rat 419 STGSPPATASSSWHTLFTAEEGQLYGPGGGGGSSSPSDAG-------- 458
Human 233 GSGSPSAAASSSWHTLFTAEEGQLYGPCGGGGGGGGGGGGGGGGGG 280
Chicken 266 ----------AEPPPGAFGCGRPENADFAA-DAWHP-MA-- 292
Zebra 255 ----------PEPPAGPA--------DCPA-DAWYP-PG-- 273
P.pustulosus ----------
X.laevis 352 ----------PYSYPR-SHGPAG----ADGEFPS-DAWYPAPTMI 380
Rat 459 ----------PVAPYGYTRPPQGLAS----QEGDFSASEVWYP-GGVV 491
Human 281 GGGGGGGEAEAVAPYGYTRPPQGLAG----QESDFTAPDVWYP-GGMV 323
Chicken 293 -RAPYAAPGSCIKSELGPWAEGYAGAYGDVRLEAGREHILPIDYYFPP 339
Zebra 274 -RAPFAAPAPGIKSELQPWVEGYAGAYGDLRLETGREHVLPIDYYFPP 320
P.pustulosus ----------
X.laevis 381 GRVPYSGP---MKTEMAPWMEGYPGAFGEMRLEGGRDHLLPIDYYFPP 425
Rat 492 NRVPYPSP-SCVKSEMGPWMENYSGPYGDMRLDSTRDHVLPIDYYFPP 538
Human 324 SRVPYPSP-TCVKSEMGPWMDSYSGPYGDMRLETARDHVLPIDYYFPP 370
Chicken 340 QKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTI 387
Zebra 321 QKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTI 368
P.pustulosus 1 ----------ASRNDCTI 8
X.laevis 426 QKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTI 473
Rat 539 QKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTI 586
Human 371 QKTCLICGDEASGCHYGALTCGSCKVFFKRAAEGKQKYLCASRNDCTI 418
Chicken 388 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGSLKTQDEAEAASSSSP 435
Zebra 369 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKAQDDMEGASSSSP 416
P.pustulosus 9 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKAQEELEGSPSQGD 56
X.laevis 474 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKAQEELDGSSVQGE 521
Rat 587 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKLQEEGENSSAGSP 634
Human 419 DKFRRKNCPSCRLRKCYEAGMTLGARKLKKLGNLKLQEEGEASSTTSP 466
Chicken 436 T-EEQAPKMVMTHVNGFECQPIFLNVLEAIEPAVVCAGHDNSQPDSFS 482
Zebra 417 T-EEQTPKLVMTRIDGYECQPIFLNVLEAIEPGVVCAGHDNSQPDSFS 463
P.pustulosus 57 G-REMTPNMSLTQLEGYSCQPIFLNVLEAIEPVVVCAGHDNNQPDSFA 103
X.laevis 522 GSKELSPGMGIPQLEGYSCQPIFLNVLEAIEPVVVCAGHDNNQPDSFA 569
Rat 635 T-EDPSQKMTVSHIEGYECQPIFLNVLEAIEPGVVCAGHDNNQPDSFA 681
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X.laevis 570 LLLSSLNELGERQLVHVVKWAKALPGFRNLHVSDQMTVIQYSWMGLMI 617
Rat 682 ALLSSLNELGERQLVHVVKWAKALPGFRNLHVDDQMAVIQYSWMGLMV 729
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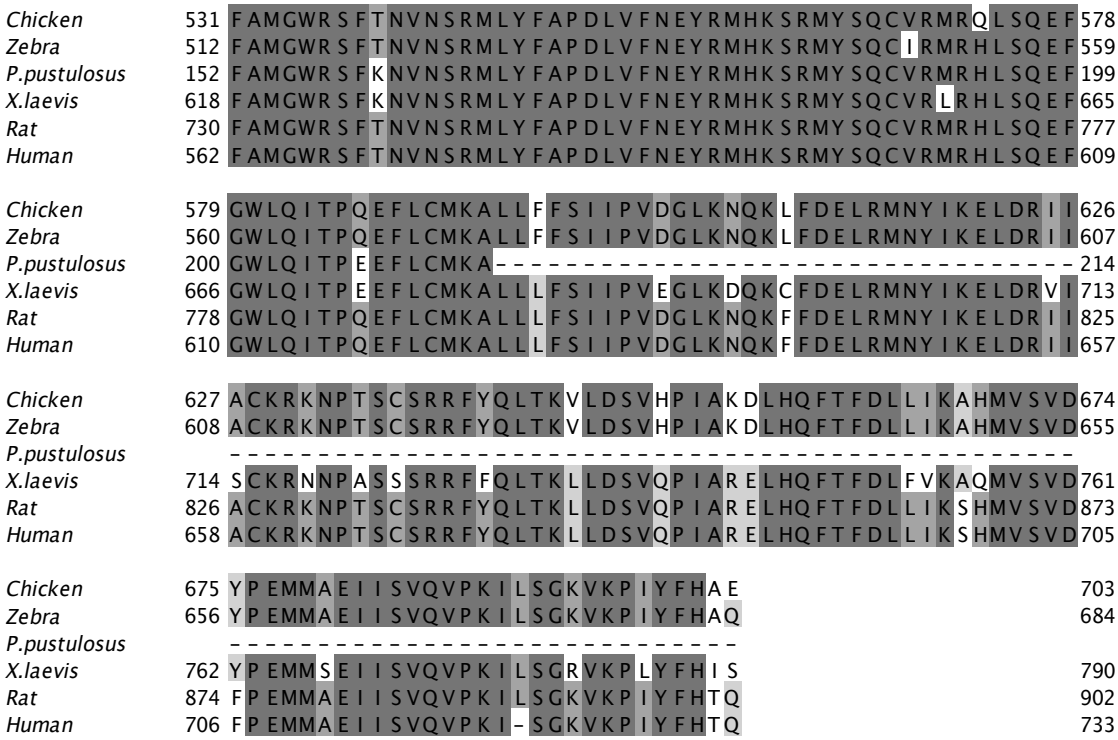
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X.laevis 618 FAMGWRSFKNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRLRHLSQEF 665
Rat 730 FAMGWRSFTNVNSRMLYFAPDLVFNEYRMHKSRMYSQCVRMRHLSQEF 777
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Zebra 560 GWLQITPQEFLCMKALLFFSIIPVDGLKNQKLFDELRMNYIKELDRII 607
P.pustulosus 200 GWLQITPEEFLCMKA---------- 214
X.laevis 666 GWLQITPEEFLCMKALLLFSIIPVEGLKDQKCFDELRMNYIKELDRVI 713
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Zebra 608 ACKRKNPTSCSRRFYQLTKVLDSVHPIAKDLHQFTFDLLIKAHMVSVD 655
P.pustulosus ----------
X.laevis 714 SCKRNNPASSSRRFFQLTKLLDSVQPIARELHQFTFDLFVKAQMVSVD 761
Rat 826 ACKRKNPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHMVSVD 873
Human 658 ACKRKNPTSCSRRFYQLTKLLDSVQPIARELHQFTFDLLIKSHMVSVD 705
Chicken 675 YPEMMAEIISVQVPKILSGKVKPIYFHAE 703
Zebra 656 YPEMMAEIISVQVPKILSGKVKPIYFHAQ 684
P.pustulosus ----------
X.laevis 762 YPEMMSEIISVQVPKILSGRVKPLYFHIS 790
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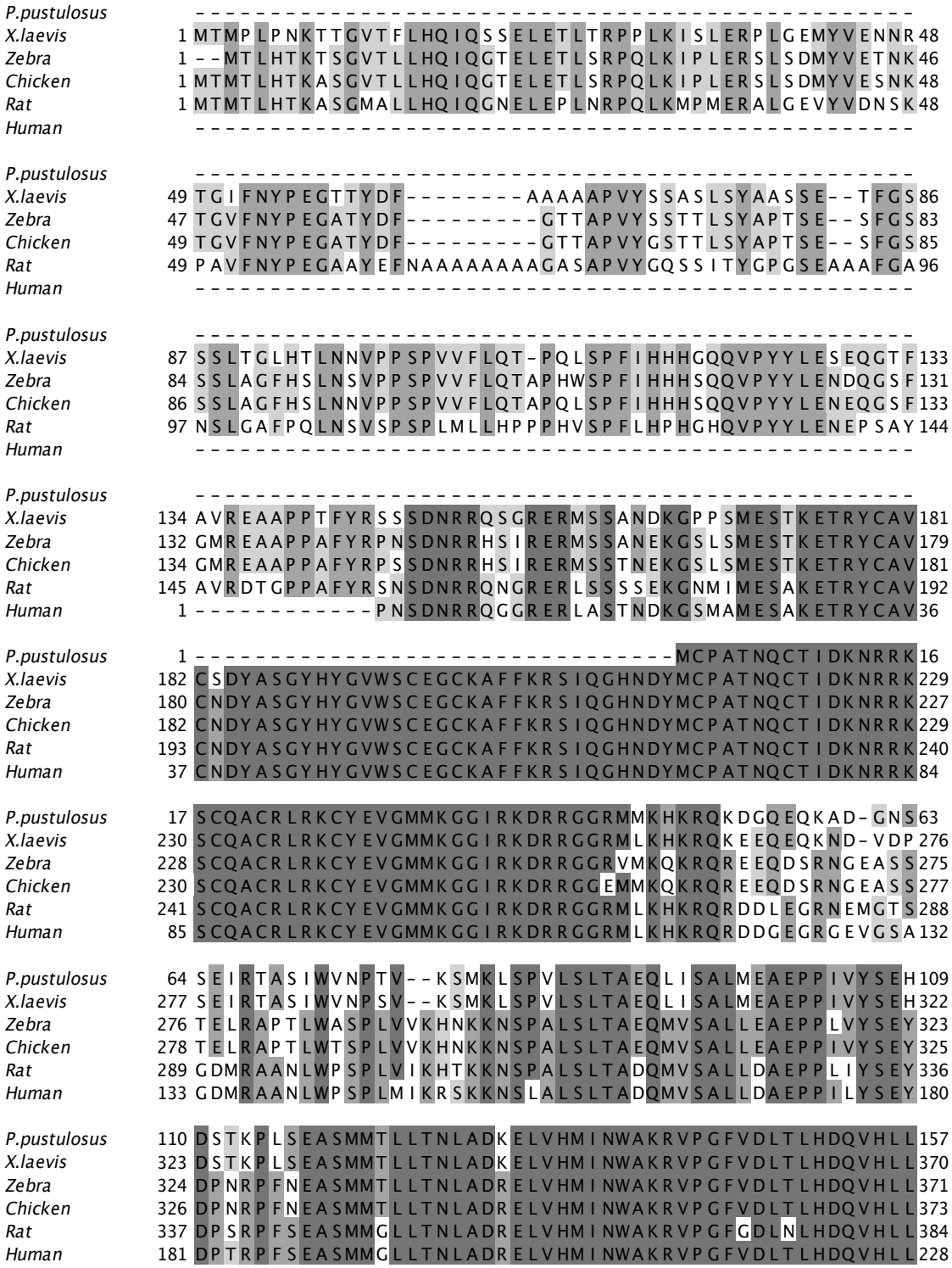
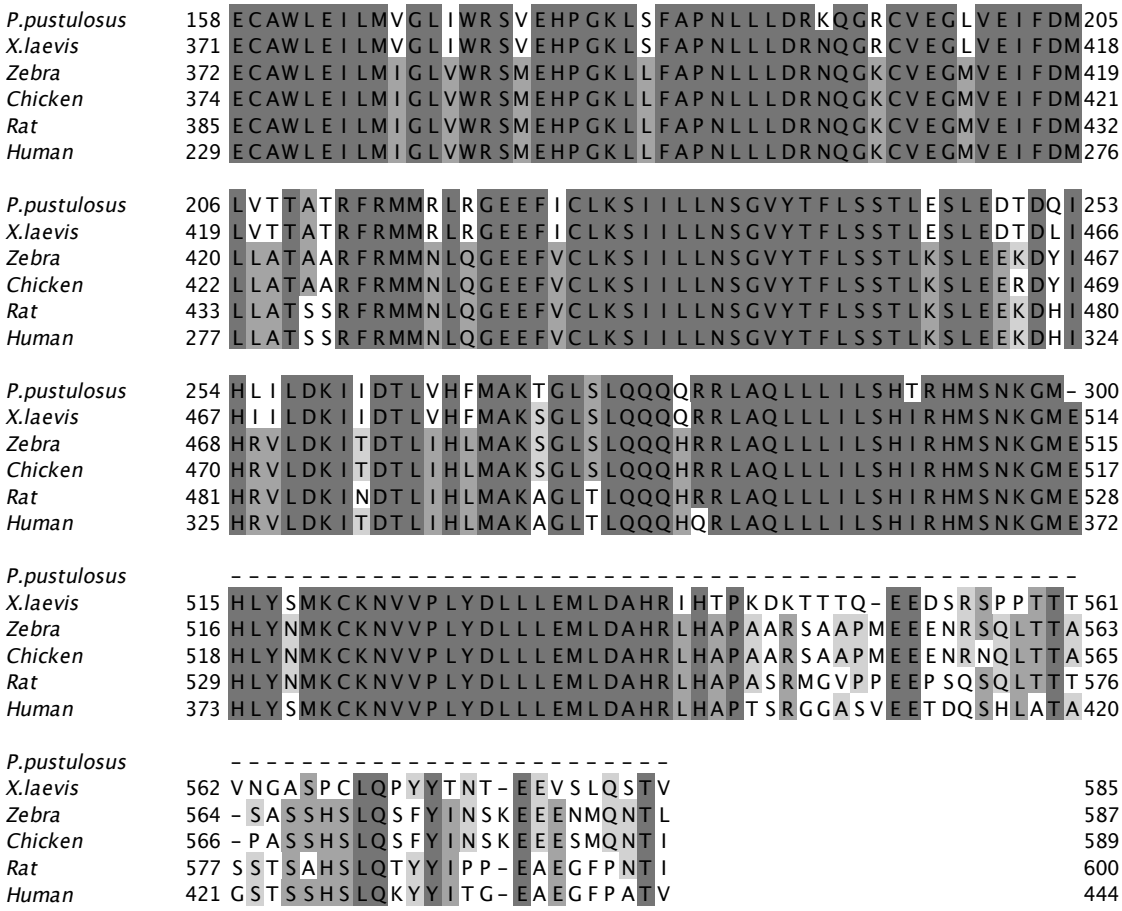
Figure 3.2B

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Human 229 ECAWLEILMIGLVWRSMEHPGKLLFAPNLLLDRNQGKCVEGMVEIFDM 276
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X.laevis 419 LVTTATRFRMMRLRGEEFICLKSIILLNSGVYTFLSSTLESLEDTDLI 466
Zebra 420 LLATAARFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDYI 467
Chicken 422 LLATAARFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEERDYI 469
Rat 433 LLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 480
Human 277 LLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHI 324
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Rat 481 HRVLDKINDTLIHLMAKAGLTLQQQHRRLAQLLLILSHIRHMSNKGME 528
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Human 373 HLYSMKCKNVVPLYDLLLEMLDAHRLHAPTSRGGASVEETDQSHLATA 420
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X.laevis 562 VNGASPCLQPYYTNT-EEVSLQSTV 585
Zebra 564 -SASSHSLQSFYINSKEEENMQNTL 587
Chicken 566 -PASSHSLQSFYINSKEEESMQNTI 589
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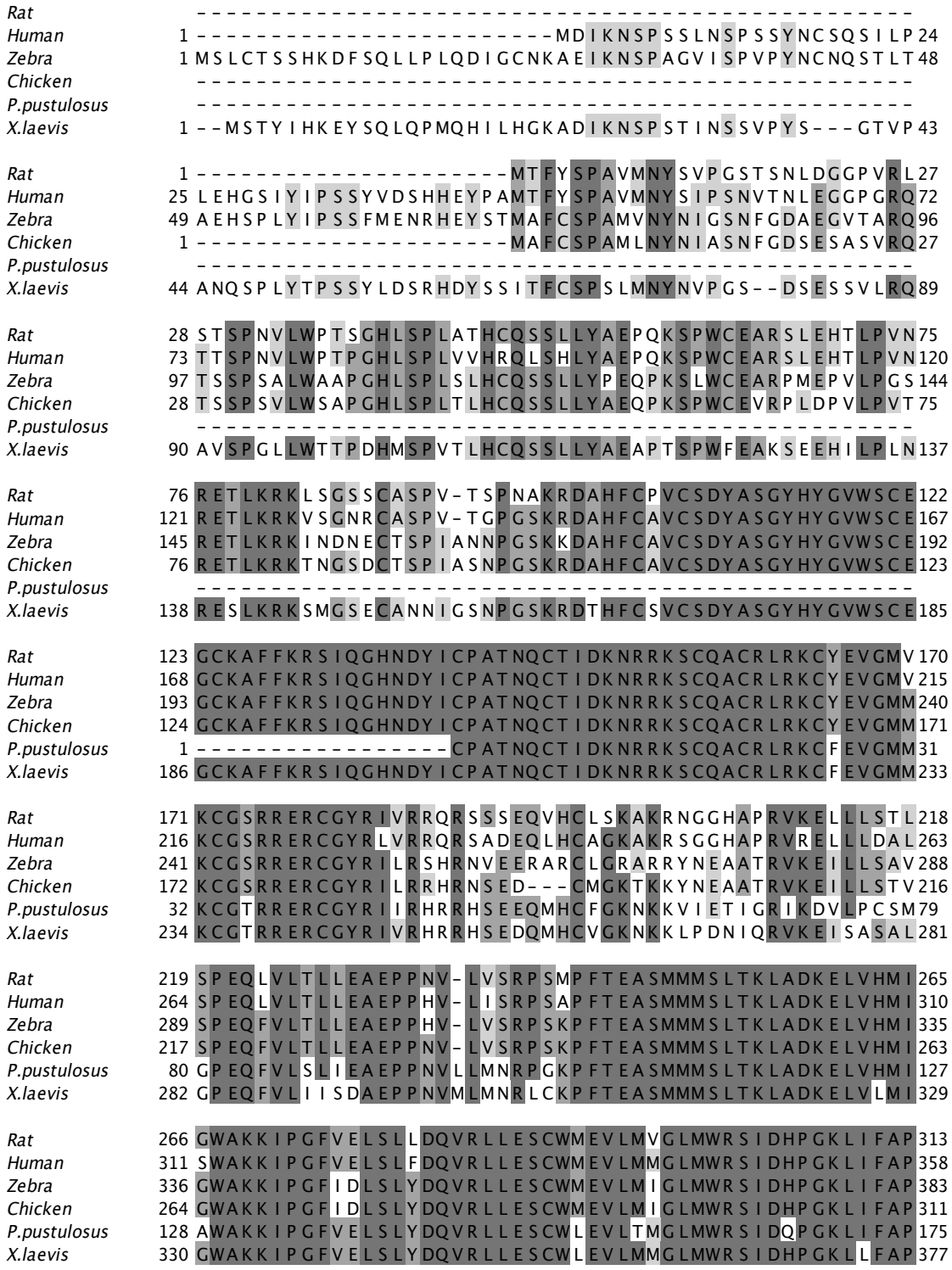
FIgure 3.2C

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Chicken 312 DLVLDRDEGKCVEGILEIFDMLLAMTSRFRELKLQHKEYLCVKAMILL 359
P.pustulosus 176 DLTLDRDEGKCVEGILEIFDMLLATTSRFRDLKLQHREYLCLKVLILL 223
X.laevis 378 DLTLDRDEGKCVEGILEIFDMLLATTSRLRELKLQHKEYLCLKVMILL 425
Rat 362 NSSMYPLASANQEAESSRKLTHLLNAVTDALVWVIAKSGISSQQQSVR 409
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Zebra 432 NSSMFPLSA--EEPESNRKLHHLLNVVTEALVWVIAKSGIPSQQQTTR 477
Chicken 360 NSSMFPLSP--EEPESNRKLHHLLNVVTDALVWVIAKSGIPSQQQTTR 405
P.pustulosus 224 NSNMFPLSTSDEESESSRKLHQLLNTVTDGLVWVIAKSGISFRQQSTR 271
X.laevis 426 NSHMFPLTSSDEESESSRKLHHLLNTVTDALVWVIAKSGIPFRQQSTR 473
Rat 410 LANLLMLLSHVRHISNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRG 457
Human 455 LANLLMLLSHVRHASNKGMEHLLNMKCKNVVPVYDLLLEMLNAHVLRG 502
Zebra 478 LANLLMLLSHVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRG 525
Chicken 406 LANLLMLLSHVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNAHTLRG 453
P.pustulosus 272 LANLLMLLSHVRHASNKGMEH---------- 292
X.laevis 474 LANLLMLLSHVRHASNKGMEHLLSMKCKNVVPVYDLLLEMLNANTLRD 521
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Human 503 C-KSSITGSECSPAEDSKSKEGSQNPQSQ 530
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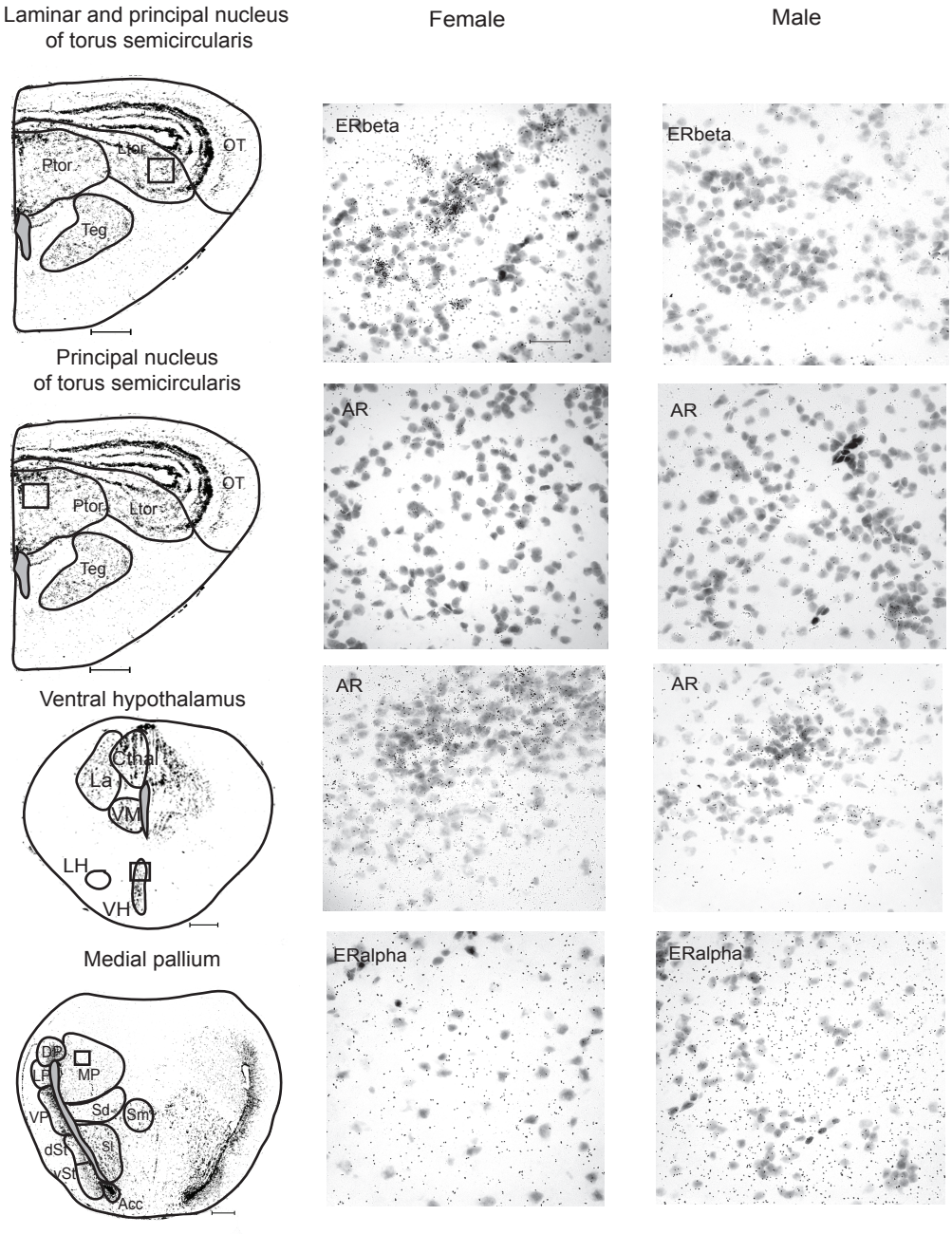
X.laevis 522 HRKPTLAISGNSSKAEGDARDTGQPTQ-- 548

Figure 3.3


Laminar and principal nucleus of torus semicircularis
Female
Male
OT
Ptor
Ltor
Teg
ERbeta
ERbeta
Principal nucleus of torus semicircularis
OT
Ptor
Ltor
Teg
AR
AR
Ventral hypothalamus
Ctha
La
VM
LH
VH
AR
AR
Medial pallium
DP
LP
MP
VP
Sd
Sm
dSt
St
vSt
Acc
ERalpha
ERalpha

## CHAPTER 4

# WIDESPREAD NEURAL PREFERENCES FOR CONSPECIFIC CALLS IN THE CENTRAL AUDITORY SYSTEM AND FOREBRAIN TARGETS


### Summary

Processing of species-specific communication signals is fundamental for finding mates. Neural representation of species-specific signals is thought to emerge at higher levels after a process of hierarchical feature detection and most studies suggest that little processing takes place at earlier stages. We investigated system wide responses to conspecific signals in túngara frog. Male túngara frogs produce mating calls that females use to discriminate between conspecific and heterospecific males. We exposed females to conspecific, heterospecific, or no sound stimuli, and measured expression of the immediate early gene *egr-1* as a marker of neural activity in the ascending auditory system, including the hindbrain, midbrain, thalamus, as well as its primary forebrain targets. With three exceptions, all auditory nuclei showed greater responses to the conspecific call than the heterospecific call, suggesting that the auditory system responds preferentially to conspecific stimuli, even in the hindbrain. This neural preference is apparent in the superior olivary nucleus and is greater in magnitude in the thalamus. Furthermore, we found that the neural preference was specific to call category (conspecific or heterospecific) rather than to the idiosyncratic acoustic traits of the mating calls we used to represent each category. Finally, the neural preference was also present in the forebrain limbic and motor areas that likely modulate behavior, including mate choice. Thus, we conclude that sensory systems

are selective towards processing species-specific stimuli even at early stages of processing.

## Introduction

Neural representation of complex stimuli emerges at higher levels of processing after a series of ever-more complex features are extracted from ascending information gathered from peripheral receptors. Thus, most investigations of the neural correlates of species-specific signal discrimination focus on higher-order processing centers in the telencephalon (e.g. Mello and Clayton, 1994; Grace et al., 2003; Petkov et al., 2008). However, studies suggest that sensory afferents are more efficient at encoding naturalistic stimuli than artificial stimuli (Reike et al., 1995) raising the possibility that enhanced responses to species-specific signals are not an inherent property of these higher brain regions, but are determined by lower brain regions.

In order to investigate system-wide sensory responses to species-specific signals, we chose the túngara frog (*Physalaemus pustulosus*) as a model species. Behavioral responses to species-specific acoustic signals, which are conveyed primarily through the acoustic domain, are well documented in túngara frogs (Ryan, 1985). In addition, neurophysiological responses to species-specific signals have been well explored in anurans. As in other vertebrates, hindbrain auditory units display simple tuning curves and the firing rates of these units tend to follow the amplitude envelope of the stimulus, suggesting that little processing occurs at these early stages in the pathway (reviewed in Narins et al., 2007). Feature detectors have been identified in the auditory midbrain and the caudal thalamus (Mudry et al., 1977; Fuzessery and Feng, 1983), although it appears that spectral and temporal processing remain separate through the thalamus (Penna et al., 1997). Cells that are truly selective for mating calls have remained elusive. Although recent functional mapping studies have demonstrated enhanced responses to species-specific

signals in a subset of auditory nuclei (Hoke et al., 2004; Hoke et al., 2007b) we know little about patterns of system wide responses to species-specific signals.

We presented reproductively active female túngara frogs with conspecific calls, heterospecific calls of an allopatric congener, *Physalaemus enesefae (fischeri)*, or no sound and assessed neural activity using expression of the immediate early gene *egr-1* (also known as *zif268* and *ZENK*). We measured *egr-1* mRNA levels in the hindbrain, midbrain, and the thalamus, as well as some of the forebrain targets of the auditory system. Based on the acoustic features of the calls, the acoustic requirements for species recognition in túngaras (Wilczynski et al., 1995), and the response properties of the anuran auditory system, we predicted that conspecific call preferences would emerge in the auditory midbrain or thalamus. In contrast, we found that conspecific calls evoked a greater response throughout the central auditory system, beginning in the superior olivary nucleus and prior to the emergence of feature detectors. This neural preference to conspecific calls was also observed in the auditory torus semicircularis (homolog of the inferior colliculus), thalamus, and in the forebrain limbic and motor targets. All but three nuclei known to receive auditory projections demonstrated greater neural responses toward conspecific calls. Thus, we conclude that, although hierarchical feature detection undoubtedly plays an important role in recognition of species-specific stimuli, sensory systems may be generally selective toward processing species-specific signals in this species.

## Materials and Methods

### Frog collection and acoustic treatment

We captured female túngara frogs in a mating clasp with males from breeding ponds over a 3-week period on the Osa Peninsula, Costa Rica in July 2007. We caught pairs between 20:00 and 24:00 hours, released the males, and brought the females to the

laboratory at the Osa Biodiversity Center. We placed the females in mesh cages inside of one of eight acoustic chambers, each equipped with a Tivoli Portable Audio Laboratory speaker (Tivoli Audio, Cambridge, MA) that was connected to an M-Audio Firewire 410 unit (M-Audio, Arcadia, CA) and Macintosh computer. After an 11-h acclimation period, we presented females with a conspecific *P. pustulosus* call (n = 11), heterospecific *P. enesefae* call (n = 11), or no sound (n = 8) for 30 minutes. We interspersed females assigned to each treatment group across days and chambers. We rapidly decapitated females 1 h after onset of stimuli, which corresponds to peak accumulation of acoustically induced *egr-1* mRNA expression in this species (Burmeister et al., 2008). After decapitation, we opened the skull in order to fix the brains for 10 min in 4% paraformaldehyde before removing them. We then rinsed the brains in phosphate buffered saline for 10 min before freezing them in liquid nitrogen in 2 ml tubes containing Tissue-Tek OCT Compound (Sakura, Finetek, Torrance, CA). We kept brains on dry ice during transportation to University of North Carolina where we stored them at – 80° C until further processing.

The University of North Carolina Institutional Animal Care and Use Committee (IACUC) approved our experimental procedures and Costa Rica's Ministerio del Ambiente Y Energia (MINAE) and Sistema Nacional de Áreas de Conservación (SINAC) permitted tissue collection and export.

Acoustic stimuli

The species-specific calls of *P. pustulosus* and *P. enesefae* are both characterized by a downward frequency sweep referred to as a whine (Fig. 4.1). The *P. enesefae* whine begins at about 1060 Hz and descends to 590 Hz in approximately 720 ms (Tárano, 2001). The *P. pustulosus* whine begins at about 1000 Hz and sweeps to 400 Hz in about 350 ms (Ryan, 1985). Thus, the two species-specific whines contain many of the same spectral components, but differ in temporal features that characterize the shape of the amplitude

envelope (Ryan et al., 2003). Behavioral analyses suggest that call recognition in the túngara frog requires frequencies within a high frequency range (900-560 Hz) during the first 100 ms of the call followed by a low frequency range (640-500) during the second part of the call (Wilczynski et al., 1995). The difference in the temporal arrangement of the spectral features of the two species' calls likely contributes to the different behavioral responses that the two calls elicit. Although the whine alone is sufficient for species recognition, *P. pustulosus* (but not *P. enesefae)* can enhance the attractiveness of their call by adding a second component referred to as a chuck (Fig. 4.1A). The chuck is a short (40 ms) burst of sound with rich harmonic structure that emphasizes frequencies greater than 1500 Hz. To represent the conspecific and heterospecific call categories, we used two call exemplars of each category (Fig. 4.1) recorded from free-living males. We recorded the *P. pustulosus* calls from breeding populations on the Osa Peninsula, while the *P. enesefae* calls were recorded by Dr. Zaida Tárano in Venezuela. We chose call exemplars that were close to the mean for the populations where they were recorded. We presented each female with one exemplar repeated every 2 seconds to reflect the calling rate of *P. pustulosus,* for 30 minutes. We played the calls using ProTools audio software (V. 7.3; Digidesign, Daly City, CA) from a Macintosh PowerBook G4. We set the peak amplitude for calls at 82 dB SPL at a distance of approximately 5 cm from the speaker.

Radioactive *in situ* hybridization

We sectioned brains in the transverse plane at 16 µm in 3 series on a cryostat. To localize *egr-1* mRNA, we used radioactive *in situ* procedures previously described in Burmeister et al. (2008). Briefly, we generated radioactively labeled sense and antisense probes from reverse transcription of a 309-nucleotide subclone of *P. pustulosus egr-1*. Before hybridization, we fixed the tissue for 10 sec in 4% paraformaldehyde before washing in phosphate-buffered saline, triethanolamine, acetic anhydride, 2× SSC, and a series of

ethanols. We hybridized the tissue with 90 µl of $3.0 \times 10^5$ cpm/ml of hybridization buffer at 65° C overnight. We removed unbound probe with a series of 65° C washes, first in 50% formamide and 2× SSC (1.25 h) followed by two washes in 0.1× SSC (30 min each). We visualized the bound riboprobe as silver grains by exposing the slides to NTB emulsion (Kodak, New Have CT) diluted 1:1 in distilled water for 14 days at 4° C, and we visualized the cell bodies by staining the tissue with thionin. Tissue incubated with the sense probe showed no significant binding above background.

Quantitative measurement of egr-1 expression

We assessed neural activity in the ascending auditory system and its primary forebrain targets (Fig. 4.2). We consider the ascending auditory system of anurans to include the dorsal medullary nucleus (homolog of the mammalian cochlear nucleus), superior olivary nucleus, midbrain torus semicircularis (homolog of the mammalian inferior colliculus) and its thalamic targets, including the posterior, central, and anterior thalamic nuclei. From the thalamus, auditory pathways converge onto limbic (medial pallium, septum, preoptic area, hypothalamus) and motor regions (striatum) of the forebrain. Unlike amniotes, in anurans, the telencephalon does not appear to contain dedicated auditory processing centers that could be considered analogous to mammalian primary auditory cortex. Because we were interested in system-wide neural activity patterns, we measured *egr-1* expression at each of these levels. Within the striatum, we sampled from the ventral part, within the septum we sampled from the ventral part of the lateral septum, and within the medial pallium, we sampled from the dorsal part.

For each brain region, we calculated an individual's mean from between two to eleven consecutive photomicrographs, captured at a magnification of 630× from one hemisphere of the brain that best represented the respective brain region morphologically. The number of brain sections we sampled from each brain region was determined by the

size of the brain region and the overall quality of the sections, which varied across individuals and brain regions, as follows: dorsal medullary nucleus, 2-7; superior olivary nucleus, 2-8; laminar nucleus of the torus, 3; principal nucleus of the torus, 3-6; magnocellular nucleus of the torus, 2-5; posterior thalamus, 3-6; central thalamus, 3-6; anterior thalamus, 2-4; preoptic area, 3-7; ventral hypothalamus, 3-11; dorsal part of the medial pallium, 6; ventral striatum, 3-4; ventral part of the lateral septum, 3-4. In addition, for each brain section of the laminar nucleus of the torus, we calculated means from photomicrographs at medial, central, and lateral positions; *egr-1* responses to mating calls did not vary with position within the nucleus (data not shown). The quality of the brain sections also influenced the sample sizes reported for each brain region, as we were unable to obtain data from all individuals in the study for each brain region.

We assessed mean *egr-1* expression as described in Burmeister et al. (2008). Briefly, we used ImageJ (http:// rsbweb.nih.gov/ij/) to quantify silver grain number in the region of interest and in a nearby area of the slide that represented the background silver grain levels for that position on the slide. We manually counted the number of cell bodies in the region of interest from separate photomicrographs. We express relative *egr-1* expression, therefore, as the number of silver grains per cell above background. To facilitate comparisons among brain regions, we also calculated the fold-change in *egr-1* levels above the no sound group.

Statistical analyses

We conducted linear mixed models for each brain region using the “lme” function in R (R Foundation for Statistical Computing, Vienna, Austria). First, in each brain region we tested for an effect of stimulus with stimulus as a fixed effect and subject as a random effect. In these analyses we could not test for an effect of call exemplar because the no sound group does not have exemplars. Second, to examine the anatomical variation in induced

*egr-1* expression in response to conspecific calls, we analyzed the fold change in silver grains relative to no sound across brain regions. For this analysis, we tested for an effect of region with region as a fixed effect and subject as a random effect. We performed separate analyses for the auditory system (auditory brainstem, midbrain, and thalamus), in which case we used the anterior thalamus as a reference brain region in the linear mixed model, and the forebrain targets of the auditory system, in which case we used the ventral part of the lateral septum as a reference brain region. Third, to determine if exemplar within a call category (conspecific or heterospecific) influenced *egr-1* expression, we tested for an effect of exemplar with exemplar as a fixed effect and subject as a random effect; we tested for exemplar effects separately for the heterospecific and conspecific groups within each brain region.

**Results**

Auditory system

Conspecific mating calls elicited robust induction of *egr-1* expression in the superior olivary nucleus, the torus semicircularis, and the thalamus, whereas heterospecific mating calls did not (Fig. 4.3, 4.4; Table 4). In fact, the *egr-1* response to heterospecific calls was no different from no sound, showing that the heterospecific calls were unable to elicit *egr-1* expression. The level of *egr-1* induction in response to conspecific calls differed across brain regions ($p = 0.06$); qualitatively, it appears that induction progressively increased from caudal to rostral positions in the brain. Although conspecific calls did not induce *egr-1* expression in the dorsal medullary nucleus, *egr-1* was expressed above background levels, suggesting that the dorsal medullary nucleus is capable of expressing *egr-1*, but that the auditory stimulation we used was insufficient to induce changes in its expression. In the superior olivary nucleus, we found that females exposed to conspecific calls had higher *egr-*

*1* expression compared to heterospecific calls, but not to no sound. In the principal and laminar nuclei of the torus, the primary afferent and efferent nuclei, respectively, of the torus semicircularis, females exposed to conspecific stimuli had higher *egr-1* expression compared to heterospecific calls. Although we observed a similar pattern of *egr-1* expression in the magnocellular nucleus of the torus, this effect was not as robust as that of the laminar and principal nuclei. All thalamic nuclei we sampled showed greater *egr-1* expression in response to the conspecific stimuli than heterospecific calls or no sound. The anterior thalamus, in fact, showed the greatest response among the primary auditory regions that we measured. Finally, we found that although call category (conspecific or heterospecific) had a strong effect on *egr-1* expression, call exemplar did not (all $p > 0.24$). Thus, the auditory system appears highly sensitive to species-specific signals, and this effect does not appear to be driven by the specific acoustic traits of the individual calls used in the study.

Forebrain targets

The neural preference toward conspecific calls that we found in the auditory system was observed in all but one of the forebrain targets (Figs. 4.5, 4.6; Table 4), and brain regions varied substantially in the magnitude of their *egr-1* response to conspecific calls ($p = 0.01$). Whereas conspecific calls induced an increase in *egr-1* expression in the preoptic area, acoustic stimuli had no effect on *egr-1* expression in the ventral hypothalamus. The greater response toward conspecific calls was also evident in the dorsal part of the medial pallium (homolog of the hippocampus), as well as auditory targets in the subpallium, including the ventral striatum and ventral part of the lateral septum. Our results indicate that the neural preference to conspecific signals in the auditory system is present in limbic and motor areas in the forebrain that are likely important in modulating behavioral responses to

conspecific signals. Once again, call exemplar had no detectible influence on *egr-1* expression (all $p > 0.18$).

## Discussion

We assessed system wide responses to species-specific signals in female túngara frogs and found that neural preferences to conspecific mating calls emerged as early as the second synapse in the ascending auditory pathway, upstream of feature detectors in the midbrain. This neural preference was also observed in the auditory midbrain, thalamus, and limbic and motor targets in the forebrain, although the response was not uniform, indicating that different nuclei vary in the magnitude of their auditory preference toward conspecific calls. The heterospecific *P. enesefae* mating call was unable to elicit an *egr-1* response, in spite of the fact that the ears of túngara frogs are sensitive to the spectral content of these calls (Wilczynski et al., 2001) and that behavioral evidence in males confirms that túngara frogs perceive *P. enesefae* calls (Bernal et al., 2007). Thus, although *P. enesefae* calls must elicit electrical activity in the túngara frog auditory system, they apparently do not activate the second messenger cascades required for induction of *egr-1*. Furthermore, we found that call exemplar was a poor predictor of *egr-1* expression patterns, indicating that system wide neural preference to conspecific calls are sensitive to call category and are not driven by the distinctive acoustic traits of individual calls. We conclude that, although hierarchical feature detection undoubtedly plays a critical role in species recognition, sensory systems are generally selective toward processing species-specific signals. However, because the conspecific and heterospecific calls we used differ in a number of acoustic features that go beyond those that are sufficient for species recognition in behavioral tests (Wilczynski et al., 1995), from our data we cannot conclude that species recognition is a consequence of the neural preference we observed. Our results are reminiscent of studies showing that sensory

systems are more efficient at processing naturalistic stimuli (Hsu et al., 2004), even at the periphery (Reike et al., 1995).

A neural preference to species-specific signals has been previously demonstrated within the superior olivary nucleus in túngara frogs (Hoke et al., 2008), but it was unknown if such responses are also present within the dorsal medullary nucleus. Thus, it was unclear if the neural preference in the superior olivary nucleus was a product of intrinsic features or whether it was triggered by the dorsal medullary nucleus. We found that, although the dorsal medullary nucleus expresses *egr-1*, mating calls do not induce *egr-1* expression there, suggesting that the responses in the superior olivary nucleus is generated intrinsically. A neural preference in the superior olivary nucleus could be the product of its sensitivity to temporal features of mating calls or a consequence of modulation by descending inputs (e.g. from the torus semicircularis). Although future studies are necessary to understand the acoustic requirements of the conspecific call preference in the superior olivary nucleus and how it is generated, our study demonstrates that the auditory system has an early preference for processing conspecific calls.

The neural preference to conspecific mating calls that emerged in the superior olivary nucleus was also observed in the torus semicircularis where feature detectors that presumably lead to the representation of mating calls are first apparent. For example, some neurons in the torus are only responsive to two-tone combinations (Fuzessery and Feng, 1983), and others are sensitive to the number of pulses in a call (Edwards et al., 2002). Feng and Lin (1991) speculated that the principal nucleus is dedicated to spectral processing and the laminar nucleus is dedicated to temporal processing. Others have proposed that the laminar and magnocellular nuclei are sites of integration of auditory, motor, and motivational systems (Endepols and Walkowiak, 2001) in part because they receive descending projections from forebrain nuclei and, in turn, project to the spinal cord (Endepols and Walkowiak, 1999). Apparently consistent with this, a recent functional

mapping study proposed that the laminar nucleus serves as a “gatekeeper” that controls behavioral selectivity to mating calls (Hoke et al., 2008) (the magnocellular nucleus was not measured). However, we found a much more general neural preference to conspecific calls in the torus than did Hoke et al. (2008). In our study, both the laminar and principal nuclei responded preferentially to conspecific calls and the pattern of *egr-1* expression in the magnocellular nucleus was similar, suggesting that all parts of the torus respond to features of conspecific calls. Future studies are needed to determine to which acoustic features each subdivision is sensitive and whether any could be acting as a call-detector.

We found that all but one of the auditory targets in the diencephalon showed a neural preference toward conspecific mating calls. All three thalamic auditory targets responded preferentially to conspecific mating calls. The central and posterior thalamic nuclei show complex processing of spectral and temporal acoustic features that are characteristic of conspecific signals (Mudry et al., 1977; Hall and Feng, 1987; Mudry and Capranica, 1987). Our results suggest that this complex processing results in auditory discrimination between conspecific and heterospecific calls. The anterior thalamus, which is clearly multimodal (Roth et al., 2003; Laberge and Roth, 2007; Laberge et al., 2008), is the primary source of ascending sensory information to the pallium (Northcutt and Ronan, 1992; Roth et al., 2003; Laberge et al., 2008), but its role in auditory processing remains unclear. In spite of the fact that the anterior nucleus is multimodal, our results demonstrate that a unimodal acoustic stimulus is sufficient to stimulate it. In fact, the *egr-1* response in the anterior thalamus was the largest of any of the thalamic nuclei. The preoptic area and ventral hypothalamus are targets of the central thalamus (Neary and Wilczynski, 1986; Allison and Wilczynski, 1991), and both are excited by conspecific mating calls (Allison, 1992). The preoptic area plays an important role in the acoustically guided behavior characterizing mate choice in female anurans (Schmidt, 1984b, 1985b), and the conspecific call preference we found there might reflect the behavioral selectivity toward these calls. In

spite of the fact that the ventral hypothalamus also contains call-responsive neurons (Allison, 1992), mating calls failed to elicit an *egr-1* response there. This might reflect a distinct role for the ventral hypothalamus in responding to mating calls, or simply that *egr-1* expression there is not responsive to acoustic stimuli.

In our study, *egr-1* expression patterns in the telencephalon revealed robust neural preference to conspecific mating calls, although the contribution of the telencephalon to conspecific call recognition is unclear. Past studies have shown that the medial pallium is acoustically sensitive (Mudry and Capranica, 1980; Mangiamele and Burmeister, 2008), is the primary pallial target of the thalamus (Neary, 1990), and may direct sexual motivation and reward seeking behaviors through its connections to the amygdala and nucleus accumbens (Northcutt and Ronan, 1992; Westhoff and Roth, 2002). Although the medial pallium receives auditory input from the anterior thalamus, it also responds to other modalities (Laberge and Roth, 2007). Given the paucity of electrophysiological recordings from the medial pallium, its function in processing auditory stimuli is obscure. However, since the anuran medial pallium is homologous to the mammalian hippocampus, we surmise that auditory responses in the medial pallium play a mnemonic role during mate choice when females assess multiple males at a breeding aggregation before choosing a mate (Ryan, 1985).

Subpallial auditory targets also responded preferentially to conspecific mating calls. The ventral striatum receives auditory inputs from the central thalamus (Marín et al., 1997a; Endepols et al., 2004), sends descending connections to the torus to modulate auditory processing (Endepols and Walkowiak, 1999, 2001), and is important in directing motor responses during acoustically guided behaviors that are important during mate choice (Walkowiak et al., 1999). The lateral septum also receives auditory inputs from the thalamus (Roden et al., 2005) and plays an important role in directing behavioral responses to mating calls (Walkowiak et al., 1999). Although we could not account for movement in the present

study (but see Hoke et al., 2007), our results indicate that motor control areas in the telencephalon respond selectively to conspecific calls and that this neural preference might reflect the behavioral selectivity toward conspecific calls.

In summary, we found widespread neural preference in the auditory system toward species-specific stimuli. Although many studies investigating the processing of species-specific signals have focused on the telencephalon (e.g., Grace et al., 2003; Petkov et al., 2008), our results suggest that at least some of these responses could be understood in terms of the inputs to these brain regions, rather than by responses that are intrinsically generated. In support of this, a recent study found that, in a songbird, a neural preference toward conspecific signals occurs in the midbrain (Poirier et al., 2009), well before the auditory forebrain regions that had been previously identified as having a preference for conspecific signals (Grace et al., 2003; Hauber et al., 2007). In anurans, the acoustic requirements for this auditory preference remain unclear, but our results suggest that the anuran auditory system is designed to respond preferentially to conspecific mating calls. Future studies are needed to understand how these auditory responses contribute to discrimination of complex biological stimuli underlying species recognition and mate choice.

**Acknowledgements**

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| Abbreviations | |
|---|---|
| Acc | Nucleus accumbens |
| AP | Amphibian papilla |
| Athal | Anterior thalamus |
| BP | Basilar papilla |
| Cthal | Central thalamus |
| DMN | Dorsal medullary nucleus |
| dMP | Dorsal medial pallium |
| DP | Dorsal pallium |
| dSt | Dorsal striatum |
| La | Lateral thalamus |
| LH | Lateral hypothalamus |
| LP | Lateral pallium |
| Ltor | Laminar nucleus of torus semicircularis |
| MCtor | Magnocellular nucleus of torus semicircularis |
| MP | Medial pallium |
| OT | Optic tectum |
| POA | Preoptic area |
| Pthal | Posterior thalamus |
| Ptor | Principal nucleus of torus semicircularis |
| SCN | Suprachiasmatic nucleus |
| Sep | Septum |
| Sd | Dorsal septal nucleus |
| Sl | Lateral septum |

| | |
|---|---|
| Sld | Dorsal part of lateral septum |
| Slv | Ventral part of lateral septum |
| Sm | Medial septal nucleus |
| SON | Superior olivary nucleus |
| St | Striatum |
| Teg | Tegmentum |
| Tel | Telencephalon |
| VH | Ventral hypothalamus |
| VL | Ventrolateral thalamus |
| VM | Ventromedial thalamus |
| vMP | Ventral medial pallium |
| VP | Ventral pallium |
| vSt | Ventral striatum |

Table 4.

Effects of acoustic stimuli on *egr-1* expression. Linear mixed models showing treatment contrasts between conspecific and heterospecific mating calls, and between conspecific mating calls and no sound; p values that were 0.05 or less are shown in bold.

| **Brain region** | **conspecific vs. heterospecific** | **conspecific vs. no sound** |
|---|---|---|
| DMN | p = 0.75 | p = 0.67 |
| SON | **p = 0.03** | p = 0.11 |
| Ptor | **p = 0.01** | p = 0.07 |
| Ltor | **p < 0.001** | **p = 0.03** |
| MCtor | p = 0.13 | p = 0.09 |
| Pthal | **p < 0.001** | **p = 0.003** |
| Cthal | **p = 0.01** | **p = 0.02** |
| Athal | **p = 0.02** | **p = 0.02** |
| POA | **p = 0.01** | **p = 0.02** |
| VH | p = 0.46 | p = 0.60 |
| St | **p = 0.01** | **p = 0.05** |
| Slv | **p = 0.01** | **p = 0.01** |
| dMP | **p = 0.01** | **p = 0.01** |

Figure Legends

Figure 4.1. Sonograms of the call exemplars presented to females. A. Conspecific *Physalaemus pustulosus* whines with one chuck. B. Heterospecific *Physalaemus enesefae* whines.

Figure 4.2. Schematic diagram of the ascending auditory system and its primary forebrain auditory targets.

Figure 4.3. Effect of acoustic treatment on *egr-1* expression in the auditory hindbrain, midbrain, and thalamus. Data are shown as mean (± SE) fold change in silver grains per cell relative to the no sound group. Sample sizes are indicated for each treatment group and horizontal lines with asterisks indicate groups that are statistically different at $p < 0.05$.

Figure 4.4. Brightfield images (left column) and inverted darkfield images of transverse sections showing *egr-1* mRNA levels within sampling windows (boxes) in response to conspecific (middle column) and heterospecific (right column) mating calls in the torus semicircularis (A - C), posterior thalamus (D - F), central thalamus (G – I), and anterior thalamus (J – L). Scale bar represents 400 μm.

Figure 4.5. Effect of acoustic treatment on *egr-1* expression in the primary forebrain targets of the ascending auditory system. Data are shown as mean (± SE) fold change in silver grains per cell relative to the no sound group. Sample sizes are indicated for each treatment group and horizontal lines with asterisks indicate groups that are statistically different at $p < 0.05$.

Figure 4.6. Brightfield images (left column) and inverted darkfield images of transverse sections showing *egr-1* mRNA levels within sampling windows (boxes) in response to conspecific (middle column) and heterospecific (right column) mating calls in the ventral part of lateral septum (A – C), ventral striatum (D – F), and the dorsal medial pallium (G – I). Scale bar represents 400 μm.

Figure 4.1

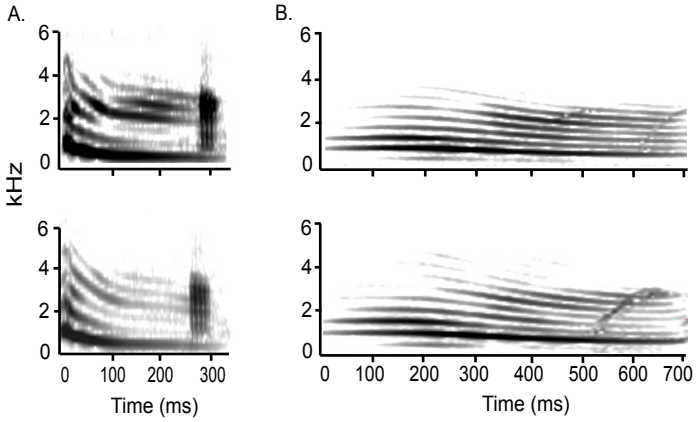


Figure 4.2

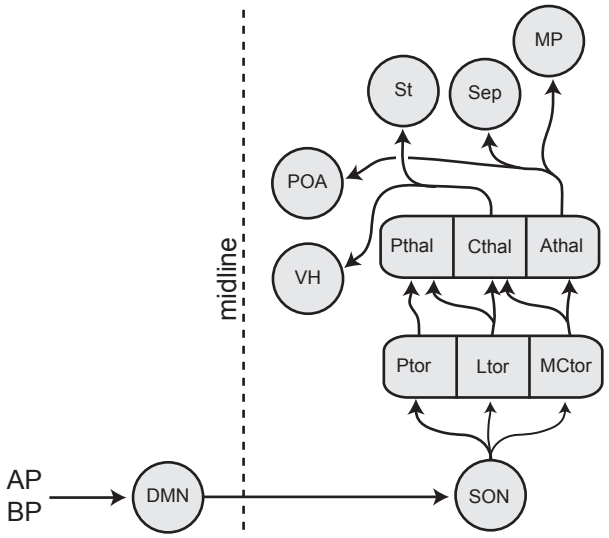

MP
St
Sep
POA
VH
midline
Pthal
Cthal
Athal
Ptor
Ltor
MCtor
AP
BP
DMN
SON

Figure 4.3

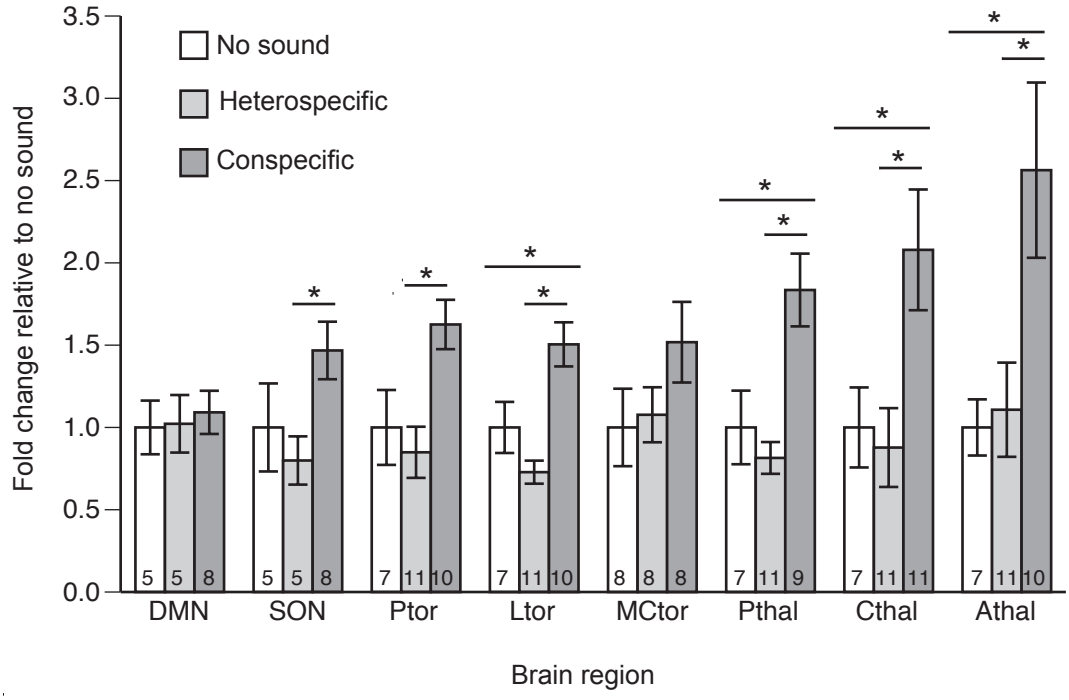

No sound
Heterospecific
Conspecific
Fold change relative to no sound
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
5 5 8
5 5 8
7 11 10
7 11 10
8 8 8
7 11 9
7 11 11
7 11 10
DMN
SON
Ptor
Ltor
MCtor
Pthal
Cthal
Athal
Brain region

Figure 4.4

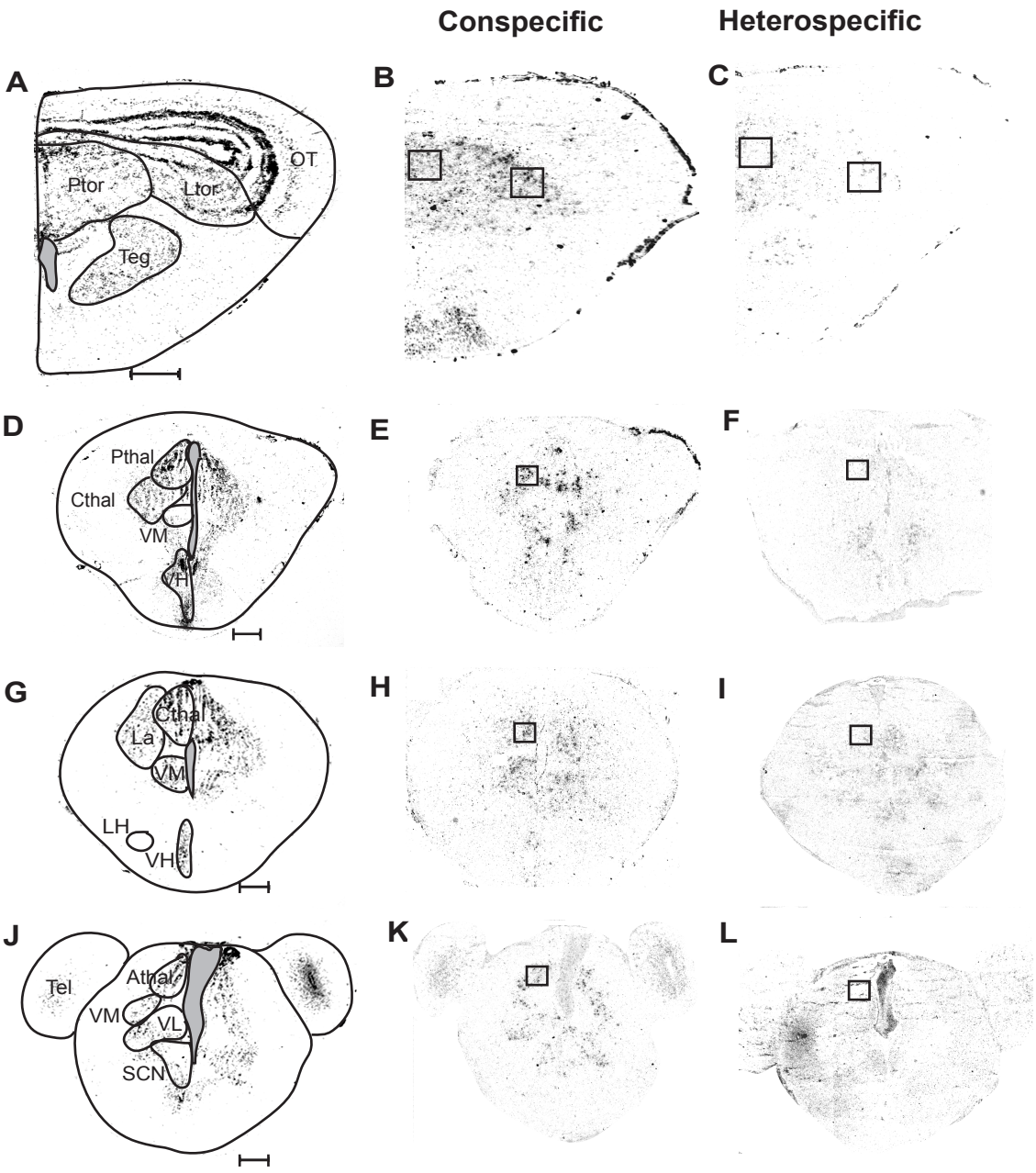
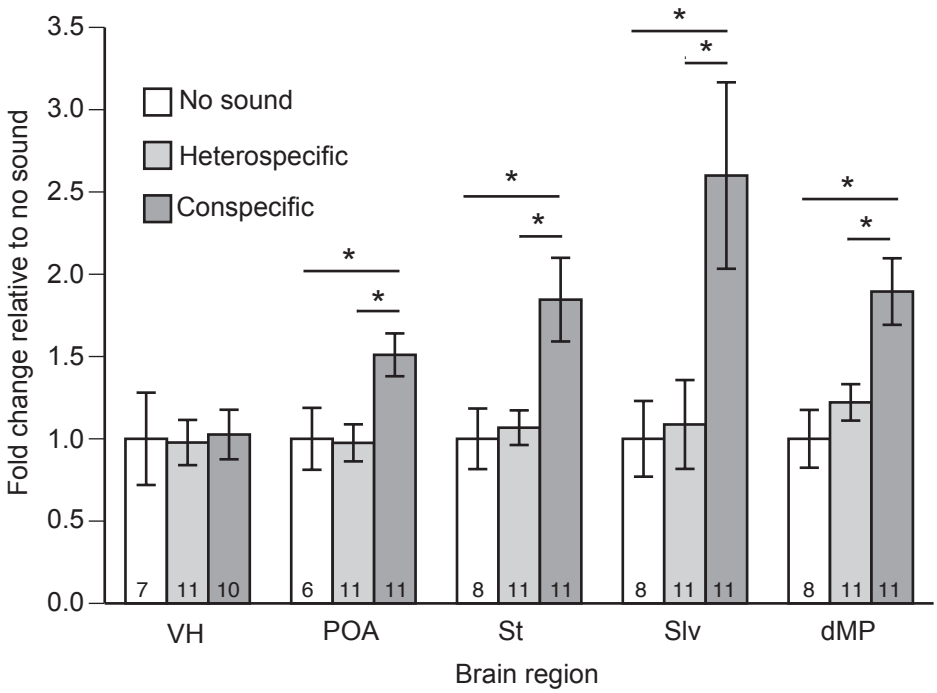

Conspecific
Heterospecific
A
B
C
OT
Ptor
Ltor
Teg
D
E
F
Pthal
Cthal
VM
VH
G
H
I
Cthal
La
VM
LH
VH
J
K
L
Tel
Athal
VM
VL
SCN

Figure 4.5


3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Fold change relative to no sound
No sound
Heterospecific
Conspecific
*
*
*
*
*
*
*
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7
11
10
6
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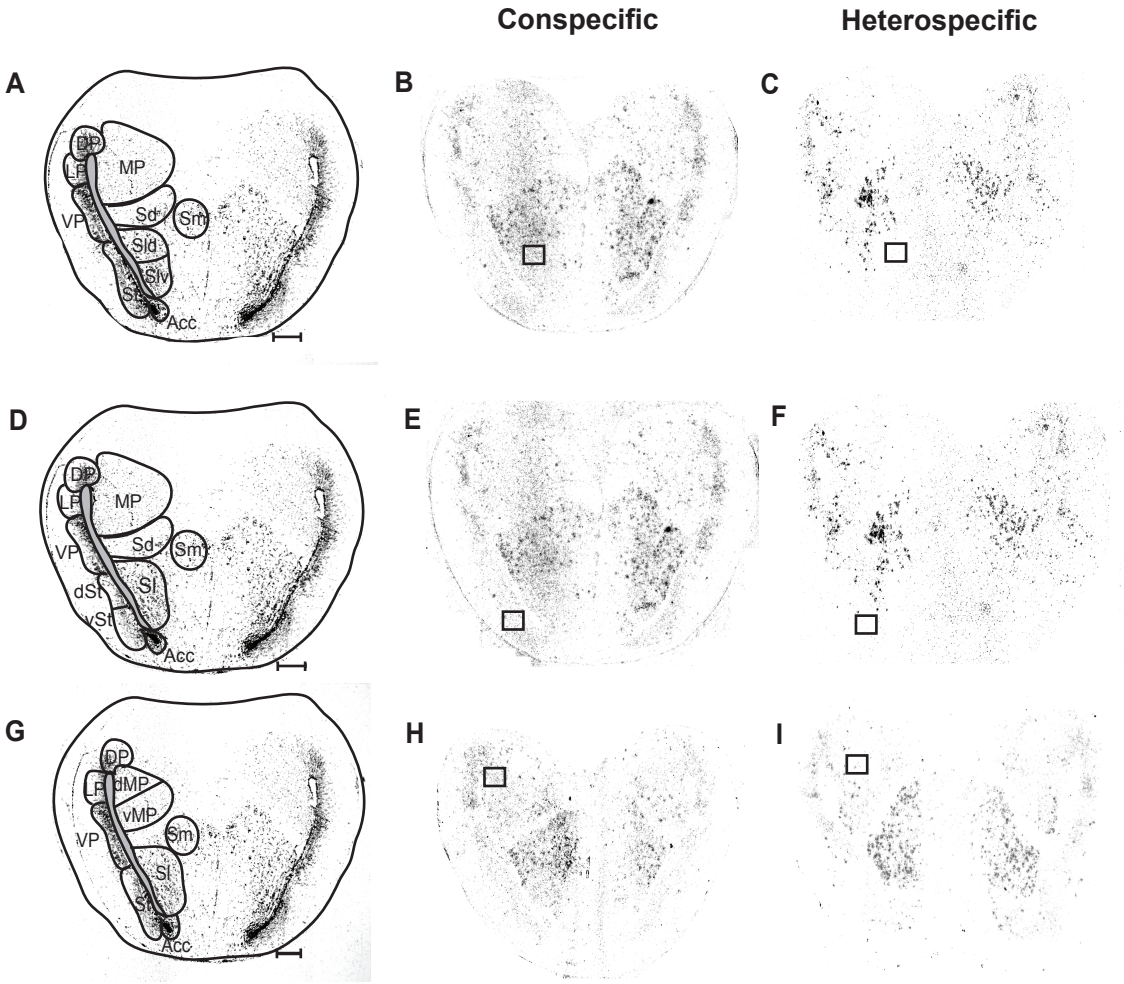
11
8
11
11
8
11
11
8
11
11
VH
POA
St
Slv
dMP
Brain region

Figure 4.6


Conspecific
Heterospecific
A
B
C
D
E
F
G
H
I
DP
LP
MP
VP
Sd
Sm
Sld
Slv
Sl
Acc
dSt
vSt
dMP
vMP

# CHAPTER 5

## ESTRADIOL ENHANCES NEURAL RESPONSES TO CONSPECIFIC SIGNALS IN TÚNGARA FROGS


### Summary

Estradiol is a potent activator of sexual behavior and is known to impact a wide variety of physiological processes, including sex differentiation, cognition, and sensory processing. Auditory processing of communication signals is fundamental for locating mates in anurans. In túngara frogs (*Physalaemus pustulosus*), estradiol is sufficient to induce behavioral responses to species-specific over heterospecific signals. In addition, parts of the ascending auditory system including the midbrain torus semicircularis, thalamus, and the forebrain limbic and motor regions are biased to respond to conspecific over heterospecific calls. The torus semicircularis, parts of the thalamus, and motor and limbic regions in the forebrain express estrogen receptors (ERs) indicating that they are targets of estrogen action. We predicted that estradiol enhances response to conspecific signals by modulating neural activity within the auditory, motor, and motivational areas that are important in sexual communication. We injected females with estradiol or fadrozole (an aromatase inhibitor) and exposed them to a conspecific, or heterospecific stimuli. We measured expression of the immediate early gene *egr-1* as a marker of neural activity in parts of the ascending auditory system including the torus semicircularis, thalamus, as well as the primary forebrain targets such as the preoptic area, striatum, lateral septum, and dorsal medial pallium. We also measured *egr-1* expression within the nucleus accumbens, an area thought to be involved

in modulating motivation and goal-directed behaviors. With four exceptions (three thalamic regions and the dorsal medial pallium), all auditory nuclei and the nucleus accumbens showed greater neural responses to species-specific over heterospecific calls in estradiol-injected females. Further analyses of these regions revealed that both estradiol and conspecific calls together induced greater neural responses than either alone, suggesting an additive effect on *egr-1* induction. We conclude that estradiol enhances neural responses to conspecific signals within brain regions that are important in sexual communication in túngara frogs.

**Introduction**

Steroid hormones are important regulators of sexual behavior and may influence female mate choice decisions by modulating sensory and motor systems. Studies indicate that the hormone estradiol plays a complex suite of roles in numerous cellular effects in vertebrates. Its effects vary from cognition, synaptic plasticity, to neuroprotection (see reviews by Björnström and Sjöberg, 2005; Edwards, 2005; Lösel et al., 2003; Parducz et al., 2006). Estradiol affects perception of sexual signals, by acting through the auditory, visual, or olfactory systems (Lacreuse and Herndon, 2003; Maney et al., 2006; Penton-Voak et al., 1999; Sisneros et al., 2004; Tremere et al., 2009; Walpurger et al., 2004). For example, steroid-dependent plasticity for species recognition is well established within the peripheral auditory system in the vocal plainfin midshipman fish (*Porichthys notatus*) where breeding females with high plasma hormone levels are known to detect higher harmonic components of courting males compared to the non-breeding season when their hormone levels are low (Sisneros et al., 2004). In female white-throated sparrows, the expression of the immediate early gene, *egr-1* in the auditory system is selective for song only when plasma estradiol levels exceed non-breeding levels (Maney et al., 2006). Thus, it appears that steroid

hormones influence sensory processing in females, thereby affect female behavioral responses to sexual stimuli. However, the mechanisms underlying such effects are largely unknown, as are the target sites in the brain in which potential interactions between sensory and endocrine systems occur.

Anurans provide a suitable model for studying the effects of hormones on auditory processing because reproductive behaviors rely on the female's ability to receive, process, and discriminate male vocalizations based on their acoustic properties (Gerhardt, 1988; Gerhardt and Huber, 2002; Ryan, 1985). Female sexual behavior can be expressed as movement towards species-specific calls (phonotaxis) (Gerhardt and Huber, 2002), by producing vocalizations to signal sexual receptivity (Shen et al., 2008; Tobias et al., 1998), or as the inhibition of release calls that are typically observed in unreceptive females (Diakow and Nemiroff, 1981; Kelley, 1982). As in other vertebrates, female receptivity to species-specific signals coincides with increases in plasma hormone levels (Chakraborty and Burmeister, 2009; Lynch and Wilczynski, 2005; Lynch et al., 2005). We investigated how steroid hormones influence sensory processing of species-specific signals in the túngara frog (*Physalaemus pustulosus*). Female behavioral responses to conspecific signals, are well established in túngara frogs (Ryan, 1985). Females exhibit strong preferences for conspecific over heterospecific calls (Ryan and Rand, 1995; Ryan et al., 2007). Estradiol injections are sufficient to induce phonotaxis towards species-specific signals (Chakraborty and Burmeister, 2009), and exposure to species-specific mating signals elevates plasma estradiol levels in females (Lynch and Wilczynski, 2006). Injections of human chorionic gonadotropins (HCG) effectively increase sexual behavior in females (Lynch et al., 2006) and modifies neural responses to mating signals within the laminar nucleus of the auditory torus semicircularis (Lynch and Wilczynski, 2008). Furthermore, exposure to species-specific signals induces robust expression of *egr-1* within parts of the central auditory system, and forebrain motor, limbic, and motivational areas that receive

auditory inputs (see Chapter 4). Most nuclei of the central auditory system, and motor and motivational areas in the forebrain express estrogen receptors (ERs) (see Chapter 3). This raises the possibility that steroid hormones enhance neural sensitivity to conspecific signals by priming sensory systems. Because estradiol clearly induces female sexual receptivity to species-specific signals, we hypothesized that estradiol may enhance sensory processing of species-specific signals by modulating call-induced *egr-1* expression within the central auditory system.

We presented females injected with estradiol or fadrozole (an aromatase inhibitor that blocks estrogen synthesis) with a conspecific call or a heterospecific call of an allopatric congener, *P. enesefae* and assessed neural activity using expression of *egr-1*. We quantified *egr-1* mRNA levels in sensory, motor, and motivational systems that are important in sexual communication including the midbrain torus semicircularis, parts of the primary thalamic and forebrain auditory targets, and the nucleus accumbens, an area thought to be important for motivation and goal-directed behaviors. We predicted that estradiol treatment would enhance *egr-1* responses to conspecific calls within parts of the central auditory system and in motor, limbic, and motivational areas that are important in modulating behavioral responses to conspecific calls. We found that estradiol treatment evoked greater *egr-1* response to species-specific over heterospecific signals within the midbrain torus semicircularis, but not within the thalamic auditory targets. This robust response was also observed in parts of the forebrain limbic, and motor targets, and within the nucleus accumbens. Further analyses of these regions revealed that both estradiol and conspecific calls together induced greater neural responses than either alone, suggesting an additive effect on *egr-1* induction. Thus, we conclude that estradiol plays a pivotal role in enhancing sensory processing of species-specific signals by modulating call-evoked *egr-1* expression within the auditory system.

## Materials and Methods

Frog collection and hormone treatment

We used adult female túngara frogs from a laboratory stock maintained at the University of North Carolina, which were originally derived from natural populations near Rio Píro on the Osa Peninsula in Costa Rica. Females were housed in 10-liter terrariums with substrate containing damp soil, and maintained under ambient conditions (light: approximately 12 h light and 12 h dark; temperature: approximately 28 °C). We sprayed females with water daily, and fed them fruit flies thrice a week. In order to ensure that all females were in the same hormonal state we injected each female with human chorionic gonadotropin (500 IU per g of body mass), which is known to induce breeding in this species (Lynch, 2005) and paired them with a male in a 3-liter terrarium. We provided the pairs with a water dish and allowed them to make nests overnight. We isolated the females that had made nests in a separate terrarium. We collected 48 females that had made nests over a period of 7 days for further hormone manipulations. Ten days after the females had made nests, we injected them with 50 μl of saline (n = 24) or fadrozole (50 μg per frog; n = 24). After 24 h we injected the saline-injected females with estradiol (0.07 μg per g body mass), and the fadrozole-injected animals with a second dose of fadrozole. Six hours after the second injections we placed females in individual mesh cages of one of eight acoustic chambers. We placed two paired females, one from each hormone treatment in individual mesh cages placed next to each other inside each acoustic chamber, and equidistant from the speaker. Each of the acoustic chambers was equipped with an audio laboratory speaker (Misco/Minneapolis Speaker Company, Minneapolis, MN) that was connected to an AMP Five series monoblock amplifier (Audiosource, Portland, OR) and a Macintosh computer. After a 18-h acclimation period, we presented females with a single exemplar of a conspecific *P. pustulosus* call (n = 24) or a heterospecific *P. enesefae* call (n = 24). We

interspersed females assigned to each acoustic treatment across days and chambers. We rapidly decapitated females 1 h after onset of stimuli, which corresponds to peak accumulation of acoustically induced *egr-1* mRNA expression (Burmeister et al., 2008). After decapitation, we opened the skull in order to fix the brains (10 min in 4% paraformaldehyde) before removing them. We froze the brains in liquid nitrogen in 2 ml tubes containing Tissue-Tek OCT Compound (Sakura, Finetek, Torrance, CA). We stored the brains at – 80° C until further processing. The University of North Carolina Institutional Animal Care and Use Committee (IACUC) approved our experimental procedures and permitted tissue collection.

Acoustic stimuli

The species-specific calls of *P. pustulosus* and *P. enesefae* are both characterized by a downward frequency sweep referred to as a whine (see Fig. 1.3). The *P. enesefae* whine begins at about 1060 Hz and descends to 590 Hz in approximately 720 ms, with a dominant frequency of 900 Hz (Tárano, 2001). The *P. pustulosus* whine begins at about 1000 Hz and sweeps to 400 Hz in about 350 ms, with a dominant frequency of 900 Hz (Ryan, 1985). Although the whine alone is sufficient for species recognition, *P. pustulosus* can enhance the attractiveness of their call by adding a second component referred to as a chuck. The chuck is a short (40 ms) burst of sound with rich harmonic structure that emphasizes frequencies greater than 1500 Hz. We recorded the *P. pustulosus* calls from breeding populations on the Osa Peninsula, while the *P. enesefae* calls were recorded by Dr. Zaida Tárano in Venezuela. We presented each female with a single call repeated every 2 seconds to reflect the calling rate of *P. pustulosus,* for 30 minutes. We played the calls using ProTools audio software (V. 7.3; Digidesign, Daly City, CA) from a Macintosh PowerBook G4. We set the peak amplitude for calls at 82 dB SPL at a distance of ~ 20 cm from the speaker.

Radioactive *in situ* hybridization

We sectioned brains in the transverse plane at 16 µm in 3 series on a cryostat. To localize *egr-1* mRNA, we used radioactive *in situ* procedures previously described in Burmeister et al. (2008). Briefly, we generated radioactively labeled sense and antisense probes from reverse transcription of a 309 nt subclone of *P. pustulosus egr-1*. Before hybridization, we fixed the tissue for 10 sec in 4% paraformaldehyde before washing in phosphate-buffered saline, triethanolamine, acetic anhydride, 2× SSC, and a series of ethanols. We hybridized the tissue with 90 µl of $3.0 \times 10^5$ cpm/ml of hybridization buffer at 65° C overnight. We removed unbound probe with a series of 65° C washes, first in 50% formamide and 2× SSC (1.25 h) followed by two washes in 0.1× SSC (30 min each). We visualized the bound riboprobe as silver grains by exposing the slides to NTB emulsion diluted 1:1 in distilled water for 14 days at 4° C, and we visualized the cell bodies by staining the tissue with thionin. Tissue incubated with the sense probe showed no significant binding above background.

Quantitative measurement of *egr-1* expression

We assessed neural activity in parts of the ascending auditory system, its primary forebrain targets, and the nucleus accumbens. Most parts of the ascending auditory system express ERs (see Chapter 3 for a detailed description of ERs). The ascending auditory system of anurans includes two hindbrain regions (the dorsal medullary and the superior olivary nuclei), the midbrain torus semicircularis (homolog of the mammalian inferior colliculus) and its thalamic targets, the posterior, central, and anterior thalamic nuclei. The dorsal medullary and the superior olivary nuclei do not express ERs. Although the inputs to the posterior and central thalamic nuclei are primarily auditory, the anterior thalamus also receives somatosensory and visual inputs. Estrogen receptors are present within the anterior and central thalamic nuclei, but absent within the posterior thalamus (see Chapter

3). From the thalamus, auditory pathways converge onto limbic (medial pallium, septum, preoptic area) and motor regions (striatum) of the forebrain all of which express ERs. In addition, we also measured expression of *egr-1* within the nucleus accumbens, an area that expresses ERs (see Chapter 3), and is thought to modulate female motivational state and goal-directed behaviors (Laberge and Roth, 2007; Marín et al., 1997a; Marín et al., 1997b). Because we were interested in localizing effects of estradiol in areas that are known to be important in sexual communication in anurans (see Chapter 4), we measured *egr-1* expression at each of these levels that also express ERs.

For each brain region, we calculated an individual's mean from three photomicrographs captured at a magnification of 630× from one hemisphere of the brain that best represented the respective brain region morphologically. The quality of the brain sections influenced the sample sizes reported for each brain region as we were unable to obtain data from all individuals in the study for all brain regions sampled. We assessed levels of *egr-1* expression using the methods described in Burmeister et al. (2008). Briefly, we used Image J (http:// rsbweb.nih.gov/ij/) to quantify silver grain number in the region of interest and in a nearby area of the slide that represented the background silver grain levels for that position on the slide. We manually counted the number of cell bodies in the region of interest from separate photomicrographs. We express relative *egr-1* expression, therefore, as the number of silver grains per cell above background.

Statistical analyses

We conducted linear mixed models for each brain region using the "lme" function in R (R Foundation for Statistical Computing, Vienna, Austria). First, in each brain region we tested for the main effect of call category and drug (estradiol or fadrozole) as fixed effects with subject as a random effect. Second, we performed separate analyses to test for an interaction between drug and call category using linear mixed models.

## Results

Torus semicircularis and thalamus

Estradiol treatment and conspecific call together elicited robust *egr-1* expression in the torus semicircularis, preoptic area, ventral part of lateral septum, striatum, and in the nucleus accumbens (Fig. 5.1-5.4; Table 5), indicating a role for estradiol in augmenting *egr-1* expression in brain regions that are important in sexual communication. We did not find any interaction between drug and call category in any of the brain regions sampled.

Both call and estradiol alone elicited increases in *egr-1* expression in the laminar and principal nuclei indicating an additive effect of hormone and species-specific signals in enhancing *egr-1* expression (Fig. 5.1; Table 5). In contrast to the toral nuclei, we found that estradiol did not have a strong effect on *egr-1* induction within any of the thalamic nuclei (Fig. 5.2; Table 5). However, conspecific stimulus alone was able to evoke greater responses than the heterospecific call within the central and anterior, but not in the posterior thalamic nuclei (Fig. 5.2; Table 5). Finally, we found no interaction between drug and call category in the toral or thalamic nuclei (Table 5). Thus, it appears that estradiol and conspecific call together produce an additive effect within the torus semicircularis to modulate *egr-1* expression.

Forebrain auditory targets and nucleus accumbens

Estradiol injections augmented *egr-1* responses within the preoptic area, ventral striatum, and ventral part of lateral septum, but not within the dorsal medial pallium (Fig. 5.3A-D; Table 5). In addition, we found that estradiol enhanced *egr-1* expression in the nucleus accumbens (Fig. 5.4; Table 5). Our results show that estradiol enhances *egr-1* responses within the limbic (preoptic area, septum), motor (striatum), and motivational (nucleus accumbens) areas in the forebrain that are thought to be important in modulating

behavioral responses to conspecific signals. With one exception (dorsal medial pallium), all other telencephalic auditory targets, and the nucleus accumbens showed similar patterns of *egr-1* expression in response to estradiol treatment to those observed within the toral nuclei. Finally, we found no significant interaction between drug and call category in any of the brain regions sampled (Table 5). This indicates that exposure to estradiol and conspecific call produce an additive effect on *egr-1* expression, so that together they induce greater *egr-1* expression than each alone. However, there was a trend for an interaction between drug and call category (Table 5) within the preoptic area ($p = 0.07$) and the ventral lateral septum ($p = 0.07$). Overall, our results suggest an important role for estradiol in modulating behavioral responses to species-specific mating calls in túngara frogs, which is presumably mediated through its effects on the auditory midbrain and forebrain limbic, motor, and motivational centers.

**Discussion**

We assessed the effects of estradiol treatment on neural responses to species-specific calls in female túngara frogs and found that estradiol augments *egr-1* responses within the auditory torus semicircularis and in the forebrain limbic, motor, and motivational areas. Furthermore, this enhanced neural response extends to the forebrain limbic, motor, and motivational pathways that are thought to modulate female behavioral responses in anurans. This suggests that the effects of estradiol are widespread across regions that are important in sexual communication in anurans. Our results show that estradiol and conspecific call together produce an additive effect within the torus semicircularis and its forebrain auditory targets so that together they induce greater *egr-1* expression than either alone. Furthermore, call alone induces *egr-1* expression irrespective of hormone treatment suggesting that neural preference for species-specific signals is present independent of

hormonal status of the female. It appears that some degree of sensitivity to conspecific over heterospecific calls remain when estradiol levels are low. This might indicate intrinsic neural biases that are possibly mediated through steroid-independent mechanisms or through other sex steroid hormone, such as progesterone. The torus semicircularis sends efferents to thalamic nuclei and parts of the subpallial telencephalon. It is also thought to be an important site of integration of auditory, motor, and motivational systems (Endepols and Walkowiak, 2001) because inputs from the forebrain nuclei converge here and they project to the spinal cord (Endepols and Walkowiak, 1999). The torus semicircularis, and its thalamic and forebrain auditory targets also express ERs indicating that estradiol could act at these target sites locally to modulate *egr-1* expression. At present, it is unclear if estradiol modification of neural responses at each of these regions occurs locally or whether inputs from the torus semicircularis alter sensory processing. Nevertheless, alteration of estradiol-dependent sensory processing may alter behavioral responses in females. Although we cannot conclude that the call-evoked *egr-1* responses observed in this study represent species recognition in túngara frogs (also see Chapter 4), our results indicate that estradiol is a potent regulator mediating neural plasticity that may underlie female behavioral preferences to conspecific signals during mate choice.

Our results parallel a number of studies from other taxa that describe an important role for estradiol in sensory processing of mating signals and of audition, generally. The role of estradiol in auditory processing has been discovered in a wide variety of species including humans, and it appears that anurans are no exception. For example, hearing thresholds and auditory event-related potentials correlate with plasma estradiol levels during the menstrual cycle in humans (Davis and Ahroon, 1982; Walpurger et al., 2004). In birds and anurans, the degree of activity-dependent gene expression that a mating signal induces within the auditory system likely reflects the behavioral relevance of that signal (Hoke et al., 2008; Hoke et al., 2004; Mello et al., 2004). For example, estradiol modulates song-induced ZENK

response in the auditory forebrain (Maney et al., 2006), and socially relevant auditory experience increases estradiol levels in the auditory forebrain in songbirds (Remage-Healey et al., 2008). Attractive species-typical signals evoke greater induction of *egr-1* in auditory areas compared to less attractive signals (Leitner et al., 2005; Sockman et al., 2002). Moreover, ZENK expression tends to be higher in individuals exposed to conspecific songs than in those exposed to heterospecific songs (Mello and Clayton, 1994; Mello et al., 1992). A recent study by Tremere et al. (2009) shows that estradiol is both necessary and sufficient to induce the expression of multiple mitogen-activated protein kinase (MAPK) genes such as ZENK, c-fos, and Arc that are thought to be necessary for synaptic plasticity. In female túngara frogs, gonadotropins increase neural responses to conspecific mating choruses within the laminar nucleus (Lynch and Wilczynski, 2008). Our findings extend those results to show that estradiol injections alone augment *egr-1* expression in the auditory midbrain and in motor, limbic and motivational areas that presumably enhance behavioral preference to species-specific mating signals. It is possible that estradiol modulates other plasticity-associated genes such as Arc and c-fos to modulate synaptic plasticity in túngara frogs. It appears that steroid-dependent behavioral responses could arise through auditory plasticity associated with the endocrine status of the animal.

We found that estradiol did not augment *egr-1* responses within the thalamic nuclei but species-specific signals alone evoked greater *egr-1* responses within the central and anterior thalamic nuclei. The central thalamic nucleus shows complex processing of spectral acoustic features that are characteristic of conspecific signals (Hall and Feng, 1987; Mudry and Capranica, 1987; Mudry et al., 1977) and exposure to conspecific signals elicits greater *egr-1* responses than heterospecific signals (see Chapter 4). Likewise, although the anterior thalamus is clearly multimodal (Laberge and Roth, 2007; Laberge et al., 2008; Roth et al., 2003), responses to conspecific signals are also evident (see Chapter 4) which suggests that thalamic nuclei respond preferentially to species-specific calls. Although we did not

observe an effect of estradiol in influencing *egr-1* responses within the thalamus, thalamic nuclei nevertheless express ERs in túngara brains (see Chapter 3) and are therefore possible targets of estrogen action.

We found robust neural responses after estradiol treatment in the preoptic area. The preoptic area expresses both *ER*$\alpha$ and *ER*$\beta$ receptor mRNA in reproductively active female túngara frogs indicating that preoptic neurons are sites of estrogen action. Furthermore, the preoptic area is a target of the central thalamus (Allison and Wilczynski, 1991; Neary and Wilczynski, 1986), is acoustically sensitive (Allison, 1992), and plays an important role in acoustically guided behaviors (phonotaxis) that are characteristic of female mate choice in anurans (Schmidt, 1984a; Schmidt, 1985; Walkowiak et al., 1999). Functional mapping studies have demonstrated that preoptic neurons respond selectively to species-specific over heterospecific calls (Chapter 4), which might reflect the behavioral bias toward these calls. Because estradiol injections alone induce this behavioral bias in phonotaxis tests (Chakraborty and Burmeister, 2009), it appears that preoptic neurons are targets of estrogenic modulation when females are actively choosing mates.

With one exception, estradiol treatment induced robust neural responses in the striatum, ventral part of lateral septum, and in the nucleus accumbens. The ventral striatum expresses ERs, receives auditory inputs from the central thalamus (Endepols et al., 2004; Marín et al., 1997a), and sends descending connections to the torus semicircularis to modulate auditory processing. The striatum is also involved in modulating motor responses to mating calls in many anuran species such as gray treefrogs (*Hyla versicolor*) (Walkowiak et al., 1999), and is therefore considered to be a part of the “audiomotor” interface in anurans (Wilczynski and Endepols, 2007). Likewise, the lateral septum is a clear target of the thalamus (Roden et al., 2005) and modulates female behavioral responses to conspecific mating calls (Walkowiak et al., 1999). Although little is currently known about the functional aspects of the anatomical connections of the nucleus accumbens, it is known to

receive significant projections from the ventral thalamus and dopaminergic connections from the posterior tubercle (Marín et al., 1997b), and is thought to be involved in regulating goal-directed behaviors. Efferent fibers of the nucleus accumbens project to the medial amygdala, preoptic area, and ventral hypothalamus (Marín et al., 1997a), indicating that the nucleus accumbens may influence the endocrine aspects of limbic functions in anurans. Because the lateral septum, striatum, and nucleus accumbens express estrogen receptors in túngara frogs they serve as prime targets for estradiol modulation. Thus, it appears that the subpallial auditory targets, the nucleus accumbens, preoptic area, and the torus semicircularis are part of an "auditory social behavior network" in anurans where steroid-dependent neural plasticity emerges to modulate behavioral plasticity towards species-specific signals.

In summary, we found widespread effects of estradiol and conspecific calls on *egr-1* expression in túngara frogs. Our results do not demonstrate that estradiol induces female sensory discrimination for mate recognition, but elucidate a pivotal role for estradiol as an important modulator of immediate early gene expression in anurans. The role of immediate early genes as important effector molecules for synaptic plasticity (see review by Mello et al., 2004) and long-term potentiation is well established (Abraham et al., 1991), and may have important implications for mate choice which has been proposed to be a complex cognitive task by Ryan et al. (2009) requiring working memory as shown in túngara frogs (Akre and Ryan, 2010). As proposed by Maney et al. (2006), estradiol-dependent modulation of immediate early gene expression may help to strengthen synaptic connections during the breeding season in brain regions that are particularly relevant for mate recognition and discrimination. In fact, estradiol has been shown to influence auditory processing through rapid changes in neuronal excitability and modulation of plasticity-associated genes such as ZENK, c-fos and Arc in birds (Tremere et al., 2009). Therefore, it is possible that estradiol may influence synaptic plasticity in anurans through similar

mechanisms. Previous studies in songbirds and fish had identified a role for estradiol in auditory processing of social signals (Maney et al., 2006; Maney et al., 2008; Sisneros et al., 2004). However, very little is known about brain regions that may serve as targets of hormonal modulation for conspecific signal processing in anurans (but see Lynch and Wilczynski, 2008). In the present study, we looked for evidence that neural responses to species-specific signals in females may be enhanced by the steroid hormone estradiol, which is known to induce female sexual responses to conspecific calls in túngara frogs (Chakraborty and Burmeister, 2009). The emergence of a modulatory role of estradiol in enhancing *egr-1* expression in what appears to be an "auditory social behavior network" containing sensory, motor and motivational areas in anurans invites further studies on how steroid-dependent neural plasticity may influence mate recognition systems critical for mate choice and speciation.

**Acknowledgements**

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Table 5.

Linear mixed models showing effects of drug treatment, acoustic stimuli, and their two-way interactions on *egr-1* expression; p values that were 0.05 or less are shown in bold.

| Brain region | Drug | Call stimuli | Drug x Call stimuli |
|---|---|---|---|
| Ptor | **p = 0.01** | **p = 0.02** | p = 0.18 |
| Ltor | **p < 0.01** | **p < 0.01** | p = 0.47 |
| Pthal | p = 0.88 | p = 0.12 | p = 0.41 |
| Cthal | p = 0.14 | **p < 0.01** | p = 0.48 |
| Athal | p = 0.72 | **p < 0.01** | p = 0.39 |
| POA | **p = 0.01** | **p < 0.01** | p = 0.07 |
| St | **p = 0.03** | **p = 0.05** | p = 0.36 |
| Slv | **p = 0.02** | **p = 0.01** | p = 0.07 |
| dMP | p = 0.17 | p = 0.26 | p = 0.19 |
| Acc | p = 0.06 | **p = 0.03** | p = 0.18 |

Figure Legends

Figure 5.1. Effect of acoustic and drug treatment on *egr-1* expression in the laminar and principal nuclei of the torus semicircularis. Data are shown as mean (± SE) silver grains/cell. Sample sizes are indicated for each treatment group.

Figure 5.2. Effect of acoustic and drug treatment on *egr-1* expression in the posterior, central, and anterior thalamic subdivisions. Data are shown as mean (± SE) silver grains/cell. Sample sizes are indicated for each treatment group.

Figure 5.3. Effect of acoustic and drug treatment on *egr-1* expression in the preoptic area, ventral striatum, ventral part of lateral septum, and dorsal medial pallium. Data are shown as mean (± SE) silver grains/cell. Sample sizes are indicated for each treatment group.

Figure 5.4. Effect of acoustic and drug treatment on *egr-1* expression in the nucleus accumbens. Data are shown as mean (± SE) silver grains/cell. Sample sizes are indicated for each treatment group.

Figure 5.1

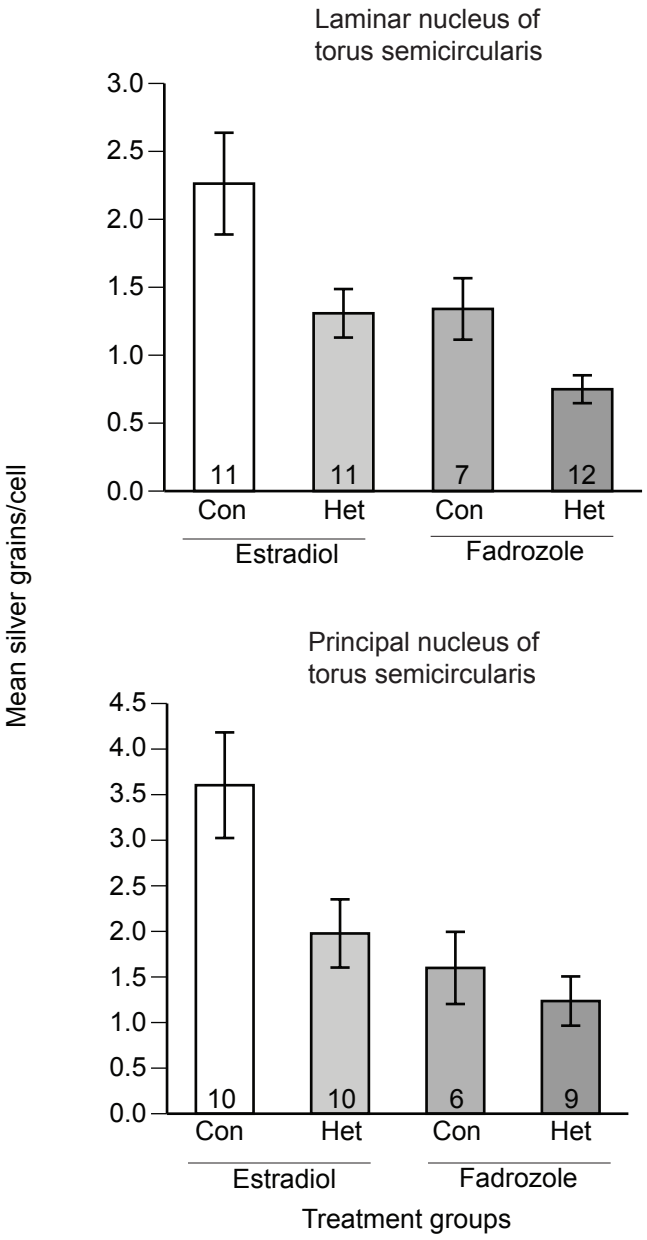

Laminar nucleus of
torus semicircularis
3.0
2.5
2.0
1.5
1.0
0.5
0.0
11
11
7
12
Con
Het
Con
Het
Estradiol
Fadrozole
Principal nucleus of
torus semicircularis
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10
10
6
9
Con
Het
Con
Het
Estradiol
Fadrozole
Treatment groups
Mean silver grains/cell

Figure 5.2

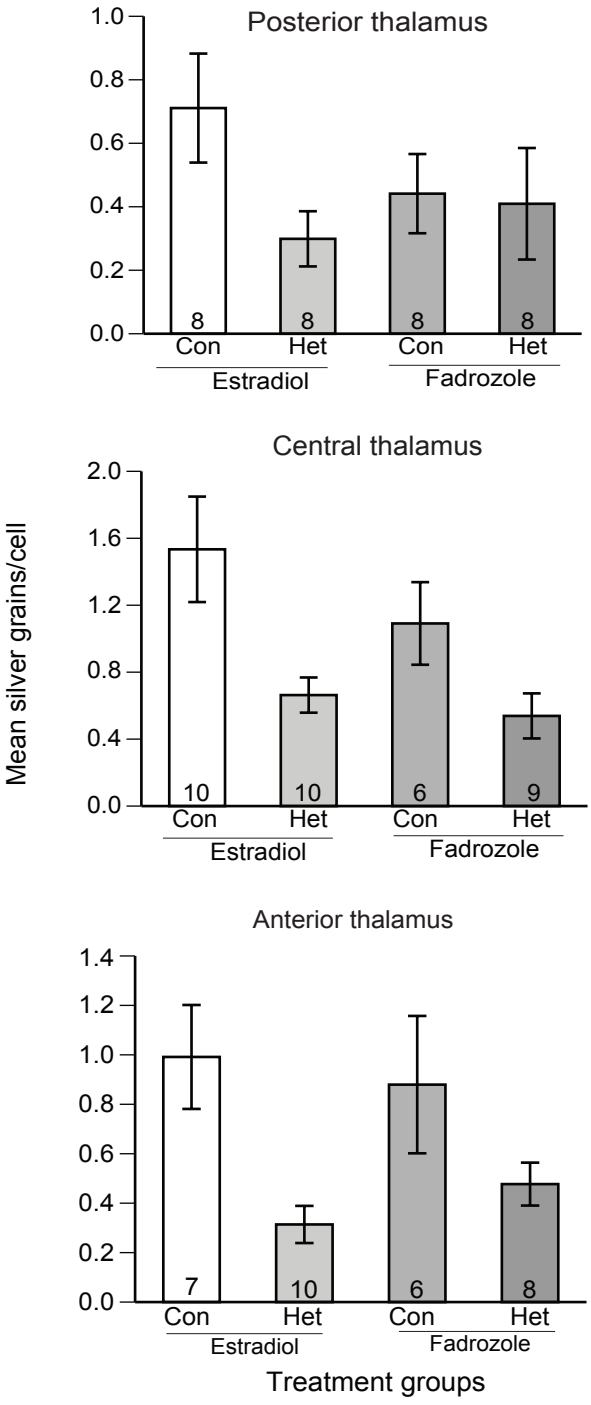

Posterior thalamus
1.0
0.8
0.6
0.4
0.2
0.0
8
8
8
8
Con
Het
Con
Het
Estradiol
Fadrozole
Central thalamus
Mean silver grains/cell
2.0
1.6
1.2
0.8
0.4
0.0
10
10
6
9
Con
Het
Con
Het
Estradiol
Fadrozole
Anterior thalamus
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7
10
6
8
Con
Het
Con
Het
Estradiol
Fadrozole
Treatment groups

Figure 5.3

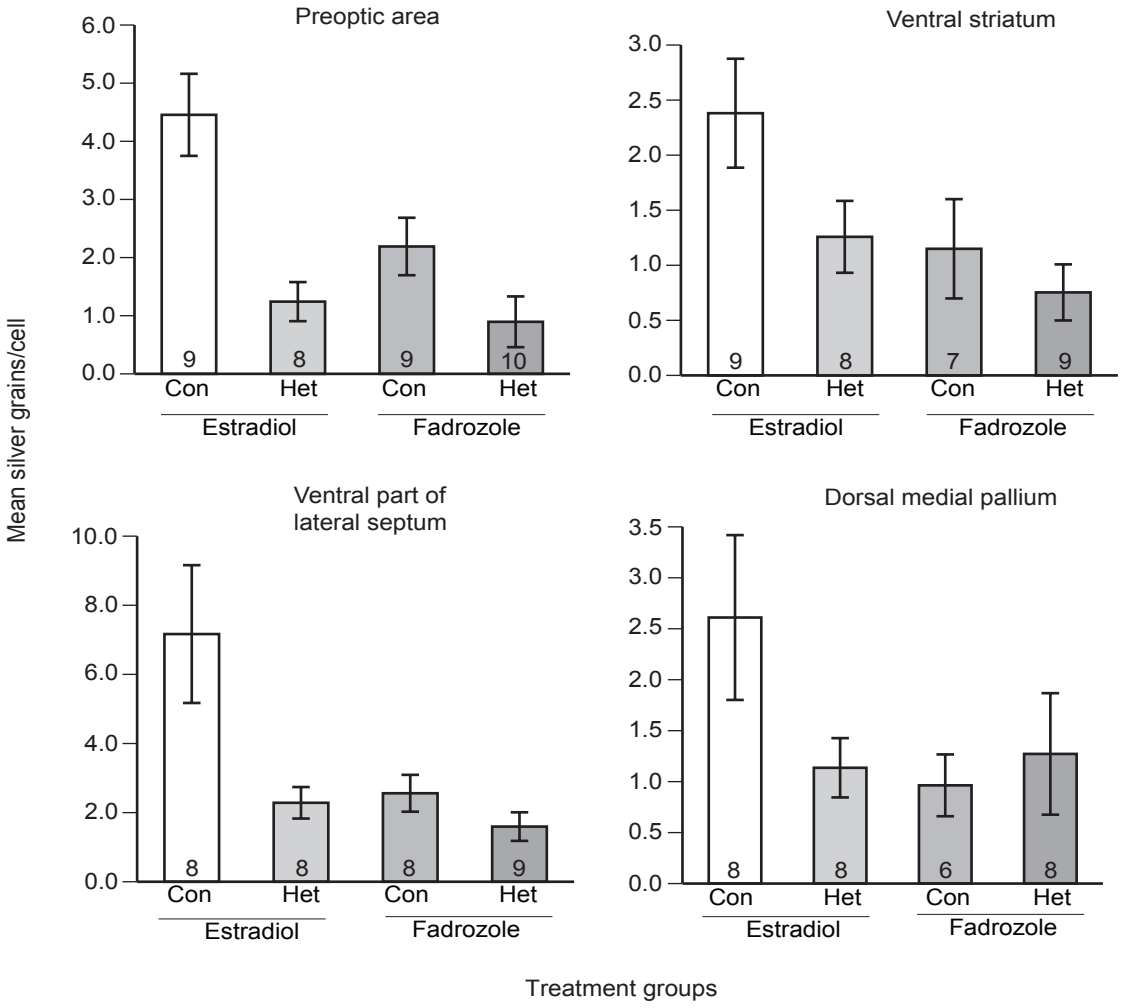
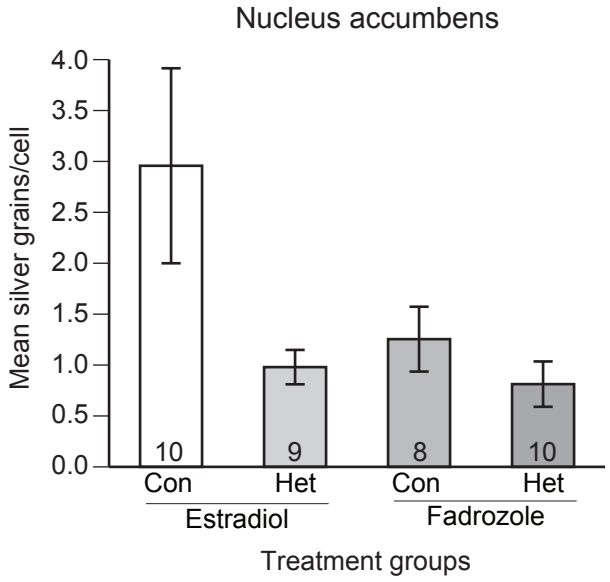

Preoptic area
Ventral striatum
Ventral part of lateral septum
Dorsal medial pallium
Mean silver grains/cell
Con
Het
Estradiol
Fadrozole
Treatment groups

Figure 5.4


Nucleus accumbens
Mean silver grains/cell
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10
9
8
10
Con
Het
Con
Het
Estradiol
Fadrozole
Treatment groups

# CHAPTER 6

## CONCLUSIONS

This research demonstrates that the hormone estradiol induces expression of phonotaxis behavior in túngara frogs, a critical feature of female anuran sexual behavior. The results also illustrate target sites of androgen and estrogen receptor action within the anuran brain, identifying sexual dimorphism in receptor expression in specific brain regions that may be key to understanding variation in sex-specific, hormone-behavior relationships. Furthermore, these experiments demonstrate that estradiol is an important neuromodulator, and influences neural processing of male courtship signals within the auditory system and in motor, limbic and motivational areas that are important in sexual communication in anurans. Overall, these results identify an important proximate mechanism that may influence mate recognition behaviors in female anurans.

These findings are significant on various levels. First, the study examining the localization of steroid receptors in the túngara brain is the first to provide a detailed description of the neuroanatomical distribution of androgen receptors (ARs) and estrogen receptor alpha (ER$\alpha$) and estrogen receptor beta (ER$\beta$) in an anuran. Second, it is the first study to report a sexual dimorphism in steroid receptor expression in the brain of any amphibians. Third, my findings suggest that estradiol is an important neuromodulator and its effects are mediated through ER$\alpha$ and ER$\beta$ receptors that are present in the auditory torus semicircularis and in the forebrain sensorimotor integration areas important in sexual communication. These results indicate steroid-dependent auditory plasticity in an anuran,

species which is largely consistent with studies from other vertebrate species that demonstrate estradiol-dependent auditory plasticity to mating signals (Maney et al., 2006; Sisneros et al., 2004). Such estradiol-dependent auditory plasticity may alter sensorimotor integration to modify behavioral responses in females during mate choice decisions.

**Summary of results**

With this series of experiments I have demonstrated that estradiol is an important neuromodulator that induces expression of female phonotaxis behavior in túngara frogs. Phonotaxis is a critical feature of female sexual behavior in anurans and is expressed as movement towards conspecific calling males (Gerhardt and Huber, 2002). Therefore, it represents recognition and assessment of potential mates based on their courtship signals. In Chapter 2, I investigated which hormonal conditions promote phonotaxis behavior in females. My results showed that estradiol alone was effective at elevating phonotaxis behavior suggesting that estradiol is sufficient to induce expression of phonotaxis in frogs. To my knowledge, this is the first study to show that a single hormone is effective in inducing phonotaxis behavior in an anuran. Intrinsic factors, such as endocrine state, can lead to variation in female sexual behavior by allowing the female to be plastic in her mate choice behavior (Lynch et al., 2005). This study demonstrates that estradiol can induce sexual behavior in female túngara frogs, which suggests that steroid hormones are capable of inducing female mate choice behavior via modulation of neural pathways that underlie phonotaxis.

Next, my goal was to determine if estradiol-injected females display the same call preferences as naturally breeding females. I found that estradiol injections induced females to exhibit similar call preferences as naturally breeding females. In addition, females injected with estradiol displayed strong preferences for the complex whine-chuck call over the simple

whine, and for a conspecific call over a heterospecific (*P. enesefae*) call. Similar to amplexed females, estradiol-injected females also failed to discriminate among calls based on the number of chucks. Thus, my data suggest that natural variation in female phonotaxis behavior that occurs over the reproductive cycle is controlled primarily by fluctuations in estradiol concentrations. Future studies will be necessary to determine whether estradiol is necessary for phonotaxis behavior and also if progesterone increases expression of phonotaxis behavior.

Previous studies by Lynch et al. (Lynch et al., 2005; 2006) have demonstrated that female permissiveness increases as she approaches oviposition when her plasma estradiol and progesterone concentrations are high. That is, the probability that a female will display any phonotaxis behavior towards a less attractive call (e.g. artificial hybrid call) increases as she approaches oviposition. This indicates that as females become more motivated to mate, she is more likely to accept a less attractive mating signal. One testable prediction is that increase in progesterone levels triggers the switch from a less permissive to a more permissive state in females. At present, it is unclear if progesterone contributes to increasing phonotaxis behavior or changes in mate preferences in females, although Lynch et al. (Lynch et al., 2005; 2006) has shown that changes in permissiveness do not influence call preferences. Therefore, hormone manipulation studies using aromatase blockers (e.g. fadrozole) and progesterone receptor blockers such as RU486 may enable us to assess the hormonal mechanisms underlying the change in permissiveness observed in phonotaxis tests.

In Chapter 3, I provide a detailed description of the neuroanatomical distribution of androgen receptors and estrogen receptors in túngara frogs. Little is known about the distribution of AR, ER$\alpha$, and ER$\beta$ receptors in the anuran brain, and therefore the variation in hormone-behavior relationships. I found *AR*, *ER*$\alpha$, and *ER*$\beta$ expression in the limbic forebrain (preoptic area, hypothalamus, nucleus accumbens, amygdala, septum, striatum),

parts of the thalamus, and in the laminar nucleus of the torus semicircularis, areas that have been reported to contain steroid concentrating cells in other anurans. In addition, I found new putative sites of *AR* and *ER* action, which includes the pallium, posterior tuberculum, locus coeruleus (*AR* only), optic tectum (*ER*$\alpha$ and *ER*$\beta$ only), and the principal nucleus of the torus semicircularis. The anuran medial pallium is thought to be homologous to the mammalian hippocampus (Kicliter and Ebbesson, 1976) and is acoustically sensitive (Mangiamele and Burmeister, 2008; Mudry and Capranica, 1980). In addition, it has been hypothesized that it may influence sexual motivation and reward seeking behaviors through its connections to the amygdala and nucleus accumbens (Northcutt and Ronan, 1992; Westhoff and Roth, 2002). Auditory responses in the medial pallium, which is likely under hormonal regulation may therefore contribute to memory formation during mate choice when females assess multiple males before choosing a mate (Ryan, 1985). Interestingly, I found *AR* and *ER*$\beta$ expression in the posterior tuberculum. The posterior tuberculum is a diencephalic region comparable to the mammalian substantia nigra pars compacta (SNc) and is acoustically responsive in túngara frogs (Hoke et al., 2005). Studies in male zebra finches have shown that neurons in the ventral tegmental area and SNc are differentially active during singing in different social contexts, and are involved in modulating the singing-related activation of the song system (Hara et al., 2007; Yanagihara and Hessler, 2006). Although, less is known about the function of the posterior tuberculum in anurans, my findings indicate that steroid hormones could regulate behavioral motivation and motor output through connections of the posterior tuberculum to the striatum.

I also found that expression levels differed in some brain regions between sexes, which may suggest mechanisms for sex-specific behaviors in anurans. This is also the first study to provide a detailed description of the neuroanatomical distribution of *AR*s and *ER*s in an anuran, and the first to report a sexual dimorphism in steroid receptor expression in the brain of any amphibian. In the auditory midbrain, females had higher *ER*$\alpha$ and *ER*$\beta$

expression than males. In the forebrain, females had higher *AR* expression than males within the ventral hypothalamus and medial pallium, whereas males had higher *ER*$\alpha$ expression in the medial pallium. Thus, sex steroid hormones are likely to have sexually dimorphic effects on auditory processing, which may have important implications for sexual communication in túngara frogs. My results suggest that the effects of estradiol in females as described in Chapter 2 are mediated through *ER*$\alpha$ and *ER*$\beta$ receptors that are present in the auditory torus semicircularis and in the forebrain sensorimotor integration areas important in regulating behavior.

One testable hypothesis is that phonotaxis behaviors are modulated in a sex-specific manner. Bernal et al. (2007) have demonstrated that males recognize and respond to a broad range of mating signals (both conspecific and heterospecific). Males also made more recognition errors than females when responding to most of the nonconspecific calls as would be predicted considering the recognition costs associated with each sex. Females are more likely to display phonotaxis behaviors in response to calls of species and ancestors that are more closely related (Ryan and Rand, 1995). Similarly, males respond to calls of closely related species suggesting an effect of evolutionary history o response to mating signals in both male and female túngara frogs. Therefore, studies investigating sex differences in neural and behavioral responses to a broad range of mating signals in hormone manipulated males and females may enable us to understand mechanisms of evolution of phonotaxis behavior and how sex differences in mate call recognition arise.

Results from Chapters 2 and 3 established that estradiol induces sexual behavior in females and that the female auditory system expresses *ER*$\alpha$ and *ER*$\beta$ receptors, indicating that the auditory system is a prime target for estrogen action in túngara frogs. In Chapter 4, my goal was to investigate the neural correlates of conspecific signal discrimination in females to identify where estrogen may act to influence behavioral responses to species-specific signals. I presented reproductively active female túngara frogs with conspecific calls

(*P. pustulosus*), heterospecific calls (*P. enesefae*), or no sound, and assessed neural activity using expression of the immediate early gene, *egr-1*. I examined *egr-1* mRNA expression in the hindbrain, midbrain, and the thalamus, as well as the forebrain auditory targets of the auditory system. My results showed that conspecific calls evoked a greater response throughout the central auditory system, including the superior olivary nucleus and upstream of feature detectors. This response-bias was also observed in the auditory torus semicircularis, thalamus, and forebrain limbic and motor targets. All but three nuclei known to receive auditory projections failed to demonstrate a bias toward conspecific calls. Thus, I concluded that although hierarchical feature detection undoubtedly plays an important role in recognition of species-specific stimuli, sensory systems may be generally biased towards processing species-specific mating signals. These results are significant since past studies have focused on investigating higher-order processing centers in the midbrain and telencephalon in birds and mammals (e.g. Grace et al., 2003; Jarvis et al., 1998; Mello and Clayton, 1994; Petkov et al., 2008), whereas a system-wide approach to understanding the neural correlates of species-specific signal discrimination was lacking. In Chapter 2, I demonstrated that estradiol induces phonotaxis behavior in females. In addition, in Chapter 3, I showed that the torus semicircularis, thalamus, and forebrain limbic and motor targets all express estrogen receptors, which makes it likely that they are modulated by estrogen inputs. Taken together, it appears that the anuran auditory system is inherently biased to respond selectively to species-specific signals. It is possible that the expression of this bias is modulated by estrogen inputs. Overall, my findings are significant since they provide the foundation to address the role of estradiol in auditory discrimination of species-specific mating signals in anurans.

In Chapters 2 and 4 I established that estradiol is sufficient to induce sexual behavior in female túngara frogs and that the auditory system is biased to respond to species-specific mating signals over heterospecific signals. In Chapter 5, I extended these results to

investigate the role of estradiol in auditory processing of species-specific mating signals. Based on previous observations, I predicted that estradiol enhances response to conspecific signals by modulating neural activity within the auditory, motor, and motivational areas that are important in sexual communication. I injected females with estradiol or fadrozole and exposed them to a conspecific, or a heterospecific stimulus. I measured neural activity using expression of *egr-1*, and assessed the levels of *egr-1* mRNA expression in the laminar and principal nuclei of the midbrain torus semicircularis, parts of the thalamus that are known to receive auditory inputs, forebrain primary auditory targets, and the nucleus accumbens. With four exceptions (three thalamic regions and the dorsal medial pallium), all auditory nuclei and the nucleus accumbens showed greater neural responses to species-specific over heterospecific calls in estradiol-injected females. Further analyses of these regions revealed that both estradiol and conspecific calls together induced greater neural responses than either alone, suggesting an additive effect on *egr-1* induction. Thus, I concluded that estradiol plays a pivotal role in enhancing sensory processing of species-specific signals by modulating call-evoked *egr-1* expression within the auditory system.

Estrogen is an important neuromodulator, and has wide-ranging physiological effects including its effects on synaptic plasticity and memory formation (Hodgson et al., 2008; Liu et al., 2008). A testable hypothesis would be that estradiol likely evolved a specialized function for synaptic plasticity to prime the female brain to respond to species-specific mating signals. My results do not demonstrate that estradiol induces female sensory discrimination for mate recognition, but elucidate an important role for estradiol as a modulator of immediate early gene expression in anurans. The role of immediate early genes as important effector molecules for synaptic plasticity (see review by Mello et al., 2004) and long-term potentiation is well established (Abraham et al., 1991), which could have important implications for mate recognition. As proposed by Maney et al. (2006) estradiol-dependent modulation of immediate early gene expression may help to strengthen

synaptic connections during the breeding season in brain regions that are particularly relevant for mate recognition and discrimination. Sockman et al. (2002) have shown that ZENK responses in the auditory forebrain in female European starlings after exposure to a preferred long song is greater in females with the preferred “long-bout” song experience than in females with the less preferred “short-bout” song experience. This indicates that response biases toward a preferred mating signal are dependent on recent experience with that category of mating signal. Recent studies in túngara frogs have demonstrated that females actively assess multiple signalers simultaneously and are sensitive to the location of preferred call types using an open-ended mate choice process that was previously unknown in anurans (Baugh and Ryan, 2010). Ryan et al. (2009) have proposed that mate choice is a complex cognitive process involving a series of decision-making rules that requires learning and memory. In addition, Akre and Ryan (2010) have shown that females retain attraction to complex calls using working memory. One prediction is that estradiol may contribute to memory formation during mate choice when females simultaneously assess multiple males before choosing a mate (Ryan, 1985). It would be useful to know if estradiol contributes to working memory in túngara frogs by influencing *egr-1* expression and synaptic plasticity-associated genes such as mitogen-activated protein kinases (MAPK) and synapsins. At present, I cannot conclude that *egr-1* response biases observed in this study represent species recognition in túngara frogs, and if that relates to estradiol effects on consolidating memory. However, the emergence of a modulatory role of estradiol in enhancing *egr-1* expression in sensory, motor and motivational areas in anurans invites further studies on how steroid-dependent neural plasticity may influence mate recognition systems critical for mate choice and speciation. I also cannot conclude if effects of estradiol observed in this study are due to rapid and local estradiol action or due to a systemic effect alone. Steroid hormones are potent neuromodulators exerting biological effects through nuclear hormone receptors (hours to days) or through membrane-bound receptors producing rapid and local

effects (seconds to minutes). A recent study has shown that estradiol levels are differentially affected during social behavior in zebra finches in a region-specific manner and in a rapid time-course similar to other traditional neuromodulators (Remage-Healey et al., 2008). Therefore, future experiments involving *in vivo* microdialysis to test whether local steroid levels fluctuate during phonotaxis may enable us to assess if estradiol actions are mediated through rapid effects in the brain.

**Future directions**

The primary goal of this series of research experiments was to understand how steroid hormones modulate auditory processing of species-specific signals in females, which may have important implications for mate recognition behaviors. The results from each of these experiments have been valuable in understanding how estradiol influences female phonotaxis and consequently mate recognition behavior. The findings from these experiments also raise interesting questions to be addressed in future investigations. In particular, Chapter 3 provides the first detailed description of AR and ER in an amphibian species, which invites future investigations to assess the mechanisms by which AR and ER may regulate sex-specific auditory processing of conspecific mating signals. The neuroanatomical distribution of AR and ER in mammals and birds has been extensively studied (e.g. Gahr, 2001; Gahr and Metzdorf, 1997; Shughrue et al., 1997; Simerly et al., 1990), and the role of steroid receptors in regulating sexual behavior is well established (Blaustein and Erskine, 2002; Ogawa et al., 2000; Rissman et al., 1997). Although, parts of the vocal and auditory pathways concentrate androgens and estrogens in anurans (Kelley, 1980; Morrell et al., 1975), little is known about the distribution of sex steroid receptors, making it difficult to compare the neural targets of sex steroid action with other vertebrates. The results from Chapter 3 attempt to fill the gaps in our understanding of the distribution of

steroid receptor and their actions in amphibians. Sexual dimorphisms in receptor expression in auditory areas in breeding adults may reflect unique specializations that could have evolved to enhance coupling of sender (males) and receiver (females) within the context of sexual communication. Furthermore, sexual dimorphism was also observed within the ventral hypothalamus with females expressing higher *AR* mRNA levels. A testable hypothesis is that higher *AR* expression may enhance the facilitatory role of estradiol in modulating sexual receptivity in females. Clearly, future studies examining the functional relevance of sexual dimorphisms in auditory, limbic and sensorimotor regions in anurans will be useful in understanding the evolution of steroid receptors, and how that relates to functional specializations of steroid receptors in other vertebrates.

The research described in Chapter 5 establishes that estradiol is an important neuromodulator for auditory processing of species-specific signals in túngara frogs and raises exciting opportunities for further advancing our understanding of steroidal regulation of female mate recognition behaviors in anurans, generally. Endocrine effects on the neural control of sexual behavior can be brought about by modulating a class of neuromodulators, known as catecholamines (Vathy and Etgen, 1989). Hormones such as estradiol can exert their facilitatory effects on reproductive and social behaviors by modulating catecholamine levels within specific brain regions (LeBlanc et al., 2007; Vathy and Etgen, 1989; Woodley et al., 2000). In songbirds, song-induced ZENK expression in different song nuclei within the brain is context-dependent and can be modulated by catecholaminergic input (Castelino and Ball, 2005; Hara et al., 2007; Lynch and Ball, 2008), suggesting that catecholamines play an important role in modulating sensory processing (see review by Sockman, 2007). Thus, although there is growing evidence in support of an interaction between the endocrine and the catecholaminergic system in modulating reproductive behavior, we do not yet fully understand how these systems are integrated to affect signal processing, and consequently aspects of female sexual behaviors.

Anurans serve as excellent model systems to study the neuroendocrine mechanisms of phonotaxis behavior, a critical feature of mate recognition. In anurans, dopamine depletion decreases acoustically guided motor responses, and impairs sensory processing and sensorimotor integration in female gray treefrogs (Endepols et al., 2004). In addition, gonadectomized adult frogs treated with exogenous androgen show elevated number of catecholaminergic cells in the forebrain compared to gonadectomized control animals (Chu and Wilczynski, 2002), indicating that steroids can regulate the catecholaminergic system in frogs. Furthermore, the anuran brain is widely innervated by catecholaminergic inputs. Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in the biosynthesis of dopamine and norepinephrine and is present in all catecholaminergic cells. Catecholaminergic cell bodies as revealed by TH staining (which stains both dopamine and norepinephrine producing cells) have been found in areas such as the posterior tuberculum, suprachiasmatic nucleus, locus coeruleus, and the midbrain tegmentum in anurans (González and Smeets, 1993). In addition, catecholaminergic fibers are known to be present in the lateral pallium, striatum, septum, amygdala, torus semicircularis, and the hypothalamus, among other areas (Endepols et al., 2004; González and Smeets, 1993). Most of the brain regions expressing catecholaminergic cells and fibers also express steroid receptors, suggesting that estradiol may affect various brain regions to influence behavior (see Chapter 3). At present, how steroid hormones and catecholamines are integrated to influence auditory, motor, limbic, and motivational systems to modulate the expression of sexual behavior remains elusive.

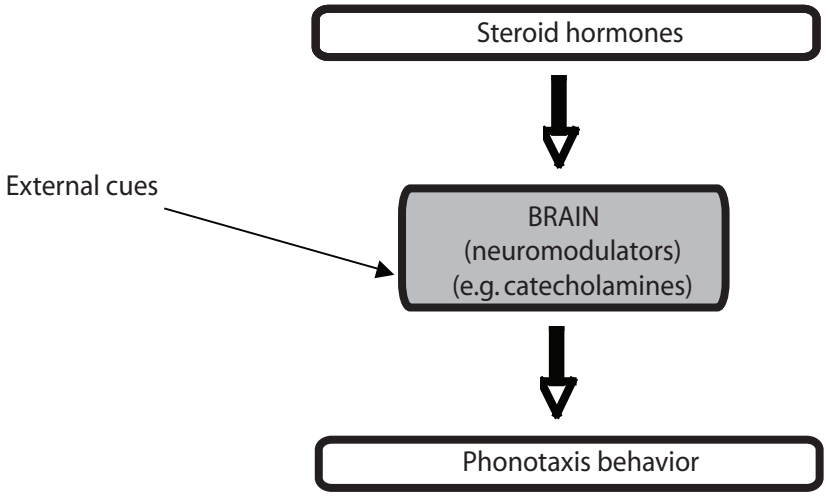
One testable hypothesis in túngara frogs would be that estradiol induces expression of female phonotaxis behavior by modulating catecholamines within auditory pathways (Figure 6). To test this, it would be important to first assess if estradiol injections elevate catecholamines in the brain. Second, to manipulate both estradiol and catecholaminergic systems and determine whether estradiol effects on the neural response to conspecific calls

in the auditory system are mediated by catecholamines. Results of the above experiments would determine if catecholamines are essential for estradiol-dependent auditory processing of conspecific stimuli during phonotaxis behavior. Manipulations of the endocrine and catecholaminergic systems concurrently and testing their effects on the neural response to conspecific stimuli offers a powerful tool to investigate how the endocrine and catecholaminergic systems are integrated to affect signal processing during expression of phonotaxis behavior. From an evolutionary perspective, an examination of the neuroendocrine mechanisms underpinning expression of phonotaxis behavior will be important in understanding the plasticity in neural processing during reproduction that is critical for female mating decisions and reproductive success.

Figure Legends

Figure 6. Model showing possible role of steroid hormones and neuromodulators in expression of phonotaxis behavior

Figure 6.


Steroid hormones
External cues
BRAIN
(neuromodulators)
(e.g. catecholamines)
Phonotaxis behavior

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