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HORMONAL MECHANISMS FOR VARIATION IN FEMALE MATE CHOICE

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HORMONAL MECHANISMS FOR VARIATION IN FEMALE MATE CHOICE

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Dissertation

Presented by the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May, 2005

Acknowledgements

The research presented here was supported by the National Science Foundation and National Institute for Health from grants awarded to Dr. Walter Wilczynski. I would also like to acknowledge the University of Texas, Society for Integrative and Comparative Biology and Sigma Xi for additional financial support. I thank my adviser, Walter Wilczynski, who rivals Guy Forsyth for the title of "coolest guy in Austin". Also, I thank Sabrina Burmeister, Eunjin Yang, Kim Hoke, Ximena Bernal, Erin O'Bryant, Mary Ramsey and other members of the Wilczynski, Crews and Ryan labs for their contribution to my eduction while I was at UT.

My family also helped me immeasurably throughout the years I chose to be a student. Among other things, they provided counseling, cheerleading, pet-sitting, and comic relief. I also thank Bozworth and Bodhi, my two best friends, who always managed to insist on a long walk just when I needed one most.

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FEMALE MATE CHOICE

Publication No. _____

Kathleen Sheila Lynch, Ph.D.

The University of Texas at Austin, 2005

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Although female mate choice has been the subject of many investigations, the causes of variation in mate choice are less understood. Theoretical models predict that individual females should show variable mate choices as a consequence of intrinsic and extrinsic factors. Empirical support for these predictions demonstrates that females show flexible mate choices over their lifetime, the breeding season and a reproductive cycle. The objective of the research presented here is to examine two intrinsic factors, reproductive and hormonal state, to determine how these contribute to individual variation in female mate choice over a reproductive cycle. I examine the link between flexibility in mate choice behavior, changes in gonadal hormones and hormonal modulation of sensory systems involved in mate choice behavior. Flexibility in mate choice was examined in female túngara frogs (*Physalaemus pustulosus*), a Neotropical species in which mate choice has been well studied. Acoustic based phonotaxis tests were used to assay mate choice behaviors such as receptivity, permissiveness and discrimination. These behaviors are respectively defined as a response to conspecific mate signals, a response to unattractive mate signals and the ability to discern the

difference between mate signals. The expression of receptive and permissive mate choices significantly fluctuates throughout different reproductive stages. The concentration of gonadal steroids, such as estrogen, progesterone and androgens, also significantly fluctuate throughout the same reproductive stages. Furthermore, hormone concentration was manipulated using human chorionic gonadotropin (HCG) and such manipulation induces flexible mate choice. Finally, I investigate whether hormones modulate a central auditory nucleus involved in phonotaxis behavior. Immediate early gene (IEG) induction, specifically *egr-1*, was used to mark neuron activity. Females were treated with either HCG or saline and exposed to either mate choruses or silence. *Egr-1* expression was quantified in an auditory midbrain nucleus, the torus semicircularis (TS). The region within the TS responsible for auditory-motor integration showed a near significant elevation in *egr-1* expression in response to acoustic exposure and a significant elevation in *egr-1* expression in response to hormone treatment, suggesting that hormones can play a role in phonotaxis response by modulating midbrain neurons that act as an auditory-motor interface.

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Chapter 1

INTRODUCTION

Although our current understanding of the causes and consequences of variation in female mate choice is limited (see Jennions and Petrie for review, 1996) it has been demonstrated that female mate choice arises due to heritable (Bakker and Pomiankowski, 1995 for review), and non-heritable causes, such as developmental, environmental or social factors (Crowley et al. 1991; Milinski and Bakker, 1992; Pruett-Jones 1992 for review of mate copying; Endler and Houde 1995; Rand et al. 1997). There is also evidence for yet another, non-heritable cause of variation in female mate choice, that is, short term changes in female condition (Thornhill, 1984; Proctor, 1991; Poulin, 1994; Simmons, 1994; Penton-Voak, 2003). For instance, female pied flycatchers (*Ficedula hypoleuca*) in good body condition will travel further to assess available mates than females in poor condition (Slagsvold et al., 1988). Female scorpionflies (*Hylobittacus apicalis*) will accept males that bring small nuptial gifts only when they are hungry. When they are satiated, however, they accept only males with large nuptial gifts (Thornhill, 1984). Parasitized female bullies (*Gobiomorphus breviceps*) accept significantly smaller males as mates relative to unparasitized female bullies (Poulin, 1994). Also, examples of short-term changes in female condition are widely reported in human females. For instance, women show stronger preferences toward masculine male faces during the fertile phase of the menstrual cycle (i.e. ovulatory stage) as opposed to the luteal stage in which they are infertile (Penton-Voak et al., 1999). Women's preferences for male behavior also shift within the menstrual cycle so that during the fertile phase of their menstrual cycle they prefer men with social presence (as measured by behaviors such as composure, eye contact, and lack of self deprecation) (Gangestad et al., 2004).

Recently, animal behavior studies have shown that condition-dependent variation in female mate choice can arise over different time scales, such as the course of the

females' lifetime (Kodric-Brown and Nicoletto, 2001; Moore and Moore, 2001), the course of a breeding season (Qvarnström et al., 2000; Veen et al., 2001) and the course of a single breeding cycle (Lea et al., 2000; Bosch and Boyero, 2004). For instance, older female cockroaches (*Nauphoeta cinerea*) were found to be less choosy in their mates; they required significantly less courtship displays from the male than did younger females. These older females also had fewer offspring per clutch and fewer clutches than younger females, indicating that mate choosiness declined with reproductive capability (Moore and Moore, 2001). Similarly, young female guppies (*Poecilia reticulata*) were found to prefer only males with large areas of carotenoid pigment, whereas older females showed no preference. This decline in choosiness was shown to change with age but not experience (Kodric-Brown and Nicoletto, 2001). Female collared flycatchers (*Ficedula albicollis*) varied their choice of males based on forehead patch size over the course of the breeding season. As the end of the breeding season approached, the females increased choosiness so that they only accepted males with the largest patch size (Qvarnaström et al., 2000). Finally, female midwife toads (*Alytes muletensis*) that were ovulating were consistent in their choice of calls, however, after mating they did not reliably discriminate between calls with low (1.5 kHz) and mean (1.8 kHz) frequencies, indicating that mate discrimination changes throughout different reproductive stages. Although these studies provide evidence that variation in mate choice can arise as a consequence of short-term changes in the females' condition, there is still much to learn about the mechanisms that can produce variation in mate choice both between and within females. The experiments presented here are aimed at understanding the mechanisms for variation in mate choice within a female throughout a single reproductive cycle.

Flexibility in Mate Choice Behavior: Ultimate Mechanisms

When males and females are searching for potential mates, females are generally the more discriminating sex. Trivers (1972) suggested this is because females have a greater gametic investment because it is more costly to produce an egg rather than a sperm. In general, females, in most taxa, invest more into the young by spending more

time raising them (Trivers, 1972). Therefore, females are more discriminating amongst potential mates than males. However, theoretical models predict that females should be flexible in their mate choices as a consequence of constraints placed upon them as they search for a mate (Real, 1990; Crowley et al., 1991). Although there are discrepancies in the direction in which theoreticians predict mate choice to change (i.e. females decrease or increase restrictions on mate choices under constraints), it is agreed that females should be flexible in their mate decisions. For instance, female sand gobies (*Pomatoschistus minutus*) increase mate selectivity later in the breeding season (Forsgren, 1997), which is consistent with Crowley's model of mate choice in which mate choosiness increases as the search progresses. Crowley et al. models mate choice behavior as a function of "expected lifetime reproductive success". This model is based on the idea that searching for mates is dangerous and the risks involved with mate searching change as predator and mate density change. Therefore, Crowley et al. predict that when those factors are held constant, mate choosiness should increase later in the season because less of the potential "expected lifetime reproductive success" will be jeopardized. That is, at the start of the season, the discriminating sex should have many mating opportunities available and therefore, should not risk predation by being choosy whereas at the end of the season, the opportunity for mating declines so the discriminating sex can afford to be choosy, especially if successfully reproduction occurred throughout the season. On the contrary, Real (1990) suggests that time constraints should cause the discriminating sex to decrease choosiness. This suggests that as the conclusion of the breeding season approaches the discriminating sex should become less choosy. Real proposes that the discriminating sex will have to make a mate choice that optimizes the balance between risk (e.g.. predation, starvation or running out of time) and mating preference. There is little empirical evidence to support Real's theory. The study presented in chapter 1 will examine the direction in which the strength of the female preference changes as she approaches the time at which she must release eggs. This study, as well as others presented here, provides empirical evidence for

theoretical models that predict that females will be flexible in their mate choices under time constraints.

Flexibility in Mate Choice Behavior: Proximal Mechanisms

Although there is a large collection of literature that describes the role of gonadal and peptide hormones in inducing female receptivity (for review see Blaustein and Erskine, 2002) in almost every taxa including birds (Noble 1973; Delville and Balthazart 1987) amphibians (Diakow and Nemiroff 1981; Schmidt 1984; 1985, Boyd 1994), reptiles (Alderete et al., 1980; Rhen et al., 1999; 2000; Rhen and Crews, 2000) and mammals (Tetel et al. 1994; Cushing and Carter 1999), few studies examine how hormones influence the female's actual mate choice. Recently, it has been shown that testosterone treated female dark-eyed juncos (*Junco hyemalis*) are less discriminating in their mate choices than control females (McGlothlin et al., 2004). Although there are no other hormone manipulation studies providing empirical support for the hypothesis that hormones serve as a proximal mechanism for mate choice flexibility, it has been shown that variation in mate choice decisions can be associated with changes in reproductive stage (Lea et. al., 2000; Bosch and Boyero, 2004) and that changes in reproductive stage can be associated with changes in hormonal state (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986; Itoh and Ishii, 1990; Harvey et al., 1997; Medina et al., 2004). The studies presented here in chapters 2 and 3 suggest that fluctuations in gonadal hormones occur as mate choice behaviors are shifting and that experimental manipulation of these same hormones can induce mate choice plasticity.

Hormones may influence mate choice behavior, specifically receptive behavior, via hormonal modulation of sensory neurons. Evidence of steroidal modulation of sensory structures originates from clinical work such as studies showing that Addison's patients (i.e. patients with insufficient adrenal gland function) with lower auditory, olfactory and taste thresholds can be treated with corticosterone to return the sensory systems to normal function (Henkin and Solomon, 1962; Henkin and Bartter, 1966; Henkin et al., 1967). There are also reports that human females experience shifts in taste,

auditory, and visual systems during natural fluctuations in the menstrual cycle (Wright and Crow; 1973; Fagan and Church, 1986; Elkind-Hirsch et al., 1992; Tasman et al., 1999; Walpurger et al., 2004). Neuromodulatory effects of peptide and gonadal hormones on sensory structures have also been reported in other taxa including fish (Meyer et al., 1984; Keller et al., 1986; Sisneros & Bass, 2003; Zakon and Smith for review, 2002) amphibians (Yovanof & Feng, 1983; Penna et al., 1992; Miranda and Wilczynski, 2004), and mammals (Bereiter & Barker, 1980). For instance, it has been shown that the receptive field of mechanoreceptor neurons on the face of female rats enlarges after treatment with estrogen, but not progesterone, as well as with natural elevations in estrogen (Bereiter and Barker, 1980). Also, when non-reproductive female midshipmen (*Porichthys notatus*) are treated with either testosterone or estrogen, the ability of the auditory system to encode the temporal pattern of the males vocalization improves, which mirrors the auditory responses of reproductive females (Sisneros and Bass, 2003). These and other studies are important for the field of animal behavior because they demonstrate the neural mechanisms by which animals can adapt to the increased demands required to successfully breed.

Reproductive Behavior and Neuroendocrinology in Amphibians: A Review

In 1960, Dodd demonstrated a causal relationship between elevations in androgen levels and the appearance of reproductive behaviors in amphibians. Since then, male amphibians have become a common model for investigations into the relationship between experience, hormones, brain and behavior. Only recently has attention been paid to female amphibians. Consequently much less is known about the experience-hormone-behavior link in female amphibians. This section will review the information known to date on the behavioral neuroendocrinology of both male and female amphibians.

Activational Effects of Androgens and Production of Mate Signals in Male Amphibians

In most anuran species, the males produce distinct advertisement signals when they are reproductively active and in the case of the anuran, the male is highly social, forming lek-like aggregations during the breeding season (Wells, 1977). The production

of the advertisement signals is androgen dependent (Wada et al., 1976; Wada and Gorbman, 1977;Wetzel and Kelley, 1983; Moore, 1987; Solis and Penna, 1997; Iwata et al., 2000; Kikuyama et al., 2002 for review) and therefore, eliminated upon castration (Dodd, 1960; Schmidt, 1966; Palka and Gorbman, 1973; Kelley and Pfaff, 1976; Deviche and Moore, 1988; Burmeister and Wilczynski, 2001). However, the specific contribution of androgens in regulating the expression of advertisement signals in anurans is not always clear. For instance, in some cases androgens are lower in calling anurans in relation to non-callers (Mendoca et al., 1985; Orchinik et al., 1988) whereas in other species androgens are higher in calling males (Marler and Ryan, 1996; Townsend and Moger, 1987). Furthermore, evoked vocalization rate and plasma androgens were not correlated in a laboratory population of breeding male *Hyla cinerea* (Burmeister and Wilczynski, 2000) whereas androgen concentration and evoked vocalization rate were correlated in a field population of *Batrachyla taeneiata* (Solis and Penna, 1997). Interspecific comparisons of breeding male anurans show that when testis mass is controlled for, vocalization effort is correlated with androgen concentration among different species (Emerson and Hess, 2001).

Studies that experimentally manipulate androgens in order to examine the effect on male reproductive behavior suggest that androgens alone are not responsible for male calling behavior. For instance, androgen replacement following castration does not fully reinstate calling behavior in some anuran species (Palka and Gorbman, 1973; Schmidt, 1966; Wada and Gorbman, 1977; Wetzel and Kelley, 1983). Additional treatment with gonadotropins reinstates courtship behavior to the level of intact males (Wetzel and Kelley, 1983) and increases sexual activity when administered to intact males (Kelley and Pfaff, 1976; Kelley, 1982). Such results suggest that gonadotropins themselves can substantially contribute to the initiation of male courtship behaviors in amphibians, perhaps through direct action in the brain. In fact, in recent studies of *X. laevis*, localization of LH receptor mRNA indicates that LH receptors are distributed in brainstem nuclei thought to be involved in regulating vocalization in male *X. laevis* as well in hypothalamic and forebrain regions (Yang and Kelley, 2004).

Seasonal elevations in androgen levels that coincide with reproductive behaviors are socially influenced. Male anurans exposed to mate choruses show elevated androgen levels and GnRH-ir cell number compared to males not exposed to mate choruses (Chu and Wilczysnki, 2001; Burmeister and Wilczynski, 2000; Burmeister and Wilczynski, 2005) and male *Hyla cinerea* with experimentally elevated androgen levels vocalize more in relation to males not receiving androgen treatment (Burmeister and Wilczynski, 2001). These studies show a clear link between social experience, androgens and behavior in male amphibians.

Thus far, it appears that androgens, specifically testosterone (T) and dihydrotestosterone (DHT), play an important role in the initiation and maintenance of advertisement signals in breeding male amphibians. The exact role that androgens play in the expression of male courtship behavior may be clarified from studies that examine androgen interactions with other steroid or peptide hormones such as corticosterone (CORT) and arginine vasotocin (AVT) and/or, as suggested by Emerson and Hess (1996), studies that examine variation in androgen activity as a consequence of life history strategies such as aggressive, territorial or parental behavior (Emerson et al., 1993; Emerson and Hess; 1996; Townsend and Moger, 1987; Townsend et al., 1991), flexibility in seasonality (Houke and Woodley, 1995; Harvey et al., 1997) and/or energy required for the species-specific advertisement call (Emerson and Hess, 1996; 2001).

Organizational Effects of Androgens and Mate Signal Production in Male Amphibians

Androgens regulate the development of the laryngeal and oblique muscles, which are primarily responsible for the production of advertisement calls in male anurans (Tobias and Kelley, 1987). Androgens have organizational effects during development of the male anuran larynx, causing sexual dimorphism in the larynx to occur so that the male larynx possesses 6-7 times more muscle fibers (Sassoon and Kelley, 1986), significantly more fast-twitch, fatigue resistant muscles (Sassoon and Kelley, 1986; Sassoon et al, 1987), 3 to 4 times higher androgen binding activity (Segil et al., 1987) and greater neuronal innervation (Robertson et al, 1994) than females in *Xenopus lavealis*. In the

bullfrog (*Rana catesbeiana*), the male's larynx has 13% more androgen receptor positive muscle nuclei than the female's larynx (Boyd et al., 1999). Oblique muscles of the body wall are also sexually dimorphic (Tiagen et al., 1985) in density of androgen receptors (Emerson et al., 1999), enzymatic capacities and fiber types (Marsh and Taigen, 1987) and contractile properties (Girgenrath and Marsh, 2003). Consequently, male oblique muscles are more resistant to fatigue and have increased contraction velocity (Marsh and Taigen, 1987) than female oblique muscles.

Androgens also affect neural targets in the central vocalization production pathway. Brainstem motor nuclei IX-X control laryngeal muscles and reportedly contain androgen receptor-ir or androgen-concentrating cells (Kelley et al., 1978; Kelley, 1980; Emerson and Boyd, 1999 for review). Also, the pretrigeminal nucleus (PTN), which is thought to play a vital role in the generation of vocalizations, receives input from the preoptic area (POA) and has been found to contain cells that concentrate androgens, specifically DHT (Kelley et al., 1975; Kelley, 1980). Such studies indicate that male vocalizations can be regulated by androgens at all levels of the vocalization production pathway.

Androgens and Male Clasping Behavior

In most anuran species the male clasps gravid females from the dorsal side during mating and waits until the female oviposits so that external fertilization can occur. This clasping is referred to as amplexus. Amplexus can last up to several days in some species (Wells, 1977). Androgens modulate the forelimb musculature used in this behavior (Sidor and Blackburn, 1998), possibly so that it can adapt to the demands of clasping. The principal forelimb flexor muscle, the flexor carpi radialis (FCR), is the forelimb flexor muscle that is sexually dimorphic, seasonally modulated, and androgen sensitive in adult males (Herrera and Regnier, 1991 for review). Testosterone has been shown to increase the size of specific FCR muscle fibers (Regnier and Herrera, 1993; Dorlöchter et al., 1994) and slow contractile kinetics (Herrera and Regnier, 1991) in some regions of the FCR.

Additional studies have also examined androgens effect on the male's motivation to clasp a female. Clasping can be induced in intact males by injections with gonadotropin (Kelley and Pfaff, 1976; Wada and Gorbman; 1977; Schmidt, 1966); however, after castration, gonadotropin is no longer effective in inducing clasping but implantation with T or DHT will reinstate clasping (Kelley and Pfaff, 1976).

Corticosterone and Male Courtship Behavior

Corticosterone (CORT) levels are seasonally modulated, (Licht et al., 1983; Pancak and Taylor, 1983; Dupont et al., 1979; Zerani and Gobbetti, 1993), socially modulated (Burmeister and Wilczynski, 2000) and contribute to the regulation of reproductive behaviors in male amphibians (Orchinik et al, 1988; Burmeister and Wilczynski, 2000). However, the relationship between male reproductive behavior and corticosterone levels in male amphibians is not well understood. For instance, in male roughed-skinned newts (*Taricha granulosa)* it is known that corticosterone rapidly inhibits male clasping behavior (Moore and Miller, 1984). On the contrary, however, in crested newts (*Triturus carnifex)* CORT levels were shown to be lower and androgen levels higher in inactive males in relation to actively courting males (Zerani and Gobbetti, 1993). Furthermore, in some amphibian species corticosterone will inhibit male calling behavior (Marler and Ryan, 1996) and is associated with low androgen levels (Moore and Zoeller, 1985; Licht et al., 1983; Marler and Ryan, 1996); however, in some male anurans species, corticosterone is elevated in calling males in relation to non-callers (Leary et al., 2004; Orchinik et al, 1988; Mendoca et al., 1985; Harvey et al, 1997) and does not seem to have an effect on androgen levels (Orchinik, 1988; Leary et al., 2004). Additional studies using interspecific comparisons show that corticosterone levels in breeding male anurans are higher in species with increased call energy and higher call rate (Emerson and Hess, 2001). These studies demonstrate the variable effects that corticosterone has on androgen levels and courtship behavior in breeding male amphibians.

Emerson (2001) proposed the Energetics- Hormone Vocalization model to explain the relationship between corticosterone and androgen levels in calling and non-calling male anurans. She suggests that corticosterone is elevated in order to meet the energetic demands of advertising but at some threshold, which may vary between species, corticosterone interferes with androgen production thereby inhibiting calling. Calling should then be reinstated when the male's energy reserve is restored, corticosterone levels are lowered and androgen is elevated once again. Leary et al. (2004) tested this model and found that although corticosterone was higher in calling males relative to non-calling, satellite males in *Bufo woodhousii* and *B. cognatus*, androgen concentrations did not differ. Instead, in order to understand the transition between calling and non-calling behavior, the authors proposed a model that includes energy reserves, androgen / corticosterone concentration and arginine vasotocin (AVT) production in neurons of the telencephalon (Leary et al., 2004).

Steroid Hormones and Reproductive Behavior in Female Amphibians

Far less is known about the relationship between hormones, experience and behavior in female amphibians. Currently, it has been shown that sex steroid levels in female amphibians are seasonally modulated (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986) and show cyclic fluctuations within the breeding season (Iela et al., 1986; Harvey et al., 1997; Medina et al. 2004). Also, fluctuations in estrogen levels are paralleled by changes in sex steroid binding proteins in *Bufo arenarum* (SSBP; Santa-Coloma et al., 1985). In both male and female *Rana esculenta*, SSBP's are low during non-breeding periods and high during breeding periods (Paolucci and Di Fiore, 1994). Hypothalamic aromatase activity, estrogen receptor activity and progesterone receptor levels also fluctuate throughout the breeding season in female anurans (Guerriero et al., 2000; Guerriero and Ciarcia, 2001). Very little is known, however, about how these hormone characteristics influence the female amphibian's behavior.

The studies that have examined hormones and behavior in female amphibians generally concentrate on hormonal induction of receptivity toward males. Sexually

receptive behavior in female amphibians can be considered an approach toward advertising males (Schmidt, 1984; 1985; Zerani and Gobbetti, 1993), emitting a vocalization (Tobias et. al., 1998), or inhibition of release calls or leg extensions (Diakow and Nemiroff, 1981; Kelley, 1982). Early studies have shown 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 F2α (Schmidt, 1984; 1985). In anuran species in which the female is able to vocalize, call production occurs when the female has mature eggs (Tobias et al., 1998), which is the same period when gonadal steroids are elevated (Harvey et al, 1997; Gobbetti and Zerani, 1999). Additionally, it is known that testosterone levels are higher in reproductive females than in males and higher than estrogen levels in many anuran species (Harvey et al., 1997; d'Istria et al., 1974; Licht et al., 1983; Iela et al., 1986; Itoh et al., 1990, Wilczynski et al., 2003; Medina et al., 2004). Although, the function of this is largely unclear, it is possible that in species where the females vocalize, testosterone may be involved in regulating this behavior (Emerson and Boyd, 1999). Furthermore, in *X. laevis*, receptivity has been induced in ovariectomized females with just E and P administration; however, maximal receptivity (i.e. vocalization production and leg extensions inhibited) was achieved with an additional HCG injection (Kelley, 1982). Similar behavioral effects have been induced in *X. laevis* using prostaglandin F2α (Weintraub et. al., 1985). On the contrary, in *R. pipiens*, E and P administration did not inhibit release calls in ovariectomized females (Diakow et al., 1978). In female crested newts, corticosterone was the only steroid that differed in concentration between receptive and non-receptive females (Zerani and Gobbetti, 1993). Perhaps these studies indicate that E and P are necessary but not sufficient at evoking maximally receptive behaviors in female amphibians.

It is thought that the male advertisement signals synchronize receptive states of males and females. Surprisingly, however, little is known about the physiological and behavioral effect that reception of male signals have on female amphibians and how changes in physiology effects reception of signals. In female túngara frogs, exposure to

natural mate choruses for 10 consecutive nights caused significant elevations in estrogen levels whereas females exposed to random tones did not have significant elevation in estrogen (Lynch and Wilczynski, 2004). In addition, female Majorcan midwife toads (*Alytes mulentensis*) exposed to conspecific mate choruses continued to ripen and mature eggs whereas females exposed to heterospecific calls or random tones reabsorbed resources from their eggs (Lea et. al., 2001). Also, in some urodele species estrogen and prolactin modulate the responsiveness of the vomeronasal epithelium to male pheromones, which in turn, increases female receptivity (Kikuyama et al., 2002 for review). Clearly, in order to understand whether male advertisement signals can function to synchronize male and female receptive states in amphibians, more studies should be conducted on the female's behavior and reproductive physiology.

***Physalaemus pustulosus* as a model system**

The ability to isolate the exact mate signal(s) females pay attention to when searching for a mate is not a trivial task. In many taxa, it is not clear which aspects of the males' signals gets the female's attention. However, in anuran amphibians, it is clear that the female bases her mate choice decision almost entirely on the acoustic signal produced by the males (Ryan 1985; Ryan and Rand 2001; Gerhardt and Huber 2002). This is the primary reason why female anurans serve as such a good model for studies on the basic principals of mate choice. In addition, when a female anuran has made a choice among potential mates, she will approach him and allow the male to clasp her from the dorsal side (i.e. amplexus). This phonotaxis behavior is relatively stereotyped and can be readily elicited in laboratory tests, which is another reason why the female anuran is commonly examined in mate choice studies. Here, we examine mate choice in túngara frogs, a Neotropical species, that have been the subject of a long series of investigations into sexual selection and mate choice. This species is a classic model for mate choice studies because male túngara frogs produce two basic types of calls that result in differential response rates from the females. The first is a simple call known as a whine, which is approximately a 400 ms call with a frequency modulated sweep from

approximately 900 Hz (the dominant frequency) to 400 Hz (Ryan, 1985). The second type of call, the complex call, is a whine with one or more chucks added to the end (referred to as the whine-chuck). Nearly all of the energy in the chuck is above 1500 Hz with an average dominant frequency about 2500 Hz (Wilczynski et al., 1999). It is also known that different components of the call stimulate different organs in the peripheral auditory system. The amphibian papilla is tuned to low frequencies (between 100 and 1200 Hz) and is stimulated by the whine whereas the high frequencies within the chuck stimulate the basilar papilla (Ryan et al., 1990).

Spectral and temporal parameters of the call have been examined to determine how they contribute to species recognition and preference. For instance, call recognition will not occur if the temporal order of the FM sweep is reversed so that it sweeps from a low frequency to a high frequency (Ryan, 1983). Call recognition will occur using stimulation in the high frequency range between 900 and 560 Hz followed by stimulation in the low frequency range between 640 and 500 Hz. Altering the temporal placement of the chuck so it is either before the whine or overlapping the end of the whine is more attractive to females than the whine alone but chucks added onto the start of the whine do not elicit phonotaxis responses (Wilczynski et al., 1999). These studies indicate that the initial portion of the whine is necessary for species recognition and that the recognition system is tolerant to variation in calls (Wilczynski et al., 1995; 1999).

It is also well understood how variation in signals influences female choice within populations and even among populations of *Physalaemus*. For instance, classic studies demonstrate that the whine is sufficient for phonotaxis and the chuck alone is not, but when placed at the end of the whine, it increases the whine's attractiveness (Rand and Ryan 1981; Ryan, 1985). Furthermore, females prefer calls with a chuck fundamental frequency around 200 Hz over a chuck with a fundamental frequency of 260 Hz. Because male size and fundamental frequency of the chuck are negatively correlated, this indicates that females use the frequency of the chuck to discriminate among males of different sizes (Ryan, 1980). Females also unanimously prefer the call of their own species to the calls of other *Physalaemus* species. In many cases, females fail to

recognize the call of other *Physalaemus* species, however, when a *P. pustulatus* call ornament is added on to the whine of a *P. pustulosus* call, females prefer the artificial ornament to a conspecific whine (Ryan and Rand, 1993a). Also, the *P. pustulosus* call can be made more attractive by adding a single frequency burst within the range of the basilar papilla, adding energy to any of the normal frequencies in the whine or the chuck or adding chucks with lower fundamental frequencies (Ryan and Rand, 1990; Wilczynski et al., 1995). These results indicate that male túngara frogs have exploited only one of a variety of calls that could attract females.

Studies in the *Physalaemus* species group also offer support for the sensory exploitation hypothesis, which posits that males evolve traits that exploit pre-existing female preferences. For instance, the calls of a closely related *Physalaemus* species, specifically *P. coloradorum* (a species in which no chucks exist), can be made more attractive by adding a *P. pustulosus* chuck on to the end of their conspecific call (Ryan and Rand, 1993b), indicating that the preference for the chuck may have evolved before the expression of the chuck. A comparison of the tuning of the basilar papilla (BP) between these two species offered further support for sensory exploitation hypothesis because there were no differences in the tuning of the BP even though only the *P. pustulosus* call has chucks that stimulate the BP (Ryan et al., 1990). Furthermore, a comparison of basilar papilla (BP) tuning in five *Physalaemus* species found that the BP was similarly tuned in all five species within the *Physalaemus* species group, even though most of the other sister species do not add ornaments to their whines. This suggests a conserved tuning pattern within this phylogeny and the *P. pustulosus* male evolved to further exploit the tuning of the BP whereas other sister species have not (Wilczynski et al., 2001). Although the male *P. pustulosus* evolved to add chucks to their calls, thereby increasing their attractiveness, the chuck can be replaced with various alternatives, such as white noise, that will elicit just as much response from females as the chuck (Ryan and Rand, 1990), indicating permissiveness in the female's preference for call adornments. Furthermore, when individual females were repeatedly tested with various call adornments in lieu of the chuck, they displayed little consistency in their choices,

suggesting that individual females display permissiveness for call adornments (Kime et al., 1998). This broad preference for call adornments can allow sexually selected call variants to evolve through sensory exploitation.

The spectral and temporal components of the túngara frog call that are responsible for species recognition as well as call attraction are well understood in this species. The sheer amount of information that is known about the males' calls as well as the females' responses provides an excellent opportunity to examine flexibility in mate choice behaviors.

Immediate Early Genes (IEG) as a Measure of Neural Activity

The expression of immediate early genes (IEG) can be used to examine neural correlates of mate choice flexibility. Immediate early genes represent evoked or immediate response within a neuron. Neuronal intracellular signal transduction begins with the activation of receptors that activate second messenger systems. Second messenger systems activate constitutively active transcription factors, which bind to the promoter regions of immediate early genes thereby inducing the transcription of immediate early genes, such as *egr-1* or *c-fos*. Immediate early gene expression is often used as a means of measuring neural activity because during an action potential there is a pulse of increased IEG gene transcription that occurs within minutes after neuronal stimulation (Clayton, 2000). The benefit of using immediate early genes to measure neuron activity is that in most parts of the brain, electrophysiological activity and immediate early gene expression are co-induced by synaptic neurotransmitter release thereby providing an opportunity to simultaneously measure neuron activity in multiple brain areas (Jarvis, 2004). Expression of immediate early genes can also indicate whether stimuli are contextually relevant or salient (Maney et al., 2003). However, it is estimated that a single neuron can express up to a hundred or so immediate early genes in response to a stimulus (Clayton, 2000). Therefore, not all neurons respond to stimulation with the same suite of immediate early gene therefore if a brain area lacks expression of a particular immediate early gene, it does not necessarily mean there was a lack of neuronal activation (Jarvis, 2004). Also, immediate early genes are only expressed during neuron

excitation and not during neuron inhibition (Clayton, 2000). Nonetheless, immediate early gene expression is effectively used in studies of avian acoustic communication (Mello et al., 1992; Gentner et al., 2001; Sockman et al., 2002; Maney et al., 2003) and song learning (Jarvis et al. 1995; Clayton, 1997). For instance, it has been shown that male songbirds exposed to conspecific songs show robust expression of ZENK (aka egr-1) in the caudomedial neostriatum (NCM) and the caudomedial part of the hyperstriatum ventrale (cmHV) when compared to control males (Mello et al., 1992). In chapter 4, we measure the expression of one immediate early gene, *egr-1*(early growth response gene 1), as our indicator of neuronal activity in the auditory midbrain of a female amphibian in animals that have been either treated with hormones or saline before exposure to mate choruses or silence.

Summary

This dissertation project is composed of a series of related studies that begin with an analysis of flexibility in female mate choice behavior over the course of a reproductive cycle. This project then moves to an investigation of possible hormonal basis for such flexibility. Finally, this project examines differences in neural activity in the auditory system as a possible mechanism for the changes induced by gonadal hormones on female responses to mate signals.

Chapter 2

Plasticity in female mate choice associated with changing reproductive states

INTRODUCTION

For the sake of convenience, mate choice studies treat female choice as if it were static. Recent studies, however, report that females are flexible in their mate decisions over the course of their lifetime (Kodric-Brown and Nicoletto, 2001; Moore and Moore, 2001), throughout the breeding season (Qvarnström et al., 2000; Veen et al., 2001) and even throughout a single reproductive cycle (Lea et al., 2000). Theoretical models of mate choice behavior suggest that it is important for females to be flexible in their mate decisions because of constraints that may be imposed upon the female as she is actively searching for a mate (Real, 1990). Such constraints may arise from both intrinsic and extrinsic factors. Extrinsic factors, such as high predation risk, can increase the cost of mate sampling (Crowley et al., 1991; Endler and Houde, 1995; Rand et al., 1997), thereby influencing a mate-searching female to use sampling strategies that have reduced costs, such as mate copying (reviewed in Pruett-Jones, 1992). Intrinsic factors, such as the approach of the time at which the breeding season will end or the time at which egg deposition is imminent, can cause time constraints on mate-searching females. As these critical times approach, the female may be faced with a trade-off in which she will have to decrease the strength of her mate preference, thereby expressing plasticity in her mate choice, or risk losing the opportunity to fertilize her eggs.

Although theoretical models predict the appearance of flexibility in mate decisions under constraints and recent empirical studies have tested these predictions, no study systematically examines different aspects of mate choice behavior to determine how each contributes to overall flexibility in mate choice. This study examines three aspects of mate choice behaviors, receptivity, permissiveness and discrimination. We consider receptive behavior to be a response to any conspecific call. Receptivity can also be thought of as indicating a female's motivation to mate so that an increase in this behavior increases the probability that she will fertilize her eggs. A permissive mate

choice is one in which a female responds to a mate signal that is normally unattractive, perhaps even at the minimum threshold for a female's response; a response to this signal will increase the female's probability of mating, but not with her preferred phenotype. There are two alternative explanations for the occurrence of a permissive mate choice. First, it is possible that the ability of the female to discriminate or discern the difference between mate signals has been reduced (Gerhardt, 1987). Alternatively, the female's ability to discriminate amongst mate signals may remain intact but she may instead lower her threshold for accepting signals (i.e. become less choosy). This may reflect a reduction in the energy and/or time she is willing to invest in mate sampling (Jennions and Petrie, 1997). We can discern between these alternative hypotheses by testing whether a female that responds to a mate signal with a reduced quality when she is given no other choice also maintains her discriminatory response to the preferred signal when it is readily available, indicating that her discriminatory ability has not been reduced.

The objectives of this study were two-fold. First, we examined whether mate choice behaviors that increase the probability of fertilization, such as receptivity and permissiveness, increase under time constraints caused by the approach of the time at which the female must oviposit. Second, we examined whether an increase in permissive mate choice was associated with either a decrease in the female discrimination or a decrease in female choosiness. An association between permissive mate choice and a decreased response to the preferred signal when it is available would support the conclusion that a decrease in discrimination contributes to permissiveness. Alternatively, an increase in permissive mate choice while discrimination in favour of the preferred call is maintained would support the conclusion that a decrease in female choosiness contributes to permissive mate choices.

Study system

We examined mate choice plasticity using anuran amphibians because anurans frequently serve as model systems for investigating basic principles of mate choice behavior (Ryan, 1985; Ryan and Rand, 2001). Most female anurans base mate choice

decisions almost entirely on the advertisement call produced by the male (Wells, 1977; Rand, 1988). Their behavior is relatively stereotyped and can be readily elicited in laboratory phonotaxis tests. Therefore, we used phonotaxis tests to assay the mate choices of female túngara frogs, *Physalaemus pustulosus*, a Neotropical species that has been the subject of a long series of investigations into sexual selection and mate choice. It is well understood how variation in male signals within túngara frog populations can influence female preferences (Ryan, 1980; Ryan, 1985; Ryan, 1997; Rand and Ryan, 1981; Ryan and Rand, 1990; Ryan and Rand, 2001; Rand et al., 1992; Wilczynski et al., 1995; Wilczynski et al., 1999) as well as how variation in auditory tuning and signals both within and between *Physalaemus* species can influence female preference (Ryan et al., 1990; Ryan and Rand, 1993a; Ryan and Rand, 1999; Wilczynski et al., 2001). The males of this species produce an advertisement call that is a frequency-modulated 'whine', to which they may add one or more 'chucks' at the end. The whine is sufficient to elicit phonotaxis, but female túngara frogs significantly prefer whines with adornments added to the end (Rand and Ryan, 1981; Rand et al., 1992; Ryan and Rand, 1990; Ryan and Rand, 1993b). When presented with a whine versus a 'whine-chuck', female túngara frogs will strongly prefer (i.e. will preferentially show phonotaxis towards) the whine-chuck. Females will show phonotaxis to a whine when it is presented by itself or paired against a noise burst, and will show a strong preference for their conspecific whine over the whines of other *Physalaemus* species. When presented only with calls that hybridize elements of conspecific and heterospecific whines, female túngara frogs will express some degree of phonotaxis towards the artificial hybrid call. In this study we used a synthetic, artificial hybrid call that had a general response rate of about 25% (Ryan et al., 2003), indicating that it is a call that is less attractive than the females' preferred call, the whine-chuck. We used this call as a stimulus to assess changes in female permissiveness.

As for many other amphibian species, female túngara frogs have distinct stages of reproductive behavior during the breeding cycle. The females ovulate and mate several times within a season during periods of approximately 4–6 weeks (Davidson and Hough, 1969; Ryan, 1985). During this time they move into breeding aggregations where males

are calling, then mate, and leave until another clutch of eggs develops and is ready for fertilization. We repeatedly tested individual females as they progressed through a single reproductive cycle. This design allowed us to assess whether mate choice plasticity occurs in the túngara frog on a timescale as short as a single reproductive cycle.

METHODS

Reproductive stages

Phonotaxis tests were repeated with individual females as they progressed through three reproductive stages: unamplexed, amplexed and postmated (Fig. 2.1). Females in the unamplexed stage were located at or near breeding ponds but before they had chosen a mate. Although Ryan (1985) reported that females come to the breeding pond only on the night they mate, it was still not possible for us to determine whether these females had already mated, were a few days from mating or were within hours of mating. Therefore, this group is considered a heterogeneous group that may be composed of females in different reproductive condition. We examined this possibility by dividing females in the unamplexed stage into two groups based on the amount of time required to oviposit after they completed phonotaxis tests in the unamplexed stage (range 0.5–100 h). These groups included: unamplexed females that mated within 24 h of testing (N = 4) and unamplexed females that mated more than 24 h after testing (N = 10). Fisher's exact test was used to compare the responses of unamplexed females in the receptivity and permissiveness phonotaxis tests to determine whether the females' responses were influenced by their readiness to oviposit.

When the female has chosen a male, she will approach him and allow him to dorsally mount her and clasp her with his forelimbs. This is known as amplexus and this was the next reproductive stage we examined. Amplexus behavior brings the cloacae nearly into contact as the two frogs simultaneously release gametes. Male and female frogs will remain in amplexus for several hours. Once the female enters into amplexus and allows the male to clasp her, this can serve as a behavioral indicator that the female is near the point at which she will release her eggs. In order to remeasure the phonotactic

responses of the same females that were tested in the unamplexed condition, we held each subject in a 10-gallon (37.85-litre) aquarium with water and a thick layer of leaf litter, which was used as shelter. A single male was also placed in the aquarium. We tested the female's mate choices again when she emerged from the leaf litter and allowed the male to amplex her, which provided us with an indication that she would soon deposit eggs. During the interim, natural mate choruses were broadcast from 1900 hours to approximately 0300 hours and all subjects were fed termites every other day until their release. It took an average ± SD of 3.3 ± 2.36 days (range 1–9 days) for females to enter into amplexus with the male that was provided to them. Variation in female attractiveness to the male did not appear to impact the time in which females entered into amplexus, because a male would amplex a female, even an unreceptive female, whenever the opportunity presented itself.

Finally, we placed the female back into the aquarium with the same male and allowed mating to proceed. Following mating, the female deposits her eggs in a foam nest (Heyer & Rand 1977), then the pair leaves with no further investment. In the present study, it took an average ± SD of 1.04 ± 0.72 days (range 0–3 days) for the female to enter into amplexus again and release her eggs. The time at which the female oviposited was recorded. In the postmated stage, we tested females' phonotaxis response approximately every other night until they became unreceptive to conspecific mate signals (3.6 ± 3.7 days). Some subjects did not become unreceptive during the postmated stage.

Field collections

Female túngara frogs were collected in Gamboa, Panama during June and July of 2001. Most female *P. pustulosus* were captured while unamplexed ($N = 31$) between 1930 and 2200 hours, but some females were captured in amplexus ($N = 13$). Snout–vent lengths of females captured while unamplexed (32.16 ± 1.46mm) were significantly larger than those of females captured during amplexus (30.99 ± 0.59 mm; $t_{42} = 2.78$, $P < 0.005$), and 61% of the unamplexed females subsequently mated. This indicates that

females in the unamplexed group can be considered sexually mature. Females were brought into the laboratory at the Smithsonian Tropical Research Institute in Gamboa for phonotaxis tests after they were captured. After each female completed all phonotaxis tests she received a unique toe clip number and was returned to the site at which she was captured. We followed the recommended toe-clipping guidelines of the Applied Ecology Research Group. Many toe-clipped females were recaptured; however, recaptured females were not used for further phonotaxis tests in this study.

Phonotaxis chamber and phonotaxis experiments

The testing chamber measured 1.8 × 2.7 m and was equipped with acoustic foam on the walls to reduce acoustic reverberation. Two ADS L2000 speakers were placed 2.7 m apart at equal distances from the centre of the chamber. The 10-cm point was marked completely around each speaker. The peak intensity of the acoustic stimulus was set at 82 dB SPL (re. 20 μPa) in the centre of the chamber where the female was released. The phonotaxis chamber contained a video camera and an infrared light so that behavioral observations could be made from outside the chamber.

Phonotaxis tests began at approximately 1900 hours and ended at approximately 0800 hours. At the start of the phonotaxis test each subject was placed in the centre of the chamber under a funnel for 3 min. During this time the acoustic stimuli were broadcast antiphonally from each speaker with a 0.5-s delay between presentations. The side on which each stimulus was presented was alternated to control for side bias. Once the funnel was lifted, the female was given 15 min to respond to either stimulus. A response was recorded if the female came within 10 cm of a speaker. If she remained stationary for at least two consecutive minutes, failed to move from the release site within 5 min, or did not approach a speaker within 15 min, she was recorded as unresponsive to the acoustic stimuli.

Each subject completed three consecutive phonotaxis tests per reproductive stage. The first and third phonotaxis tests were used to measure receptivity. In each test, the female heard a conspecific whine from one speaker and a conspecific whine-chuck from

the opposing speaker. A response to either of these stimuli was sufficient to label the female as receptive, however, the female needed to respond in the first and last phonotaxis tests to be labelled as receptive. Therefore, this test only measured response or no response to any conspecific call. Females that did not respond in either the first or last test were recorded as unreceptive. Females that approached a speaker in only one of the two tests were also considered nonreceptive, because we could not be sure that an apparent response in only one test indicated receptivity or was simply a random movement towards one of the speakers. All subjects completed the remaining phonotaxis tests regardless of their receptive state. The next phonotaxis test was used to measure female permissiveness. During this test, the female heard a synthesized artificial hybrid whine from one speaker and white noise of equal duration and amplitude from the opposite speaker. This synthesized artificial hybrid call had previously been determined to elicit a 25% response rate from female túngara frogs, which indicates that it is a less attractive advertisement call than the conspecific mate call (Ryan et al., 2003). Females that approached the speaker broadcasting the hybrid whine were recorded as responding permissively, whereas females that did not approach the speaker were recorded as nonpermissive responders. We used the last phonotaxis test to measure receptivity and discrimination. Females that consistently responded to the whine-chuck in both the first and last phonotaxis tests were considered discriminatory responders, whereas females that were not consistent in their choice of the whine-chuck were considered nondiscriminatory responders.

We also recorded the latency to respond (time from raising of the funnel to the female arriving within 10 cm of a speaker) in each of the tests. Subjects that did not respond received a latency score of 900 s, which was the maximum time allowed for each female to make a choice.

All stimuli were synthesized on a Dell computer with unpublished software produced by J. Schwartz. Previous phonotaxis tests using synthesized conspecific calls versus natural calls showed that female *P. pustulosus* do not discriminate between the two call types, indicating that the synthetic call captures the salient features in the

conspecific call that are necessary to elicit phonotaxis behavior (A. S. Rand and M. J. Ryan, unpublished data). The synthesized call is based on the average signal parameters and has been used in a large number of phonotaxis experiments with this species, providing a baseline of response. The artificial hybrid call was a synthetic call with parameters that were intermediate between the calls of *P. pustulosus* and *P. enesefae* (see Ryan et al., 2003).

Statistics

The number of females that completed the phonotaxis tests in each stage varied: unamplexed ($N = 31$), amplexed ($N = 34$) and postmated ($N = 30$). The total number of subjects in each group included females that repeated phonotaxis tests in two or three reproductive stages and some females that completed tests in only a single reproductive stage. Therefore, we used descriptive statistics to examine the behavioral pattern for all of the females tested (Table 2.1).

Responses of some females that were collected in the unamplexed stage were not measured in the amplexed stage, because they released eggs rapidly once they were amplexed, and responses of some females that were collected in the amplexed stage were only measured in the amplexed and postmated stages. Consequently, there were 37 females that completed the phonotaxis tests in at least two reproductive stages and only 11 females that completed the phonotaxis tests in all three reproductive stages. Therefore, we analysed the data using all possible pairwise comparisons, which included: females that completed both the unamplexed and amplexed stages ($N = 20$), females that completed both the amplexed and postmated stages ($N = 22$), and females that completed both the unamplexed and postmated stage ($N = 16$).

Proportion of responsive females

We used a chi-square goodness-of-fit test to examine whether the proportion of females that responded in the receptivity and permissiveness tests differed significantly between reproductive stages. The number of responses in one reproductive stage was

used as the expected response frequency and the number of responses from the same females in a different reproductive stage was used as the observed frequency. This allowed us to test females against their own responses. We used the amplexed reproductive stage as our expected group whenever possible. Separate analyses were done for receptivity and permissiveness tests. The alpha value was set at 0.05.

Responses of individual females

We also used a chi-square test to examine whether individual females significantly decreased their responsiveness in the receptivity and permissiveness tests between stages. Females that were responsive during the receptivity and/or permissiveness test in the first reproductive stage were examined to determine whether they were still responsive during these phonotaxis tests in the second reproductive stage. This analysis was conducted as described above. In addition, we used a paired samples *t* test to examine whether the female's response time during the receptivity and permissiveness phonotaxis tests was significantly different between stages. We compared females' latency to respond during the receptivity tests by averaging response time in both the first and last phonotaxis test for each group of females. Females that did not respond received a score of 900 s, which was the maximum time allowed for each female to make a choice.

Discrimination

Because we assessed discriminatory responses of each female that completed one reproductive stage, we used the total number of females responding to the whine-chuck in both the first and the third phonotaxis tests (conspecific call tests) to determine the percentage of discriminatory response within each reproductive stage. We used a Fisher's exact test to determine whether the proportion of unamplexed or postmated females displaying discriminating responses differed from the proportion of females displaying discriminating responses in the amplexed stage.

Association between receptivity and permissiveness

We used two analyses to examine whether more motivated females, as measured by their latency to respond to a conspecific signal, were also more likely to respond permissively. First, we divided the total number of amplexed females into permissive ($N = 12$) and nonpermissive ($N = 22$) responders, then compared each group's latency to respond to conspecific mate calls using a Student's *t* test. In addition, we examined whether the time to respond to a conspecific call in the receptivity test significantly explained the variation in the time to respond to an artificial hybrid call in the permissiveness test using simple linear regression. Non-responses were given a score of 900 s.

Latency to respond to mate signals and time to oviposit

We recorded the time from the last phonotaxis test to oviposition for 25 females. Periodic checks (0.5–5 h) were made to note whether the female released eggs. Because we did not have a continuous method for checking the time of egg release, we used an analysis in which we divided females according to whether they required more ($N = 11$) or less ($N = 14$) than the median time to oviposit. The median time was 14.5 h after their last phonotaxis test. We compared the latency to respond in both the receptivity and permissiveness phonotaxis tests using a Student's *t* test. All alpha values were set at 0.05 for these comparisons. Values are given as means ± SD unless stated otherwise.

RESULTS

Receptivity

Descriptive statistics for all females that completed receptivity phonotaxis tests in at least one stage showed that the time to respond to conspecific calls decreased from the unamplexed to the amplexed stage and increased again in the postmated stage. Similarly, the frequency of response to these calls increased from the unamplexed to the amplexed stage and declined again in the postmated stage (Table 2.1).

Proportion of receptive females

Among females that completed both the unamplexed and amplexed reproductive stages, three females responded in both conspecific call tests in the unamplexed stage. When those same females entered into amplexus, nine females responded each time they were tested with a conspecific advertisement call ($N = 20$; $\chi_1^2 = 7.27$, $P = 0.007$; Fig. 2.2a); thus, the proportion of females that expressed receptivity at these two stages differed significantly. Fourteen females responded to the conspecific calls while in the amplexed stage but when those same females entered the postmated stage, only four responded ($N = 22$; $\chi_1^2 = 19.64$, $P < 0.001$; Fig. 2.2a), again indicating that the proportion of receptive females changed significantly between the two stages. Six females responded in both conspecific call tests while in the unamplexed stage but once those same females mated, four were responsive to conspecific calls ($N = 16$; $\chi_1^2 = 1.07$, $P = 0.30$; Fig. 2.2a).

Receptivity of individual females

Of the three females that were receptive during the unamplexed stage, two were still receptive in the amplexed stage ($\chi_1^2 = 0.392$, $P = 0.53$; Table 2.2), indicating that the increase in receptivity was due to nonreceptive females changing to a receptive state when they reached the amplexed stage. In addition, of the 14 females that were receptive during the amplexed stage, three were still receptive during the postmated stage ($\chi_1^2 = 23.77$, $P < 0.001$; Table 2.2), indicating that the decrease in receptivity was due to receptive females changing to a nonreceptive state when they reached the postmated stage. Finally, of the six females that were receptive during the unamplexed stage, two were still receptive during the postmated stage ($\chi_1^2 = 4.27$, $P = 0.04$; Table 2.2). These results show that there was a significant decline in the receptive responses of individual females from the unamplexed to the postmated stage; however, there was no significant difference in the proportion of receptive females between these stages. Together, these analyses indicate that the receptive females in the unamplexed stage significantly reduced their receptivity while other females began to express receptivity entering the postmated stage.

Further examination of receptivity in individual females showed that there was a significant decrease in the latency to respond to a conspecific mate signal for females that completed the receptivity phonotaxis test in both the unamplexed and amplexed condition (728.02 ± 242.36 s and 449.55 ± 341.70 s, respectively; $t_{19} = 3.339$, $P = 0.003$; Fig. 2.2b). The latency to respond to a conspecific mate signal significantly increased for the females that completed the receptivity phonotaxis test in both the amplexed and postmated condition (324.02 ± 333.44 s and 668.20 ± 275.27 s, respectively; $t_{21} = -5.18$, $P < 0.0001$; Fig. 2.2b). There was no difference in the latency to respond to a conspecific mate signal for females that completed the receptivity phonotaxis test in both the unamplexed and postmated conditions (507.93 ± 343.95 s and 652.9 ± 309.73 s; $t_{15} = -1.325$, $P = 0.205$; Fig. 2.2b).

Permissiveness results

Descriptive statistics for all females that completed a permissiveness phonotaxis test in at least one stage showed that the time to respond to an artificial hybrid call decreased from the unamplexed to the amplexed stage and increased again in the postmated stage. Similarly, the frequency of response to the hybrid call increased from the unamplexed to the amplexed stage and declined again in the postmated stage (Table 2.1).

Proportion of permissive females

Among females that were measured in the unamplexed and amplexed reproductive stages, no female had responded to the artificial hybrid call in the unamplexed stage but when those same females entered the amplexed stage, four responded to the artificial hybrid call ($N = 20$; $\chi_1^2 = 5.0$, $P = 0.025$; Fig. 2.3a). Ten females responded permissively while in the amplexed stage, which decreased to four after they had mated ($N = 22$; $\chi_1^2 = 6.6$, $P = 0.01$; Fig. 2.3a). Finally, four females responded to the artificial hybrid call while they were in the unamplexed and postmated stages ($N = 16$; $\chi_1^2 = 0.0$, $P = 1.0$; Fig. 2.3a).

Permissiveness of individual females

Clearly, none of the permissive females in the amplexed stage were the same females that had made permissive mate choices in the previous stage ($\chi_1^2 = 0.0$, $P = 1.0$; Table 2.2). Therefore, the increase in permissiveness was due to nonpermissive females changing to a permissive state when they reached the amplexed stage. In addition, of the 10 females that were permissive in the amplexed stage, two were still permissive in the postmated stage ($\chi_1^2 = 11.73$, $P < 0.001$; Table 2.2), indicating that the decrease in permissiveness was due to permissive females changing to a nonpermissive state when they reached the postmated stage. Finally, of the four females that were permissive during the unamplexed stage, only one was still permissive during the postmated stage ($\chi_1^2 = 3.0$, $P = 0.08$; Table 2.2). The marginally significant difference between the groups suggests that there was a decline in the permissive responses of individual females from the unamplexed to the postmated stage; however, there was no significant difference in the proportion of permissive females between these stages. Again, these analyses indicate that the permissive females in the unamplexed stage were significantly reducing their permissiveness as females in the postmated stage began to express permissiveness. Further examination of permissiveness in individual females showed that there was a significant decrease in the latency to respond to an artificial hybrid signal for females that completed the permissiveness phonotaxis test in both the unamplexed and amplexed condition (900 ± 0.0 s and 742.75 ± 327.48 s, respectively; $t_{19} = 2.15$, $P = 0.045$; Fig. 2.3b). The latency to respond to the artificial hybrid signal significantly increased for the females that completed the permissiveness phonotaxis test in both the amplexed and postmated condition (529.81 ± 415.99 s and 785.23 ± 275.9 s, respectively; $t_{21} = -2.67$, $P = 0.014$; Fig. 2.3b). There was no difference in the latency to respond to the artificial hybrid signal for females that completed the permissiveness phonotaxis test in both the unamplexed and postmated conditions (709.0 ± 349.9 s and 739.87 ± 320.5 s; $t_{15} = -0.282$, $P = 0.782$; Fig. 2.3b).

Discrimination

Females that responded to the whine-chuck in both the first and last phonotaxis tests were considered discriminating. Females that were inconsistent in their responses between these two tests were considered to have nondiscriminating responses. No female consistently chose a whine alone in both tests. The results for each reproductive stage are given in Table 2.3. Nine females responded in both conspecific call tests in the unamplexed stage. Seven of those females consistently chose the whine-chuck. Of the 18 females that responded in both conspecific call tests, 14 consistently chose the whine-chuck in the amplexed stage. In contrast, six females chose the whine-chuck in both conspecific call tests in the postmated stage, but only one female consistently chose the whine-chuck in both tests. The results of a Fisher's exact test revealed that the proportions of discriminatory responses in the unamplexed and amplexed stages were not significantly different ($P = 1.0$). On the contrary, there were a significantly greater proportion of discriminatory responses in the amplexed stage compared with the postmated stage ($P = 0.015$).

We also examined whether the females that responded to the artificial hybrid call were also more likely to show inconsistent responses during the conspecific call tests. Five females behaved permissively in the unamplexed stage (16% permissiveness; $N = 31$). Three of the five showed discriminatory behavior, one did not, and one did not consistently respond in the discrimination tests. Twelve females behaved permissively in the amplexed stage (35% permissiveness; $N = 34$). Seven of the 12 showed discriminatory behavior, three did not and two did not consistently respond in the discrimination tests. Finally, five females behaved permissively in the postmated stage (16.7% permissiveness; $N = 30$). Three of the five females showed no discriminatory response and two did not consistently respond in the discrimination test (Table 2.3).

Heterogeneity within the unamplexed stage

All females that oviposited within 24 h from the time they completed their unamplexed phonotaxis test ($N = 4$) were receptive to conspecific calls. Only one female

out of the 10 that required more time to oviposit were receptive to conspecific calls (Fisher's exact test: $P = 0.005$). In addition, three of the four unamplexed females that oviposited immediately after testing responded to the artificial hybrid call, whereas only one female that required more than 24 h to oviposit responded to the artificial hybrid call (Fisher's exact test: $P = 0.04$).

Association between receptivity and permissiveness

We rarely observed permissive mate choice in females that did not show receptive behavior in both conspecific call tests (3.2% of 31 females, 5.9% of 34 females and 6.7% of 30 females in the unamplexed, amplexed and postmated groups, respectively). Therefore, we examined whether increases in receptivity, as measured by latency to respond to a conspecific signal, were accompanied by an increased likelihood to respond permissively. We divided females according to whether they responded permissively (Fig. 2.4). Permissive responders required a mean of 196.83 ± 178.33 s ($N = 12$) to respond to a conspecific call, which was significantly less time than the 517.07 ± 355.56 s that nonpermissive responders required to choose a conspecific call ($N = 22$; $t_{32} = 3.49$, $P = 0.001$). Furthermore, linear regression revealed that some of the variation in time to respond to a hybrid call was significantly explained by the female's time to respond to a conspecific call ($N = 34$; $r^2 = 0.21$; $F_{1, 32} = 8.3$, $P = 0.007$).

Latency to respond and time to oviposit

The amount of time that a female requires to respond to any mate signal may be influenced by how much time she has remaining before she must oviposit. Therefore, we examined the relationship between latency to respond in both the receptivity and permissiveness tests and time to oviposit. We divided females according to whether they required more ($N = 11$) or less ($N = 14$) than the median time (14.5 h) to oviposit after their last phonotaxis test. All females that laid eggs within 14.5 h of their last phonotaxis test displayed receptivity, whereas 27% of females that laid eggs more than 14.5 h after their last phonotaxis test displayed receptivity. Females that oviposited within 14.5 h

required an average of 86.78 ± 50.38 s to respond to a conspecific advertisement calls, whereas females that oviposited after 14.5 h required 533.7 ± 308.75 s to respond ($t_{23} = -4.7$, $P = 0.001$; Fig. 2.5). Seventy-one per cent of females that laid eggs within 14.5 h displayed permissive mate choices, whereas 36% of females that laid eggs after 14.5 h displayed permissive mate choices. Females that oviposited within 14.5 h required an average of 310.5 ± 388.74 s to respond to the hybrid call, whereas females that oviposited after 14.5 h required 632.09 ± 380.78 s to respond ($t_{23} = -2.1$, $P = 0.05$; Fig. 2.5).

DISCUSSION

The results of this study show that two aspects of mate choice behavior, receptivity and permissiveness, are low during the unamplexed stage but increase in the amplexed stage and decrease again in the postmated stage (Fig. 2.6). This pattern was shown both in the proportion of females in different reproductive stages expressing receptive and permissive behavior and in the behavioral changes of individual females moving from the unamplexed to the amplexed state. Furthermore, the mean time to respond to both a conspecific call (receptivity test) and an artificial hybrid call (permissiveness test) significantly declined from the unamplexed to the amplexed stage, then significantly increased again between the amplexed and postmated stages. Overall, these analyses show that the proportions of receptive and permissive females, the receptive and permissive states of individual females and the time to respond to mate signals all change throughout a single reproductive cycle

If amplexus is considered as a behavioral indicator that females are near the point of releasing eggs, these data suggest that female anurans increase receptivity and permissiveness as time to oviposit approaches. That is, the probability that a female will display any phonotaxis behavior, the probability that she will display phonotaxis behavior towards a less attractive mate signal (i.e. artificial hybrid call), and the speed at which she will respond to either signal all increase as time to oviposit approaches. This increase in permissive mate choice, however, is not a result of a decrease in female discrimination.

Females retain their ability to discriminate between whines and whine-chucks but are willing to accept a normally unattractive hybrid call when it is the only signal available. Furthermore, our results show that both female receptivity and female permissiveness are correlated and simultaneously increase as females approach the time at which their eggs are ready to be released. This conclusion was further supported by results showing that females that required less time to respond to an attractive conspecific mate call also required significantly less time to respond to the artificial hybrid call compared with females that required more time to respond to a conspecific call. This suggests that as a female becomes more motivated to mate, as measured by her latency to respond to a conspecific call, she will also become more likely to respond permissively. Additional analyses also support the conclusion that both female receptivity and permissiveness increase near the point of oviposition. First, within the group of unamplexed females, those that oviposited within 24 h of capture were more likely to be receptive and to respond permissively. Second, females that oviposited within 14.5 h of their last phonotaxis test showed higher receptivity than those that took longer than 14.5 h to release their eggs.

Ryan et al. (2003) points out that when female túngara frogs are tested in two-choice phonotaxis tests, they will show strong discrimination in favour of a conspecific call over a heterospecific call; however, when tested in one-choice phonotaxis tests, females will often display some degree of recognition towards the heterospecific call. This suggests that a female will always prefer the conspecific call to the hybrid call in a two-choice test. Therefore, we examined the females' responses to our hybridized (heterospecific/conspecific) call in a single choice test, and we measured the females' overall discrimination in a two-choice test. This type of design allowed us to determine the point at which females begin to accept the hybrid call and whether there is an overall breakdown in female discrimination. The results of our study show that if females do respond to less attractive signals in a single choice phonotaxis test, it does not indicate a lack of discriminatory response, because when we tested the same females in a two-choice phonotaxis test, they still displayed phonotaxis towards the preferred conspecific

call. Thus, our results suggest, instead, that the increase in permissive mate choices during the amplexed stage is probably explained by females lowering their threshold for accepting calls, thereby broadening the range of mate signals that were acceptable rather than losing an ability to discriminate among signals. Murphy and Gerhardt (1996) reported a similar result in a study conducted with female barking treefrogs, *Hyla gratiosa*. They tested females with calls that differed either in fundamental frequency or in repetition rate and they found that discrimination of females tested before and after they entered amplexus did not differ.

Interestingly, females in the postmated stage continued to display phonotaxis behavior despite having released their eggs. There is no clear adaptive explanation as to why females should continue to be receptive to male mate signals when they no longer have eggs to fertilize. The behavior may instead be the residual effects of whatever mechanism increased female receptivity as time to oviposit approached. For example, it may indicate the gradual clearing of some endocrine product that peaked near oviposition.

During the postmated stage, both receptivity and permissiveness fell to levels seen in the preamplexus stage, again indicating some link in these two processes. Females in this stage, however, were the only ones that did not discriminate between the whine and the whine-chuck. This result is consistent with Lea et al.'s (2000) finding for midwife toads, *Alytes muletensis*, that females show positive phonotaxis after mating but do not reliably discriminate between calls with low (1.5 kHz) and mean (1.8 kHz) frequencies. We do not know whether the lack of consistent preference in our study was because females were unable to distinguish between the two test stimuli or because females perceive the differences as unimportant; that is, whether the differences in the test stimuli were ‘just noticeably different’ or ‘just meaningfully different’ (Nelson and Marler 1990). Nevertheless, the results from the postmated stage support the conclusion that changes in permissiveness are not strictly tied to changes in the female's ability or willingness to discriminate between signals.

Both an increase in receptivity and permissiveness may increase a female's probability of having her eggs fertilized, thereby increasing the probability that the high cost of creating and maintaining her oocytes will not be wasted. Current theoretical models of mate choice behavior make opposing predictions about the direction of change in female choosiness under time constraints. Real (1990) presented a model predicting that the criteria determining the acceptance of a potential mate should be relaxed if mate choice occurs under time constraints. Moore and Moore (2001) recently provided empirical support for this model by showing that time constraints due to reproductive ageing cause female cockroaches, *Nauphoeta cinerea*, to reduce choosiness during mate choice tests. Alternatively, however, Crowley et al. (1991) presented a model predicting that females under time constraints caused by the approach of the end of the breeding season should increase choosiness. This model makes these predictions for animals that have only a single breeding season during which they repeatedly reproduce. Qvarnström et al. (2000) recently provided support for this model by showing that female collared flycatchers, *Ficedula albicollis*, display flexibility in their preference for the size of male forehead patch over the course of the breeding season. Their results showed that female preference becomes more marked, rather than less marked, as time becomes limited near the conclusion of the season. They also reported that large-patched males adjust their behavior to allocate more effort into postmating reproductive activities later in the season and as a consequence, females that mate with them late in the season benefit through increased reproductive success. In addition, Forsgren (1997) showed that female sand gobies, *Pomatoschistus minutus*, become more selective later in the breeding season. The opposing predictions of theoretical models as well as evidence provided by empirical studies indicate that life-history traits of an animal, such as the number of breeding seasons the adults will experience and/or whether the adults provide parental investment to the offspring, should be considered when making predictions about the directional change of female choosiness. However, regardless of whether female choosiness is relaxed or intensified under time constraints, the significant result in these mate choice

studies is that females do show flexibility in their mate decisions over time, indicating that mate choice is in fact not static.

The results of our study indicate that reproductive stage can influence the expression of three aspects of female mate choice behavior: receptivity, permissiveness and discrimination. Furthermore, it appears that receptivity and permissiveness may be associated so that changes in one behavior are paralleled by changes in the other behavior. One explanation for this result is that these behaviors may be influenced by a common mechanism. Because these behaviors appear to vary according to reproductive stage, it is possible that they are under the influence of the same neuroendocrine mechanisms. The neuroendocrine mechanisms that contribute to female receptivity are well understood in a variety of taxa including birds (Noble, 1973; Delville and Balthazart, 1987) amphibians (Diakow and Nemiroff, 1981; Schmidt, 1984; Schmidt, 1985; Boyd, 1994) and mammals (Tetel et al., 1994; Cushing and Carter, 1999). One mechanism by which hormones could modulate permissive behavior is by direct action on sensory neurons. For instance, Yovanof and Feng (1983) found that intraventricular injections of oestradiol increase average midbrain auditory-evoked responses in *Rana pipiens*. Additionally, Bereiter and Barker (1980) showed that injections of oestradiol cause receptive field areas of trigeminal mechanoreceptive neurons to enlarge in female rats. Furthermore, these authors found that receptive field area varied significantly throughout the oestrous cycle, with the largest area appearing during oestrus and the smallest area appearing in dioestrus.

The results of our study provide support for theoretical models that predict that female mate choice should be plastic if it occurs under time constraints and indicate that changes can occur on a timescale as short as a single reproductive cycle. We found that, as the time to deposit eggs approached, females increased receptive and permissive mate choice behaviours. Furthermore, these two behaviours fluctuated in parallel throughout the breeding cycle. We also report that the increase in permissive mate choices was not due to a decrease in discriminatory responses but rather indicates a change in a female's threshold for accepting unattractive calls. It is possible that receptivity and

permissiveness share a common mechanism so that changes in one behavior are accompanied by changes in the other behavior. Future studies may reveal whether a common mechanism can influence these behaviors.

Receptivity	**Unamplexed**	**Amplexed**	**Post-Mated**
Percent response	29%	53%	20%
Mean time to respond	616.72 sec	404.04 sec	673.33 sec
s.d.	323.41 sec	339.38 sec	280.16 sec
Permissiveness			
Percent response	16%	35%	16.7%
Mean time to respond	785.83 sec	618.18 sec	786.76 sec
s.d.	273.82 sec	390.12 sec	279.41 sec
N	31	34	30

Table 2.1. Descriptive statistics provide the general pattern of response for all females that were tested in each reproductive stage. Additional repeated measures comparisons support this pattern by showing that the proportion of responsive females changes among the stages and that individual females do fluctuate in their tendency to perform each behavior throughout the reproductive cycle.

Reproductive stages	Response ⬆	Response ⬇	Response None	Response Both Stage	Total N	P
Receptivity						
Unamplexed / Amplexed	7	1	10	2	20	P = 0.53
Amplexed / Post-mated	1	11	7	3	22	P < 0.001
Unamplexed / Post-mated	2	4	8	2	16	P = 0.03
Permissiveness						
Unamplexed / Amplexed	4	0	16	0	20	P = 1.0
Amplexed / Post-mated	2	8	10	2	22	P < 0.001
Unamplexed / Post-mated	3	3	9	1	16	P = 0.08

Table 2.2. This comparison examines whether the responses of individual females fluctuates throughout a single reproductive cycle. Chi squared Goodness of fit was used to test whether the responsive females in the first reproductive stage were still responsive in the following stage. The large probability values in the unamplexed/amplexed comparisons indicate that the females responding in the first stage of the comparison do not stop responding in the second stage of the comparison. However, the proportion of females showing a response increases from the unamplexed to amplexed stage (figures 1a and 2a), indicating that females are changing from a non-responsive state to a responsive state as they move from the unamplexed to the amplexed stage. The small probability values in the unamplexed/amplexed comparisons indicate that the females in the first stage of the comparison stop responding in the second stage of the comparison. However, the proportion of females showing a response in each of these stages is not different, indicating that these are different females responding in each stage of the comparison.

Frequency of discrimination	# discriminatory responses	N	% discriminatory behavior
Unamplexed stage	7	9	78%
Amplexed stage	14	18	78%
Post-mated stage	1	6	17%
Permissive responders only			
Unamplexed stage	3	4	75%
Amplexed stage	7	10	70%
Post-mated stage	0	3	0%

Table 2.3. The top rows show the probability of expressing discriminatory behavior in each reproductive stage. Females were considered as having a high probability of discrimination if they consistently maintained their preference for a "whine-chuck" during the two-choice receptivity tests. Only females that responded in both conspecific call tests were considered. No female consistently chose a "whine" alone. The bottom rows show the number of females that chose the artificial hybrid call in the permissiveness test yet consistently responded to the preferred "whine-chuck" when it was available in the discrimination tests.

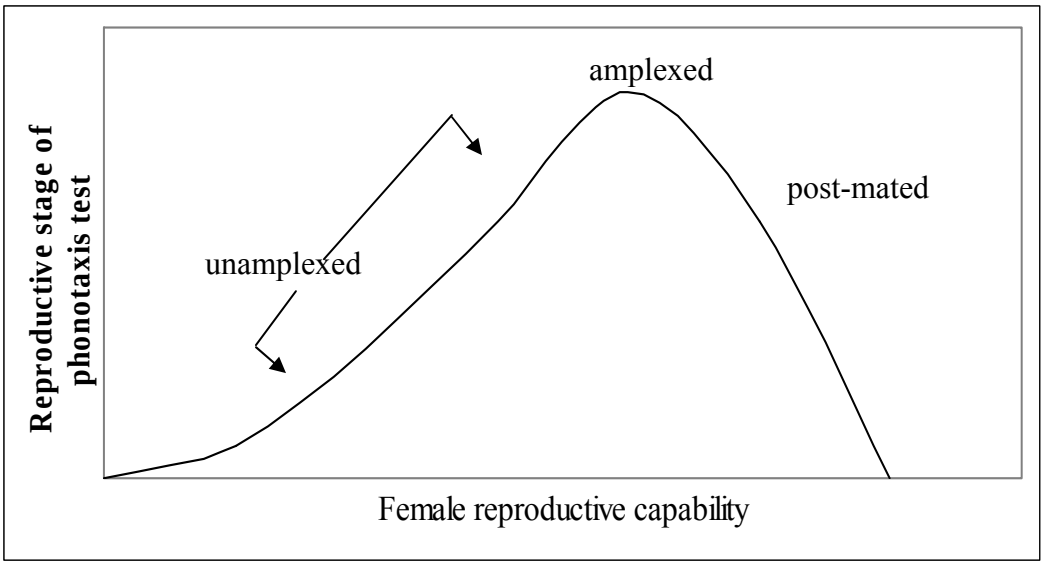


Fig. 2.1. Female mate choice was repeatedly measured in three reproductive stages as the female approached the time at which she must release eggs. These stages include the unamplexed, amplexed and post-mated stages. We measured receptivity (response to a conspecific mate signal), permissiveness (response to signals that are less attractive than conspecific signals) and discrimination (ability to discern differences among signals).

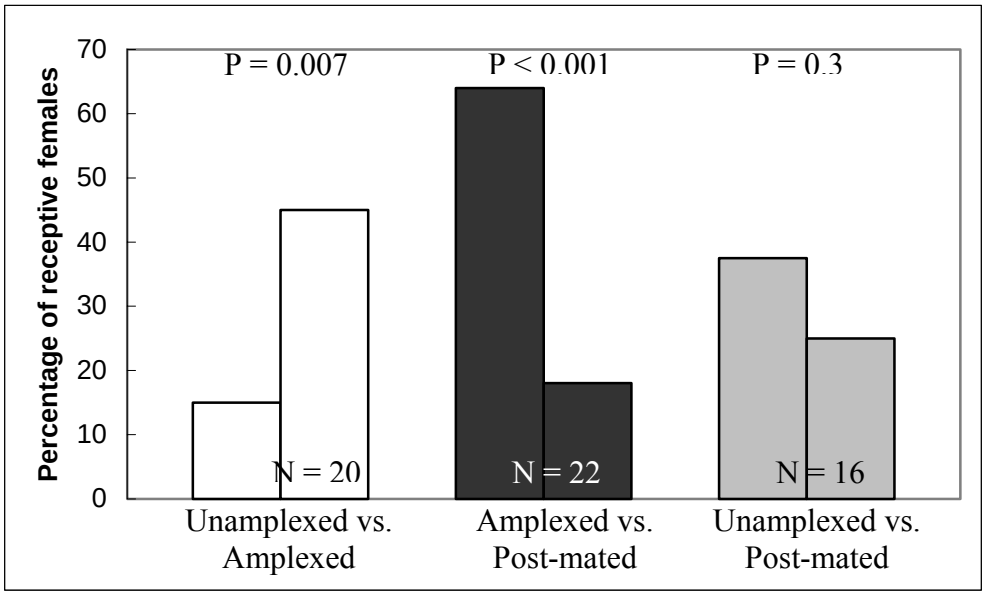


Fig. 2.2a. Pairwise comparisons were used to examine whether female receptivity fluctuates within three reproductive stages. The three within-individual comparisons include only females that completed phonotaxis tests in each of the two reproductive stages being compared. Alpha value is 0.05.

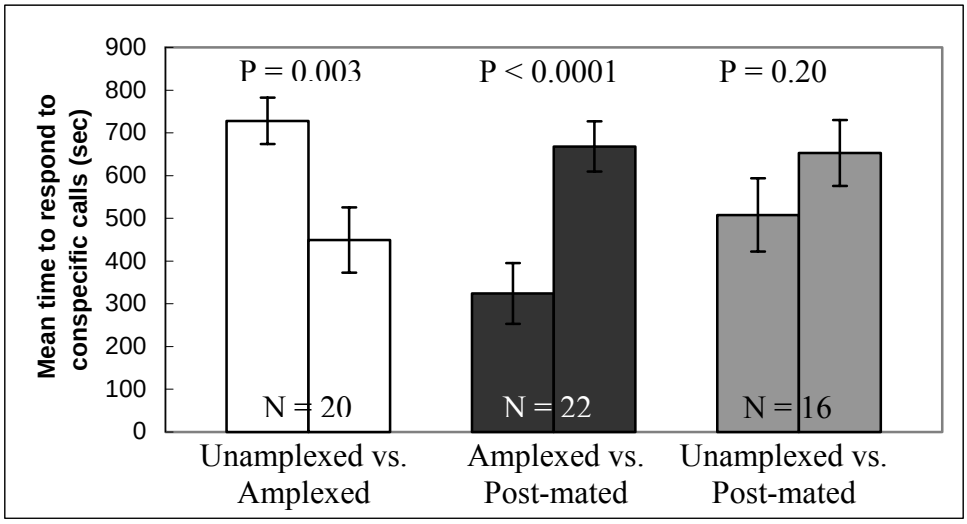


Figure 2.2b. Pairwise comparisons were used to determine whether the mean time to respond to a conspecific call during the receptivity test fluctuated within a single reproductive cycle. A paired t-test was used to examine whether individual females change the latency in which they respond to mate signals.

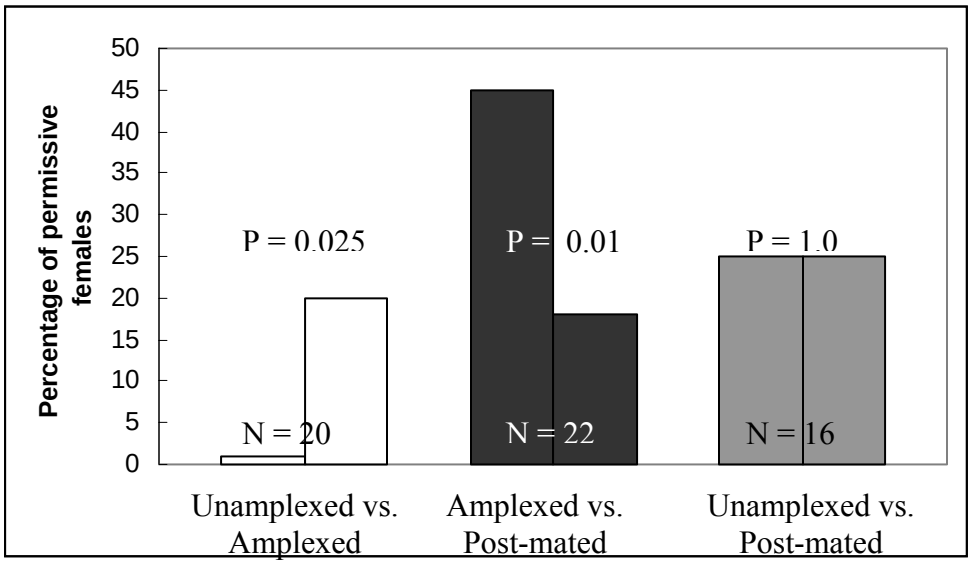


Fig. 2.3a. Pairwise comparisons were used to examine whether the probability of responding to a less attractive mate signal (permissiveness) fluctuates within a single reproductive cycle. The three pairwise comparisons include only females that completed phonotaxis tests in each of the two reproductive stages being compared.

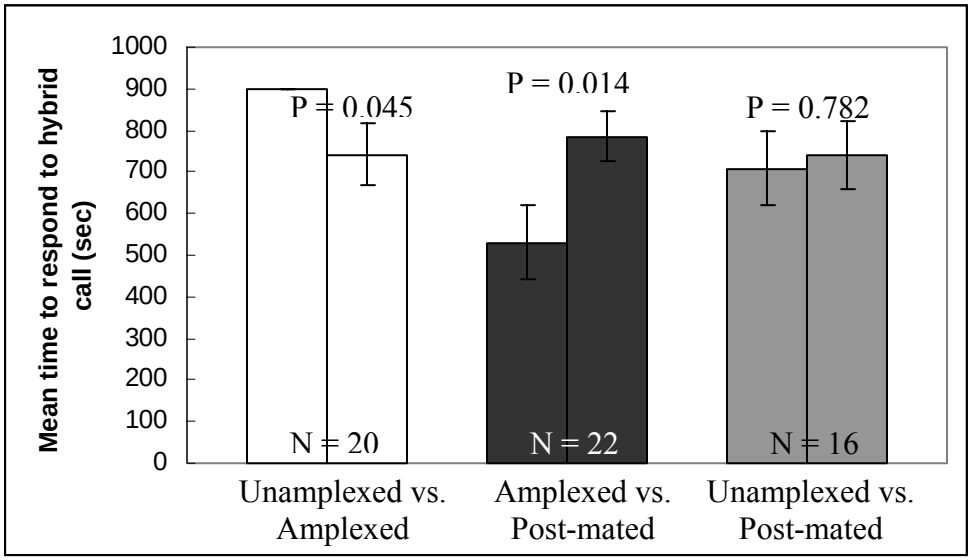


Figure 2.3b. Pairwise comparisons were used to determine whether the mean time to respond to an artificial hybrid call during the permissiveness test fluctuated within a single reproductive cycle. A paired t-test was used to examine whether individual females change the latency in which they respond to mate signals.

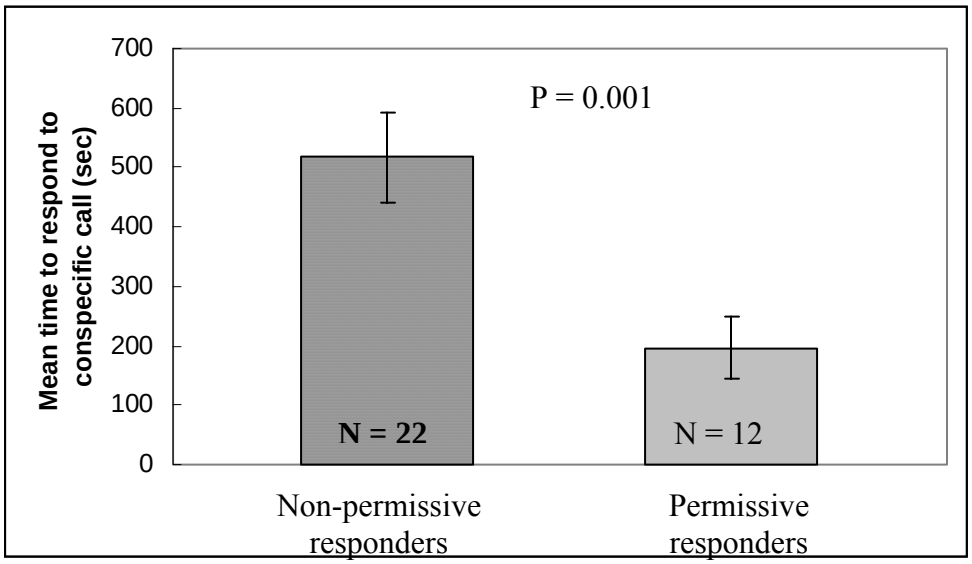


Fig. 2.4. A large majority of the permissive responders first expressed receptive behavior whereas only a very small number of females responded permissively without responding receptively. This analysis was used to determine the relationship between motivation to mate (i.e. receptivity) and permissive mate decisions to determine whether time to respond to a more attractive conspecific mate signal can be used to predict whether a female will respond permissively. Females within the amplexed reproductive stage were divided into permissive and non-permissive responders. The latency to respond during the conspecific call tests were averaged and compared between the two groups using a student's t-test.

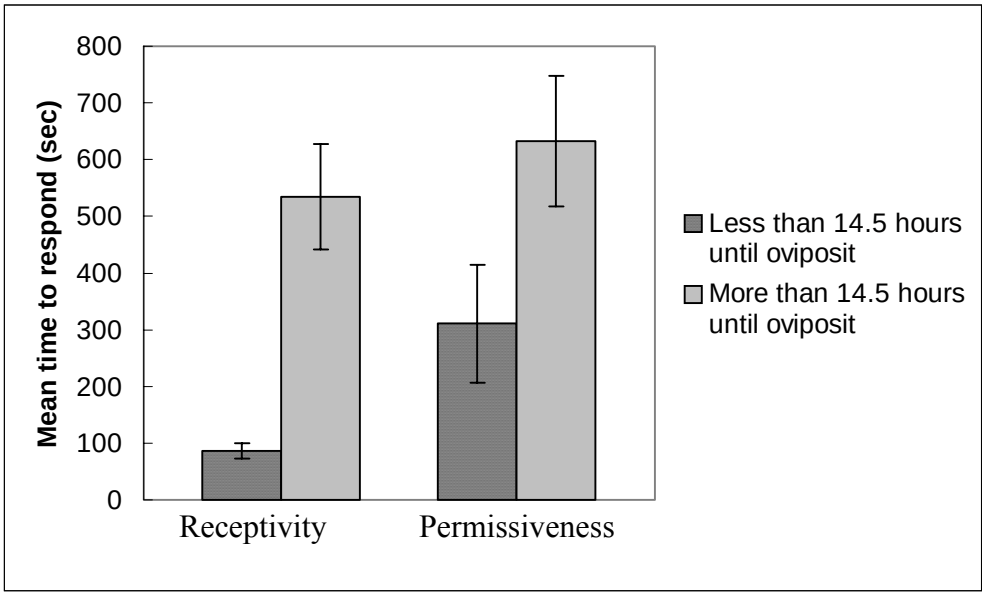


Fig. 2.5. The time required to oviposit was recorded for 25 females after the last phonotaxis test. The median time to oviposit after the last phonotaxis test was 14.5 hours. A student's t-test was used to compare the time to respond to a conspecific call and a hybrid call between females that had more and less time before they ultimately had to release their eggs.

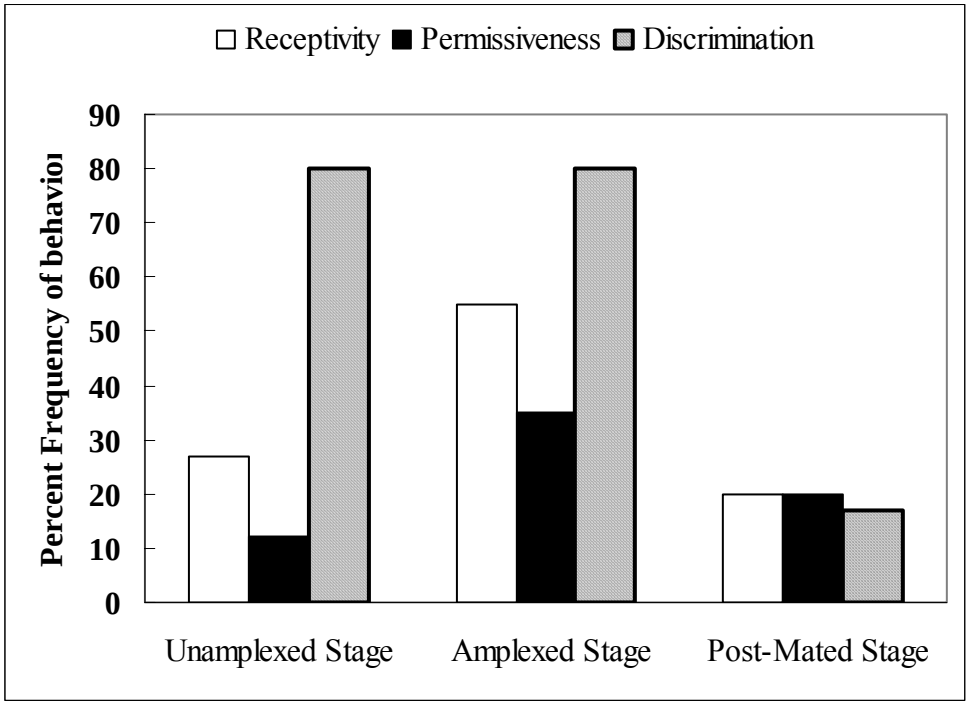


Fig. 2.6. The overall pattern of receptivity, permissiveness and discrimination that was observed in the three reproductive stages is illustrated.

Chapter 3

Gonadal Steroids Vary with Reproductive Stage in a Tropically Breeding Female Anuran

INTRODUCTION

The link between reproductive behavior and hormones is fundamental to questions in vertebrate behavioral endocrinology and has been studied in nearly every vertebrate taxon. The classic hormone-reproductive behavior paradigm is one in which a rise in gonadal hormone production immediately proceeds the expression of reproductive behavior. This pattern occurs in most vertebrates. In female rodents, for instance, there is a surge of estrogen shortly followed by a surge of progesterone, which leads to ovulation, estrous behavior and mating. Estrogen concentrations rapidly decline thereafter, but there is a gradual decline in progesterone concentrations (Nelson, 2000). Variations on this hormone-reproductive behavior pattern do exist, however, and they are thought to be a consequence of the diverse environmental conditions in which reproduction must occur (Crews and Moore, 1986). Well known variations of this classic hormone-reproductive behavior pattern have been discovered in taxa showing a dissociated mating pattern (Godwin and Crews, 2002), or a constant mating pattern (Immelmann, 1971; Hahn, 1998; Bentley et al., 2000) or a mating pattern in which there is more flexibility and less seasonality in the onset of reproductive behaviors (Harvey et al., 1997; Leitner et al., 2003).

There are also other variations in the classic reproductive behavior-hormone pattern, such as the exact role that gonadal hormones play in initiating reproductive behaviors. Crews and Moore (1986) suggested that in animals with unpredictable, aseasonal environments, gonadal hormones could have a permissive, rather than an activating role in the expression of mating behavior. The breeding seasons of some tropical amphibians have elements of this. There, breeding coincides with the rainy season, and is therefore

not totally unpredictable. Within a tropical breeding season, however, heavy rain may be sporadic, and therefore it is not as reliable a cue as temperate zone environmental cues, such as changes in day length. In many tropical amphibians, reproductive behavior must occur immediately after a rainstorm while standing water remains available. Animals must therefore maintain readiness to mate for a long time and be capable of switching on reproductive behavior quickly when local conditions are favorable. For instance, Orchinik et al. (1988) examined the role of androgens and corticosterone in the reproduction of male marine toads (*Bufo marinus*), a tropical species that exhibit bouts of breeding behavior when the appropriate environmental stimulus occurs, which is a large rainfall. Because the occurrence of a local heavy rainfall is unpredictable, even within the rainy season, these anurans are subject to unpredictable breeding conditions and therefore must be ready to breed at any time. Orchinik et al. showed that the bursts of reproductive activity during heavy rainfall were associated with elevated corticosterone levels with no change in androgen levels, suggesting that androgens played a permissive role whereas corticosterone played an activating role in the initiation of reproductive behavior. Other steroid or peptide hormones can also play an activational role (Diakow and Nemiroff, 1981; Schmidt, 1984, 1985; Weintraub et al., 1985; Moore et al., 1992; Penna et al., 1992; Boyd, 1994; Marler et al., 1995; Cushing and Carter, 1999). It is also possible that gonadal hormones are entirely responsible for the activation of breeding behavior, but different species vary in the threshold at which reproductive behaviors become activated (Emerson and Hess, 1996). Little is know about which hormones permissively or actively promote reproductive behavior in the different taxa, nonetheless, it is clear that a wide variety of hormonal strategies exist in the reproductive behavior-hormone paradigm.

In 1960, Dodd reported a causal relationship between reproductive hormones and reproductive behaviors in anuran amphibians. Since then, there have been many studies of breeding male anurans (Rastogi et al., 1978; Licht et al., 1983; Iela et al., 1986; Mendonça et al., 1985; Itoh and Ishii, 1990; Emerson et al., 1993; Emerson and Hess, 1996; Marler and Ryan, 1996; Houck and Woodley, 1995) and it has been reported that

androgen levels of breeding male frogs show substantial variation both between and within species (Licht et al., 1983, Emerson et al., 1993; Emerson and Hess, 1996). However, only a few studies have examined the relationship between natural fluctuations in reproductive hormones and reproductive behaviors in female anurans (d'Istria et al., 1974; Pierantoni et al., 1984; Iela et al., 1986; Harvey et al., 1997; Gobbetti and Zerani, 1999; Medina et al., 2004). In addition, most of these studies are conducted in anurans that do not breed in the tropics, so that little is known about the hormone patterns in female tropical anurans, which are widely subject to unpredictable environments in which they must breed. The objectives of this study are to determine the pattern of estrogen, progesterone and androgen production in a female Neotropical anuran in three reproductive stages within a single reproductive cycle and to examine whether fluctuations in these hormones occur during the reproductive stages in which plasticity in mate choice behaviors has been previously shown to occur.

We examined gonadal hormone cycles in female túngara frogs (*Physalaemus pustulosus*), a Neotropical species that requires standing water for successful reproduction, thereby making breeding periods within a rainy season unpredictable. Túngara populations in their natural environment (Ryan, 1985) as well as in the laboratory (Davidson and Hough, 1969) will show bouts of breeding after a rainfall or simulated rainfall. It has been noted that these females will even lay eggs during the dry season months if an adequate rainfall occurs (S. Rand, pers. comm.), even so, the rainy season months is when most reproduction occurs. Davidson and Hough (1969) reported that female túngara frogs retain oocytes in various stages of development, from stage I to stage VI, with stage VI being the most mature oocytes. This pattern of asynchronous oogenesis is advantageous because it allows the females to be ready to deposit mature eggs whenever breeding conditions are favorable. During the breeding season (i.e. rainy season) females are capable of mating approximately every four to six weeks (Davidson and Hough, 1969) but will do so only when local conditions are favorable.

Our previous study showed that reproductive behaviors, such as receptivity to male mate signals, fluctuated throughout the gravid / non-gravid times. Furthermore,

aspects of mate choice behavior, such as permissiveness (the likelihood of responding to unattractive mate signals), also showed periodic fluctuation between gravid and non-gravid times (Lynch et al., 2005). It is not clear, however, what the pattern of gonadal hormone production is as females fluctuate in and out of reproductive stages. It is possible that the pattern of gonadal hormones follows the classic paradigm in which the production of sex steroids is temporally associated with periods of reproductive behavior and reproduction. On the contrary, it is also possible that both asynchronous oogenesis and unpredictability of rainfall during the tropical rainy season may require more flexibility in the pattern of steroid hormone production so that the hormonal pattern will not conform to the classic paradigm. It is possible, for instance, that gonadal hormones express a general elevated level during the breeding season so that they play a permissive role in reproductive behaviors, suggesting that some other factor may be responsible for the activational role in reproductive behavior, as in the male tropical anuran, the marine toad (Orchinik et al. 1988). This study will explore these alternative hormone patterns as well as determine if elevated hormone levels occur during the same reproductive stage in which it has been reported that females express reproductive behaviors.

METHODS

Hormone levels were measured in similar reproductive stages as described in Lynch et al. (2005). These stages include the unamplexed, amplexed and post-mated stage. Females that were found at the breeding pond but had not yet chosen a mate were placed in the unamplexed group. Females that had chosen a mate and allowed the male to clasp them from the dorsal side were considered amplexed. The presence of an amplexed male serves as a behavioral indicator that the female is approaching the time at which she will release eggs. In this study, females 10 days after releasing eggs were the post-mated group, however, in Lynch et al. the post-mated group was 1-2 days after mating.

Females were collected in Gamboa, Panama in June and July of 2002 and 2004. All females were caught in either an unamplexed or amplexed state and immediately brought to the laboratory at the Smithsonian Tropical Research Institute. Blood was

collected via the orbital sinus from unamplexed females on the same night of capture. Females capture while in amplexus were randomly placed into one of two groups. Some had blood collected on the night of capture so that the sample was taken before oviposition occurred. Some were allowed to oviposit and held in a 10 gallon aquarium for 10 nights so that blood was collected for the post-mated group. The blood was centrifuged and the plasma layer frozen in liquid nitrogen. Samples were then shipped to the University of Texas on dry ice where they were stored at -20 degrees until assayed.

The plasma collected in the 2002 breeding season was used to assay estrogen concentrations during the three reproductive stages (unamplexed, n = 3; amplexed, n = 16; post-mated, n = 20). The plasma collected in the 2004 breeding season was used to assay progesterone (unamplexed, n = 6; amplexed, n = 14; post-mated, n = 13) and androgens (unamplexed, n = 4; amplexed, n = 14; post-mated, n =17). Because we can only collect a small volume of blood from each individual, estrogen measurements were done from one set of subjects whereas progesterone and androgens were both measured from a different set of subjects. Our method of measuring testosterone (described below) had 27.4% and 18.9 % cross reactivity with 5α-dihydrotestosterone and 5β-dihydrotestosterone respectively, therefore, we will refer to testosterone measurements simply as androgens.

The volume of plasma used in each assay ranged from 5 µl to 20 µl. Plasma samples were spiked with 20µl of tritiated estrogen, progesterone or testosterone and extracted using 3mL of diethyl ether. The extraction procedure resulted in a mean recovery of 74% +/- 11% for estrogen, 95.8 % +/- 13.4% for testosterone and 28 % +/- 1.9% for progesterone. Recovery values were used to correct the concentration of hormone estimated in each sample. Hormone assays were done using enzyme immunoassay (EIA) kits purchased from Caymen Chemical. These kits were validated prior to use in this study by extracting hormone from a pooled sample of frog plasma then repeatedly measuring the concentrations at three to four dilutions. The assay estimated the hormone concentration in each dilution to be within 20% of each other. We assayed each sample in this study in duplicate (estrogen and progesterone) or triplicate

(androgens) and each sample was measured at a minimum of two dilutions. Inter-assay variation was 9.06%, 2.34% and 5.08% for estrogen, progesterone and androgens respectively. Intra-assay variation was 9.47%, 10.03% and 6.71% for estrogen, progesterone and androgens respectively. Cross reactivity in the estrogen kit was 0.1% for testosterone and 5 α-dihydrotestosterone, 0.07 % for 17 α-estradiol, and 0.03% for progesterone. Cross reactivity in the progesterone kit was 7.2% for 17 β- estradiol and 0.01% for 17-α estradiol.

Samples that were not diluted enough to fall within the sensitive area of the standard curve (i.e. too much hormone in the sample) were removed from the analysis due to unreliable estimation of hormone concentration. Samples that were measured at the lowest dilution, yet still not within the sensitive area of the curve (i.e. not enough detectable hormone in the sample) received the lowest detectable amount for that particular assay (lowest detectable amount; E = 0.023 ng/mL and P = 0.013 ng/mL). No values in the testosterone assay needed this substitution. The concentration of hormones during the three reproductive stages was compared using Kruskall-Wallis tests with an alpha value of 0.05. All statistics were conducted between individuals in the three reproductive stages. All values are reported as the mean +/- standard error.

RESULTS

Fluctuations in Plasma Estrogen Levels

Levels of estrogen in the plasma did significantly change between the three reproductive stages ($X^2 = 6.93$; DF = 2; P = 0.03; fig. 3.1). The mean concentration of estrogen in unamplexed females was 1.46 ng/mL +/- 0. 43 ng/mL. This mean concentration increased to 2.76 ng/mL +/- .87 ng/mL during the amplexed stage. There was a significant decline in the mean concentration of estrogen in the post-mated stage (mean = 0.805ng/mL +/- 0.14ng/mL).

Fluctuations in Plasma Progesterone Levels

Levels of progesterone in the plasma did significantly change between the three reproductive stages ($X^2 = 18.57$; DF = 2; $P < 0.001$; fig. 3.1). The mean concentration of

progesterone unamplexed females was 3.01 ng/mL +/- 0.70 ng/mL. This mean concentration increased sharply to 22.87 ng/mL +/- 7.2 ng/mL during the amplexed stage. There was also a sharp decline in the mean concentration of progesterone in the post-mated stage (mean = 2.54 ng/mL +/- 0.46 ng/mL).

Fluctuations in Plasma Androgen Levels

Levels of androgen in the plasma did significantly change between the three reproductive stages ($X^2 = 14.06$; DF = 2; P = 0.001; fig. 3.1). There was a peak in the mean concentration of androgen in unamplexed females (x = 11.78 ng/mL +/- 2.2 ng/mL). This mean concentration of plasma androgen declined sharply to 2.52 ng/mL +/- 0.35 ng/mL during the amplexed stage. The plasma level of androgen did not change in the post-mated stage relative to the amplexed stage (mean = 1.91 ng/mL +/- 0.82 ng/mL).

DISCUSSION

This study showed significant changes in levels of plasma gonadal steroids in three different reproductive stages within a single reproductive cycle in a tropically breeding female anuran. In the different individuals that were measured, levels of both estrogen and progesterone peaked in the amplexed stage, which is the stage nearest to the point of ovipositing. On the contrary, androgen concentrations were highest in the unamplexed stage and significantly declined in the amplexed stage. Although these hormones were measured in different individuals, the cyclic hormone pattern reported here in the female túngara frog is similar to patterns reported in other female anurans that breed in temperate or desert zones, such as the desert spadefoot toad (*Scaphiopus couchii*) and *Rana esculenta* (Harvey et al, 1997; Gobbetti and Zerani, 1999).

Because túngara frogs have asynchronous oogenesis, in which they are constantly producing and maintaining oocytes in the ovaries and oviducts, it could have been possible that estrogen showed a constant elevated level throughout the breeding season. This would have been a reasonable alternative prediction because in vertebrates with oviparous reproduction estrogen stimulates the liver to create vitellogenin, which is

released into circulation and used by maturing oocytes to create proteins needed for egg yolk production (Wallace, 1985). Paolucci et al. (1988) showed that plasma concentrations of estrogen and vitellogenin fluctuated in synchrony throughout a reproductive cycle in *R. esculenta*. Although túngara frogs use asynchronous oogenesis as a breeding strategy, they still show periodic fluctuations in estrogen, progesterone and androgen levels, as is the case for nearly all female vertebrates. These results are paradoxical and raise the question as to why estrogen levels show cyclic changes when vitellogenin should be regularly produced. Studies that examine hormone fluctuations throughout the entire breeding season as well as between breeding and non-breeding seasons would help to provide a better understanding of hormonal control of asynchronous breeders.

Because females in this study were in captivity for 10 days after mating, it is possible that the low levels of sex steroids reported in the post-mated stage are stress induced. Licht et al. (1983) reported, however, that short-term captivity in vitellogenic female bullfrogs (*Rana catesbeiana*) caused no significant change in testosterone or estrogen levels and Coddington and Cree (1995) also report that short-term captivity did not impact levels of testosterone and estrogen in female whistling frogs (*Litoria ewingi*). However, this issue remains to be resolved in the tùngara frog.

We have previously reported fluctuations in female mate choice behavior throughout three reproductive stages and the present study shows that levels of plasma sex steroids also fluctuate across the same stages. Although we were unable to measure hormone concentrations in the same individuals, this pattern suggests a simultaneous increase in estrogen and progesterone during the same reproductive stage at which females express maximal receptivity and permissiveness, the amplexed stage. Androgen levels peak just before the expression of these behaviors but then decline when receptivity and permissiveness are highest. Furthermore, as the plasma levels of estrogen and progesterone decline within a reproductive cycle, receptive and permissive behaviors also decline. Comparing the hormone pattern reported in this study and the behavioral pattern reported in Lynch et al. suggests that a temporal relationship exists between

surges in estrogen and progesterone and the appearance of receptive behaviors in the túngara frog.

Previous studies that experimentally manipulate hormone levels have shown that estrogen and progesterone facilitate the expression of reproductive hormones in female anurans. Kelley (1982) showed that neither estrogen or progesterone alone were able to induce receptivity however, receptivity was reinstated in ovariectomized female South African clawed frogs (*Xenopus laevis*) treated with a combination of estrogen then progesterone. Schmidt (1985) showed significant increases in phonotaxis responses in female American toads (*Bufo americanus*) when progesterone was administered prior to prostaglandin, but not when prostaglandin was administered alone. The present study only shows that estrogen and progesterone concentrations are elevated during the same reproductive stages in which reproductive behaviors are reported to be elevated. The results of the hormonal manipulation studies, however, suggest that estrogen and progesterone are able to induce the expression of reproductive behaviors in female anurans and our results are consistent with this.

Our study shows that total androgen levels are elevated in the unamplexed stage. We are unable to discern whether this is an elevation of testosterone, dihydrotestosterone or both. However, Medina et al., (2004) reported that testosterone exhibits significantly higher circulating levels in female *Bufo arenum* than dihydrotestosterone during all months, including a 10-fold difference during the breeding months. Wilczynski et al. (2003) also reported elevated testosterone levels in relation to dihydrotestosterone levels in a laboratory population of *Rana pipiens* and Harvey et al. (1997) reported the same pattern in wild-caught spadefoot toads. This suggests that much of the increase in androgen concentration in mating female frogs is likely due to a significantly elevation in testosterone. Levels of testosterone in some female anurans are close to the testosterone levels reported in males and are generally higher than the concentration of estrogen in the plasma (d'Istria et al., 1974; Licht et al., 1983; Harvey et al.,1997; Wilczynski et al., 2003; Medina et al., 2004). The behavioral implications of elevated testosterone levels are largely unknown.

This study shows that gonadal hormones, specifically estrogen, progesterone and testosterone, do fluctuate throughout three reproductive stages within a single reproductive cycle in a tropical frog. The pattern of hormone fluctuation is similar to the classic paradigm in which there is a clear temporal relationship between the appearance of reproductive hormones and reproductive behaviors. Peaks in estrogen and progesterone occur at the same stage in which maximal expression of both receptive and permissive behaviors have been reported so that it is possible that these hormones contribute to the expression of these behaviors. Further experiments in which hormone levels are manipulated and behavior is measured are needed.

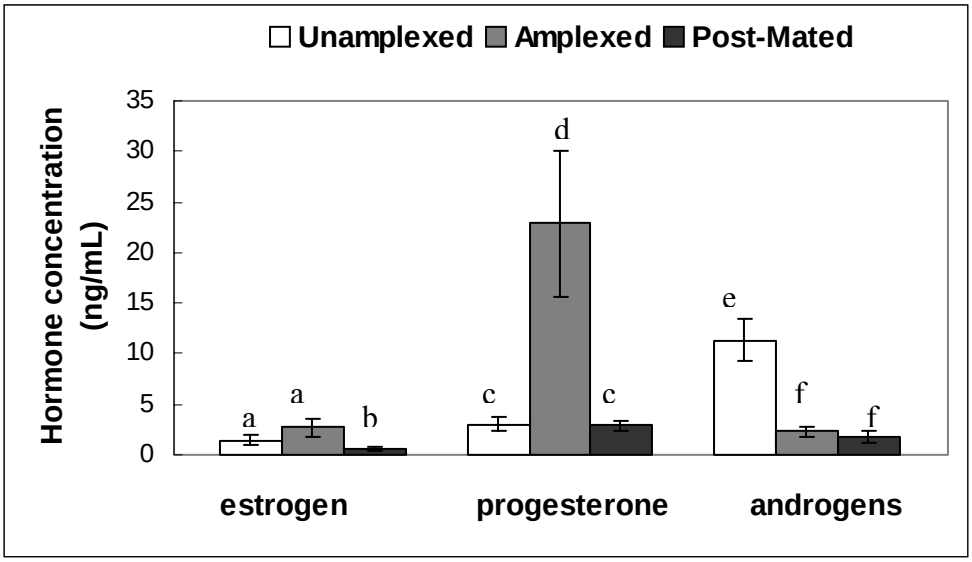


Fig. 3.1. The mean concentration of estrogen, progesterone and androgen (± s.e) throughout three different reproductive stages measured in 2002 (estrogen) and 2004. It has previously been reported that mate choice behaviors also vary throughout the same time frame in the female túngara frog. Comparisons were made using Kruskal Wallis tests with alpha value set at 0.05.

Chapter 4

Gonadotropins Induce Flexibility in Female Mate Choice in the Túngara Frog (*Physalaemus pustulosus*)

INTRODUCTION

Flexibility in female mate choice potentially influences the strength of directional selection on male advertisement signals and possibly even the strength of selection on the choice itself (see Jennions and Petrie for review, 1996). The mechanisms, however, for generating such flexibility are not well understood. Theoreticians suggest that the physiological state of the female while she is actively searching for a mate may influence the outcome of her choice (Real, 1990). For instance, hungry female scorpionflies (*Hylobittacus apicalis*) will accept males that bring small nuptial gifts but not when they are satiated (Thornhill, 1984). One aspect of the female's physiology, hormonal state, has been shown to be critical for the induction of female receptive behaviors in a variety of taxa including birds (Noble, 1973; Delville and Balthazart, 1987) amphibians (Diakow and Nemiroff, 1981; Schmidt, 1984; Schmidt, 1985; Boyd, 1994) mammals (Tetel et al., 1994; Cushing and Carter, 1999) and reptiles (Aldrete et al., 1980; Rhen et al., 1999; 2000; Rhen and Crews, 2000). It is not clear, however, if fluctuations in hormonal state may influence the female's mate selectivity such that the female lowers the threshold for mate acceptance. In one study, testosterone treated female dark-eyed juncos (*Junco hyemalis*) are less discriminating in their mate choices than control females (McGlothlin et al., 2004). Although there are no other hormone manipulation studies that provide empirical support for such predictions, it has been shown that variation in mate choice decisions can be associated with changes in reproductive stage (Lea et. al., 2000; Bosch and Boyero, 2004; Lynch et. al., 2005) and that, in a few anuran species, changes in reproductive stage are associated with changes in hormonal state (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986; Itoh and Ishii, 1990; Harvey et al., 1997; Medina

et al., 2004; Lynch and Wilczynski, *in press*). Such studies suggest that fluctuation in hormonal state may serve as a proximal mechanism for flexibility in mate choice.

Anurans are often used as a model in studies of female mate choice because the females base their mate choices almost entirely on the male's acoustic advertisement signal (Wells, 1977). Their reproductive behavior is relatively stereotyped, therefore making it easy to observe female mate choice both in the field and in the lab (Ryan, 1985). We assayed individual variation in mate choice by repeatedly testing mate preferences of female túngara frogs, a Neotropical species in which differences in male signals within a population influence female preference (Ryan 1980; Rand & Ryan 1981; Ryan 1985; Ryan & Rand 1990; Rand et al. 1992; Ryan 1997; Ryan & Rand 2001; Wilczynski et al. 1995, 1999). We used phonotaxis tests to assay three aspects of acoustic-based mate preferences in the túngara frog; receptivity, permissiveness, and discrimination. Receptivity is considered a positive phonotactic response to any conspecific male signal, permissiveness is a measure of the female's selectivity and is assessed by a response to an artificial hybrid call that is known to be less attractive than the conspecific call, and discrimination is the female's ability to discern the difference between a conspecific call and the artificial hybrid call (Lynch et al., 2005). We examined these aspects of mate choice behavior both before and after hormone manipulation to determine whether hormonal state acts as a proximal mechanism for flexibility in mate choice behavior.

Hormonal modulation of mate choice behavior may occur at any level of the hypothalamic-pituitary-gonadal (HPG) axis. It is possible, for instance, that gonadotropins can act in the central nervous system so that they alone are necessary and sufficient for modulation of mate choice behavior. Alternatively, it is possible that pituitary peptides are needed to stimulate the production of gonadal hormones, which in turn, modulate mate choice behavior. In this study we first examine whether activation of the HPG axis with human chorionic gonadotropin (HCG), a gonadotropin that is a ligand for luteinizing hormone (LH) receptors, can induce the same pattern of mate choice behaviors observed in wild-caught female túngara frogs (*Physalaemus pustulosus)* as

they transitioned through different reproductive stages. That is, as wild-caught females neared the time to oviposit, they increased motivation to mate and became less selective in their mate choices (Lynch et al., 2005). We then examined whether elevated estrogen levels are necessary and sufficient to induce such a behavioral pattern by administering HCG with an aromatase inhibitor that blocks the synthesis of estrogen. Overall, our objective in this study was to determine whether general activation of the hypothalamic-pituitary-gonadal axis contributes to the mate choice flexibility we have observed in wild-caught female túngara frogs. This initial broad approach allows us to determine whether fluctuations in the female's physiological state can act as a constraint that influences her mate choice decisions.

METHODS

All female túngara frogs used in this study were from a colony maintained at the University of Texas at Austin. Frogs in the colony were maintained in five or ten-gallon aquariums with damp moss in groups of five and fed 1-week old crickets three times per week. The frogs were housed with simulated, but clock-shifted, equatorial light/dark cycles so that dusk began at 14:00 and dawn began at 02:00.

All females had previously mated with a male (or released eggs) at least once before used for phonotaxis tests in this study, which allowed us to be sure that all females were reproductively able. Phonotaxis tests in the HCG experiment (experiment one) were conducted in July, 2003 and phonotaxis tests for HCG plus fadrozole (experiment two) were conducted in December, 2003. In experiment one, the mean weight of females was 2.45g and the mean snout-vent-length was 29.36mm. The mean weight and snout-vent-length for females in experiment two was smaller (1.146g; 25.05 mm); however, the females in experiments one and two were not different in their probability of a receptive response (Fishers exact $P = 0.32$) or a permissive response (Fishers exact $P = 1.0$) before hormone treatment.

During the phonotaxis test, the subject was placed into an acoustic chamber measuring 1.8 x 2.7 m with acoustic foam on the walls to reduce reverberation. Two

speakers were placed 2.7 m apart at equal distances from the center of the chamber. The peak intensity of the acoustic stimulus was set at 80 dB SPL (re 20 μP) in the center of the chamber where the female was initially released. All acoustic stimuli were synthesized on a Dell computer with unpublished software produced by J. Schwartz. Phonotaxis tests were conducted from approximately 13:00 to 19:00.

To initiate the phonotaxis test, the female was placed under a funnel in the center of the chamber for three minutes while acoustic stimuli are antiphonally broadcast from opposing speakers with a 0.5 second interval between the stimuli. The side on which each stimulus is presented was alternated to control for side bias. After the funnel was lifted the female was allowed 15 min to approach either speaker. A response was recorded if the female came within 10 cm of a speaker. If the female remained stationary for at least two consecutive minutes, failed to move from the release site within five minutes, or did not approach a speaker within 15 minutes, she was considered unresponsive to the acoustic stimuli. In total, each female completed four phonotaxis tests during each trial: the first receptivity test, a permissiveness test, a discrimination test and a final receptivity test.

Receptivity phonotaxis tests

The female's receptive state was examined in the first phonotaxis test. The female was given a choice between two conspecific mate signals, a whine alone and a whine-chuck. A response to either of these signals is sufficient to consider the female as receptive to mate signals. The same stimuli were repeated in the last phonotaxis test and the female needed to respond to a conspecific call in both the first and last test in order to be considered fully receptive. Females that approached a speaker in only one of the two tests were not considered receptive, as we could not be sure that an apparent response in only one test indicated receptivity or was simply a random movement toward one of the speakers.

Permissiveness phonotaxis test

Immediately following the first receptivity test, the female was re-tested to determine if she would approach an artificial hybrid call. The artificial hybrid whine was synthesized by varying spectral and temporal components of the *P. pustulosus* whine so that the call would be intermediate between the calls of *P. pustulosus* and *P. enesefae*, a closely related species. Details describing the synthesis of this call are discussed in Ryan et al. (2003). This artificial hybrid whine has previously been shown to elicit a response from only 25% of females (Ryan et al., 2003), indicating this call is less attractive to *P. pustulosus* females than are conspecific mate calls. The hybrid whine was paired against white noise with equal amplitude and duration. Females that respond to white noise were excluded from statistical analyses. This single-choice design was used because female túngara exhibit strong discrimination in favor of a conspecific signal when present, yet display some degree of recognition toward heterospecific calls when the conspecific call is not available (Ryan et al., 2003). This type of design allowed us to examine how hormonal state influences the strength of female preferences.

Discrimination phonotaxis test

Immediately following the permissiveness test we examined the female's ability to discern the difference between the conspecific whine and the hybrid whine. A response to a conspecific whine over the hybrid whine indicates the female is able to discern the difference between the two whines; that is, she is maintaining her discrimination. Conversely, a response to a hybrid whine indicates that the female has not maintained discriminatory responses.

Experiment One: Effects of HCG administration on mate choice behaviors

In the first experiment, females completed phonotaxis assays (pre HCG tests) and were randomly placed into one of five dose groups in which they received human chorionic gonadotropin (HCG; Sigma). HCG is a ligand for luteneizing hormone (LH) receptors, which causes gonadal activation and therefore, the production of gonadal

hormones. Doses of HCG include: 0 (control; N = 8), 10 (N = 8), 100 (N = 8), 500 (N = 16), or 1000 IU (N = 20). HCG was dissolved in 0.9% saline solution and given in a subcutaneous injection in a volume of 50 µl. The same phonotaxis tests described above were repeated approximately 20 – 24 hours after HCG administration (post HCG tests).

Experiment Two: Effects of fadrozole administration on mate choice behaviors

In the second experiment, females completed phonotaxis assays and received 50 µg of Fadrozole (Novartis; 4-(5,6,7,8- tetrahydrimidazo[1,5a]pyridine-5-yl)benzonitrile monohydrochloride), a potent and specific aromatase inhibitor of estrogen synthesis. The following day females received another subcutaneous injection of fadrozole in addition to either a saline injection (N = 5) or 500 IU of HCG (N = 5). Approximately 20 hours later, females were retested in phonotaxis assays to examine whether HCG caused a similar behavioral pattern to that seen in the first experiment or whether the absence of estrogen inhibited the behavioral pattern.

In both experiments, the observer scoring the behavior was unaware of the treatment each subject received.

Hormone Assays

Upon completion of phonotaxis tests blood samples were collected via the orbital sinus from five subjects in each dose group. These procedures were approved by IACUC. The blood was centrifuged and the plasma layer stored at -20° C until assayed. Plasma volumes ranged from 5 µl to 20 µl. Twenty µl of tritiated estrogen or testosterone (approximately 1000cpm) was added to each plasma sample for recovery determination. Plasma samples were extracted using 3mL of diethyl ether. The mean recovery after extraction was 71 +/- 0.09% and 95 +/- 0.16% for estrogen and androgens respectively. Hormone assays were conducted with enzyme immunoassay (EIA) kits purchased from Caymen Chemical. These kits were validated prior to use in this study by extracting hormone from a pooled sample of frog plasma then repeatedly measuring the concentrations at three to four dilutions. The assay estimated the hormone concentration

of the different dilutions to be within 20%. Each sample was assayed in duplicate and each sample was measured at a minimum of two dilutions. Inter-assay variation was 12% for estrogen whereas androgens were measured on a single plate. Intra-assay variation was 9.9 % and 9.5% for estrogen and androgens respectively. Cross reactivity in the estrogen kit was 0.1% for testosterone and 5 α- DHT, 0.07 % for 17 α-estradiol, and 0.03% for progesterone and the detection limit is 8 pg/mL. The detection limit for testosterone EIA kits is 6 pg/mL. Testosterone EIA kits have a 27.4% and 18.9 % cross reactivity with 5α-dihydrotestosterone and 5β-dihydrotestosterone respectively. Therefore, we refer to testosterone measurements simply as androgens.

In experiment two, plasma samples were collected from all subjects (N = 10) to confirm that estrogen concentrations were effectively reduced by fadrozole. The mean estrogen recovery in this experiment was 78 +/- 0.1 %. All estrogen samples were run on a single plate in this experiment. The intra-assay variation was 6.5%.

Statistical Analyses

In experiment one, we used two analyses to assess variation in receptive and permissive behaviors. First, we tested whether the probability of a response to a mate signal changed between the pre and post HCG tests using Chi-Squared Goodness of Fit. We used the frequency of responses in the post HCG condition as the expected and the frequency of response in the pre HCG condition as the observed. These tests were not used in the lower dose groups (0, 10 and 100 IU) because low response frequencies yielded low expected values. Second, we tested whether individual females changed their responses between the pre and post HCG phonotaxis tests by using a Kruskal-Wallis test to analyze the difference in the female's response time before and after HCG treatment. These scores were recorded in seconds and non-responsive females received the maximum number of seconds (900 s). These analyses were done for both the receptivity and permissiveness phonotaxis tests.

In experiment two, we examined the female's response in the receptivity phonotaxis test by using a mixed ANOVA with repeated measures to determine whether

there were significant differences in latency to respond to a conspecific mate signal after treatment with fadrozole + HCG (or saline). We also used a student t-test to determine if latency to respond to a conspecific mate signal was significantly different between females treated with fadrozole + HCG and females treated with 500 IU HCG.

In experiment two, our approach to examine the permissiveness data needed to be modified due to reduced sample sizes and non-normal distributions. Therefore, we examined whether there was a significant difference between the post-treatment groups in response time to an artificial hybrid call using a Mann Whitney Test.

We tested responses of females after treatment with HCG to determine whether hormonal manipulation influenced discrimination. We used a binomial test to examine whether the probability of a discriminatory response was significantly different from the probability of a random response.

We examined whether HCG administration significantly altered the plasma concentration of estrogen and androgens using one-way ANOVA with a Tukey's post hoc comparisons. Alpha values were set at 0.05 for all statistical tests and all reported values are mean +/- s.e.

RESULTS

Experiment One: Effects of HCG administration on mate choice behaviors

At the highest doses of HCG (500 and 1000 IU), receptivity and permissiveness were increased but there was no change in discrimination. Female receptivity among the five doses of HCG was examined by determining if females increased the number of responses after treatment and whether females responded in less time after treatment (figures 4.1a and 4.1b respectively). No females responded to a conspecific call in the pre HCG test in the control group and one responded after saline injection (N = 8). Three females responded before HCG administration and one responded after HCG administration at the 10 IU level (N = 8). At the 100 IU level, two females responded both before and after HCG treatment (N = 8). At 500 IU of HCG four females responded to a conspecific call before treatment whereas ten females responded to a conspecific call

after treatment (N = 16; $X^2 = 9.6$; DF = 1; $P < 0.002$). At 1000 IU of HCG two females responded to a conspecific call before treatment whereas 14 responded after treatment (N = 20; $X^2 = 34.28$; DF = 1; $P < 0.001$). These data show that the probability of a receptive response (i.e. positive phonotatic response to a conspecific call) increases at the 500 and 1000 IU levels.

The Kruskal-Wallis test showed a significant difference in the amount of time to respond to a conspecific mate call between the pre and post HCG tests ($X^2 = 21.1$; DF = 4; $P < 0.001$) among the five doses. The mean differences in response time were -47.5 sec ± 55.1 sec, 81.75 sec ± 90.1 sec, -15.93 sec ± 107.4 sec, 372.22 sec ± 73.3 sec and – 431.2 sec ± 79.2 sec for the 0 IU, 10 IU, 100 IU, 500 IU, and 1000 IU dose groups respectively. These data show that female receptivity is significantly higher at 500 and 1000 IU doses and that the 500 IU dose is the threshold for which receptive behaviors appear.

Female permissiveness among the five doses of HCG was examined by determining if females were more likely to respond to the less attractive hybrid call after treatment and whether treatment influenced latency to respond (figures 4.2a and 4.2b respectively). Among all the dose groups, nine females responded to white noise and were therefore excluded from this analysis. No females responded to the hybrid call in the pre or post HCG test in the control group (N = 7). One female responded before HCG administration and none responded after HCG administration at the 10 IU and 100 IU levels (N = 7). At 500 IU of HCG four females responded permissively before treatment whereas eight females responded permissively after treatment (N = 13; $X^2 = 1.23$; DF = 1; $P = 0.26$). At 1000 IU of HCG one female responded permissively before treatment whereas eight responded permissively after treatment (N = 17; $X^2 = 10.21$; DF = 1; $P = 0.001$).

A Kruskal- Wallis test showed a significant difference in the amount of change in a females' latency to respond to the hybrid call between the pre and post HCG tests ($X^2 = 9.7$, DF = 4, $P = 0.046$) among the five doses. The mean differences in response time were 0 sec ± 0 sec, 48.43 sec ± 48.43 sec, 54.71 sec ± 54.71 sec, -194.23 sec ± 87.42 sec

and –251.53 sec ± 96.88 sec for the 0 IU, 10 IU, 100 IU, 500 IU, and 1000 IU dose groups respectively. These results show that female permissiveness is significantly higher at 500 and 1000 IU doses and that the 500 IU dose is the threshold for which permissive behaviors appear.

We examined whether hormone manipulation influenced discriminate between the conspecific and hybrid whine (figure 4.3). At the three lower doses of HCG (0, 10, 100 IU) few females responded during the discrimination test, making it difficult to detect the probability of a non-random response. At the two highest doses (500 IU, N = 10; 1000 IU, N = 11) females responded to the conspecific whine over the hybrid whine significantly more often than chance (two-tailed binomial test P = 0.012 and P = 0.001 respectively).

Hormone Assays

Estrogen concentration significantly increased in a dose-dependent manner after HCG treatment (figure 4.4; N = 19; DF = 4, 14; F = 12.55; P < 0.0001). The estrogen concentration in the control group (0 IU) was 0.89 ± 0.32 ng/mL (N= 4). The 10 IU (N = 4), 100 IU (N = 4), 500 IU (N = 3) and 1000 IU (N = 4) dose groups had a mean estrogen concentration of 1.39 ± 0.30 ng/mL, 3.64 ± 0.92 ng/mL, 6.77 ± 1.05 ng/mL, 6.48 ± 1.40 ng/mL respectively. Tukey's post hoc tests revealed that both 500 and 1000 IU dose groups had significantly elevated estrogen concentrations in relation to the control group (P = 0.001 for both 500 and 1000 IU). These high doses of HCG also had significantly elevated estrogen levels in relation to the 10 IU group (P = 0.003 and P = 0.002 for 500 and 1000 IU). The estrogen concentration in the 100 IU group was marginally different from the 500 IU group (P = 0.097). There was no difference in estrogen concentration between 500 and 1000 IU dose groups (P = 0.99).

There was no significant difference in the mean concentration of androgen among the different dose groups (figure 4.5; N = 18; DF = 4, 13; F = 2.108; P = 0.14). The control group (0 IU) had a mean androgen concentration of 8.15 ± 3.15 ng/mL (N = 3). The 10 IU (N = 3), 100 IU (N = 2), 500 IU (N = 5) and 1000 IU (N = 5) dose groups had

a mean androgen concentration of 2.56 ± 0.91 ng/mL, 6.67 ± 1.19 ng/mL, 3.85 ± 2.26 ng/mL and 1.17 ± 0.41 ng/mL.

Experiment Two: Effects of fadrozole administration on mate choice behaviors

Fadrozole administration was effective at reducing circulating estrogen to undetectable levels. Each female tested in experiment two had less than the lowest detectable limit of 0.08 ng/mL of estrogen.

The mean time to respond to a conspecific call during the receptivity test was 842.5 ± 81.3 sec before treatment whereas after treatment with fadrozole + HCG female response time showed a mean of 442.6 ± 158.4 sec (N = 5). On the contrary, in the control group the mean time to respond to a conspecific call was 745.9 ± 122.2 sec before fadrozole + saline administration and response time after saline injection was 608.1 ± 169.2 sec (N=5). Repeated measures mixed ANOVA showed a within-subjects effect arising from the decreased response time in the post fadrozole + HCG treatment group (N = 10; $F_{1, 8} = 5.85$; P = 0.04). There is neither a significant main effect between groups ($F_{1, 8} = 0.10$; P = 0.76) nor a significant interaction ($F_{1, 8} = 1.4$; P = 0.27) indicating that both groups show a decrease in time to respond to a conspecific stimulus but only one (fadrozole + HCG) responded significantly faster (figure 4.6).

The mean time to respond to the artificial hybrid call during the permissiveness test was 837.3 ± 54.3 sec before treatment and 614 ± 286 sec after treatment with fadrozole + HCG (N = 3). In the control group, response time prior to fadrozole + saline administration was 720.5 ± 160.5 and 740.7 ± 159.2 sec after treatment (N = 4). There was no significant difference in the time to respond to an artificial hybrid call between the post-treatment groups (U = 5.0, P = 0.857).

The difference in the time to respond to a conspecific mate signal was calculated using pre and post treatment response times in the group receiving fadrozole + 500 IU HCG and the group receiving 500 IU HCG. A student t-test revealed that there was no significant difference in the latency to respond to a conspecific mate signal during the receptivity test between these two groups (DF = 19; t = -0.173; P = 0.86). This suggests

that the receptive state of females is similar when they are treated with 500 IU of HCG with and without pretreatment with fadrozole.

DISCUSSION

Human chorionic gonadotropin simultaneously induces a significant elevation in circulating estrogen levels while evoking receptive and permissive mate choice behaviors, indicating that activation of the HPG axis can induce flexibility in mate choice behavior. HCG activates LH receptors as well as stimulates the production of gonadal hormones so that it is possible that either of these events is responsible for the behavioral effects. The simultaneous increase in both estrogen levels as well as mate choice behaviors suggests that the effects of HCG may be due to the production of estrogen, which occurs in a dose-dependent manner but plateaus at 500 IU. Furthermore, the mean estrogen concentration with high doses of HCG is 6.5 to 6.8 ng/mL, which is within physiological range of wild-caught females that are actively reproducing (i.e. amplexing; Lynch and Wilczynski, *in press*). In addition, wild-caught females in amplexus also show maximal receptive and permissive behaviors (Lynch et al., 2005). Consequently, evidence from both hormone manipulation studies and natural hormone fluctuation studies show a peak in estrogen when receptive and permissive behaviors are maximal, suggesting that estrogen contributes to the expression of these behaviors. When we administer HCG + fadrozole, however, we still observe a significant increase in receptivity. That is, females that are pre-treated with fadrozole before HCG administration still respond to a conspecific call significantly faster during the receptivity phonotaxis tests. Such a result indicates that activation of LH receptors with HCG is may play a role in evoking receptive behavior. This may be because HCG can evoke receptive behaviors by directly acting on LH receptors in the brain. However, this conclusion is not consistent with the results presented by Kelley (1982) in which she showed that luteinizing-hormone releasing hormone (LHRH), but not HCG, was effective at maximizing receptive behavior in steroid-primed, ovariectomized female *X. laevis*. Alternatively, it is possible that HCG exerts behavioral effects by elevating peptide

hormones. For instance, Schmidt (1984) showed that the action of HCG could be inhibited if given with indomethacin, an inhibitor of prostaglandin synthesis, suggesting that HCG may exert behavioral effects by elevating prostaglandin levels.

Induction of receptive behavior in the absence of estrogen does not allow us to conclude that increased estrogen cannot produce such a result, only that estrogen is not necessary. It is possible that estrogen acts in parallel with some other factor, possibly progesterone. For instance, it has long been known that sexual behavior in female mammals can be reinstated in ovariectomized females by mimicking the cyclic release of estrogen and progesterone with exogenous hormone treatment. These experiments indicate that sexual behavior in female mammals is dependent on the action of both estrogen and progesterone (Blaustein and Erskine, 2002). Also, in female *X. laevis* neither estrogen or progesterone alone were effective at reinstating receptive behaviors in ovariectomized females, however, when administered together females showed an increase in receptive behavior. The frequency of these behaviors, however, did not increase significantly until there was an additional treatment of gonadotropin (Kelley, 1982). Also, progesterone alone or in combination with arginine vasotocin (AVT) primes females before the administration of other drugs used to induce receptivity in a female anuran (Schmidt, 1984; 1985). In addition, other studies have found peptide hormones such as AVT, prostaglandins, and LHRH to be effective at inducing receptivity in female amphibians (Diakow and Nemiroff, 1981; Kelley, 1982; Boyd, 1994; Schmidt, 1985).

Although our results indicate that HCG activation of LH receptors, without elevated estrogen, is necessary and sufficient to induce receptive behavior in female túngara frogs, it is likely that there are endogenous peripheral influences as well. For instance, female leopard frogs (*Rana pipiens*) must also ovulate and pass eggs through the oviduct in order to display receptive behavior (Diakow et al., 1988). This change in receptive behavior can be induced in ovariectomized leopard frogs by artificially distending the female with fluid (Diakow et al., 1978). These studies indicate that there are peripheral neural responses, especially in the oviduct, that contribute to the induction of receptivity. A closer examination of our data shows that females that received high

doses of HCG (500 or 1000 IU) yet never laid eggs after HCG treatment did not significantly increase receptive and permissive behaviors after hormone treatment. On the other hand, females that received high doses of HCG and laid eggs after treatment did significantly increase receptive (Chi Squared Goodness of Fit test; $N = 25$; $X^2 = 52.25$; $P < 0.0001$) and permissive behaviors ($N = 20$; $X^2 = 5.0$; $P = 0.025$) after hormone treatment. In other words, the significant increase in receptive and permissive responses observed in females treated with high doses of HCG occurs only in females that are retaining at least some mature eggs. The mechanism by which these peripheral factors influence behavior is unclear. It is possible that eggs retained within the oviduct can initiate hormone release, such as prostaglandin, which is needed for receptive behaviors. It is also possible that retained eggs cause distention of the oviduct and the somatosensory stimulation facilitates receptivity. In sum, hormonal and physiological factors may work together to influence female mate choice behavior. Our results show that activating the HPG axis is important and that activation without elevated estrogen is sufficient. The influence of estrogen and other factors, alone or in combination with gonadotropins, remain to be determined.

Theoretical models of mate choice behavior suggest that constraints can influence the outcome of a female's decision on which male to accept (Real, 1990; Crowley et al., 1991). Fluctuations in the female's physiological or hormonal state may act as a constraint that causes a mate-searching female to adjust the range of mate signals she is willing to accept. In this study the experimental manipulation of hormonal state indicates that hormones can act as an intrinsic factor capable of constraining female mate choice. Specifically, we show that the probability of response to a conspecific and an artificial hybrid signal increases and the latency to respond to these signals decreases after treatment with high doses of HCG. The increase in receptivity is consistent with studies of wild-caught, reproductively active female anurans, which show receptive behaviors are associated with natural fluctuations in LH or gonadal steroids (Itoh and Ishii, 1990; Harvey et al., 1997; Medina et al., 2004). There are few empirical studies, however, showing that hormone manipulation can alter the range of mate signals a female is

willing to accept. In this study the threshold at which this happens is not immediately obvious because females that were already responding permissively were randomly placed into the pre HCG group at the 500 IU level, which likely explains the lack of a significant increase after HCG treatment at this level. However, there is a clear increase in the frequency of permissive responders after HCG treatment between the 500 IU level and the control group. Also, examination of the time required to respond to the hybrid call during the permissiveness test indicates that females start responding significantly faster at the 500 IU level. In addition, we show that even though females broaden the range of mate signals they will accept when treated with high doses of HCG, there is no change in the female's ability to discriminate between the conspecific whine and the hybrid whine. This suggests that a decline in female choosiness is likely responsible for increased permissive mate choices, rather than a decrease in discrimination. Overall, our results suggest that flexibility in female mate choice that occurs throughout different reproductive stages may be a consequence of fluctuations in the HPG axis.

There is no significant pattern in androgen concentration among the HCG dosages, however, the trend suggests that androgen levels decline as dosage increases. Although our assays are unable to discern whether our androgen pattern is due to a change in testosterone, dihydrotestosterone or both, previous studies have reported that reproductively active female anurans exhibit significantly higher circulating levels of testosterone than dihydrotestosterone (Harvey et al., 1997; Wilczynski et al., 2003; Medina et al., 2004). In addition, levels of testosterone in some female anurans are comparable to male levels and are generally higher than the concentration of estrogen in the plasma (d'Istria et al., 1974; Licht et al., 1983; Harvey et al.,1997; Wilczynski et al., 2003; Medina et al., 2004). Although the behavioral consequences of elevated testosterone in female anurans are unclear, our results show that when females express peak receptive and permissive behaviors, androgen levels decline. This is consistent with our previous finding in which androgen levels significantly decline in wild-caught females when they go from an unamplexed to an amplexed state (Lynch and Wilczynski, 2005). Such a result can be explained if testosterone is primarily responsible for our

estimation of circulating androgens because a decline in testosterone levels may reflect aromatization of testosterone into estrogen.

Our results provide empirical support for theoretical models of mate choice behavior, which suggest that females should be flexible in mate choice decisions (Real, 1990; Crowley et al., 1991; Sullivan, 1994). Although the direction in which mate choice is predicted to change is different in different models (i.e. females decrease or increase restrictions on mate choices under constraints), theoretical models agree that females should be flexible in their decisions on which males to mate with. Our study demonstrates that activation of the HPG axis can act as a mechanism for flexible mate choice behavior in females that are maintaining oocytes and are ready to reproduce.

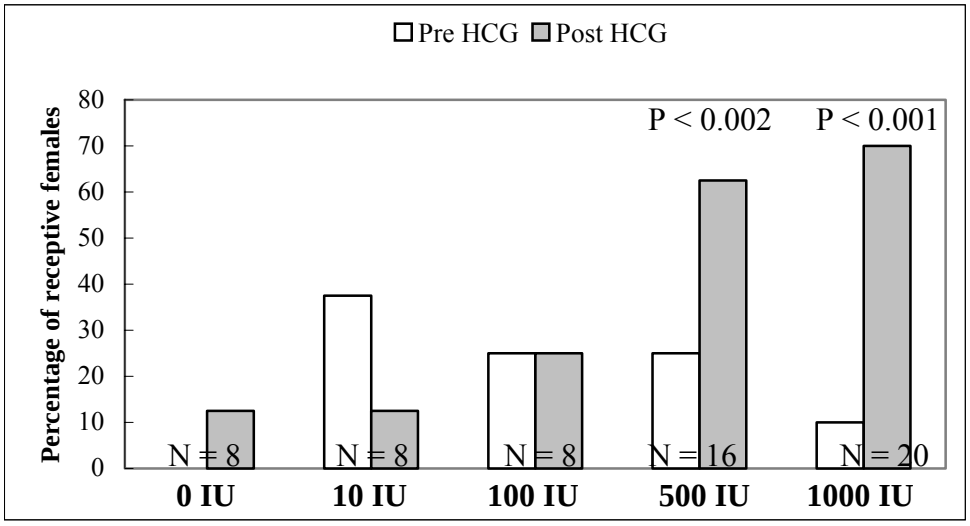


Fig. 4.1a. Responses to a conspecific mate signal during the receptivity phonotaxis test were compared before and after treatment with HCG. The probability of response was compared between pre and post HCG tests using Chi-Squared Goodness of Fit.

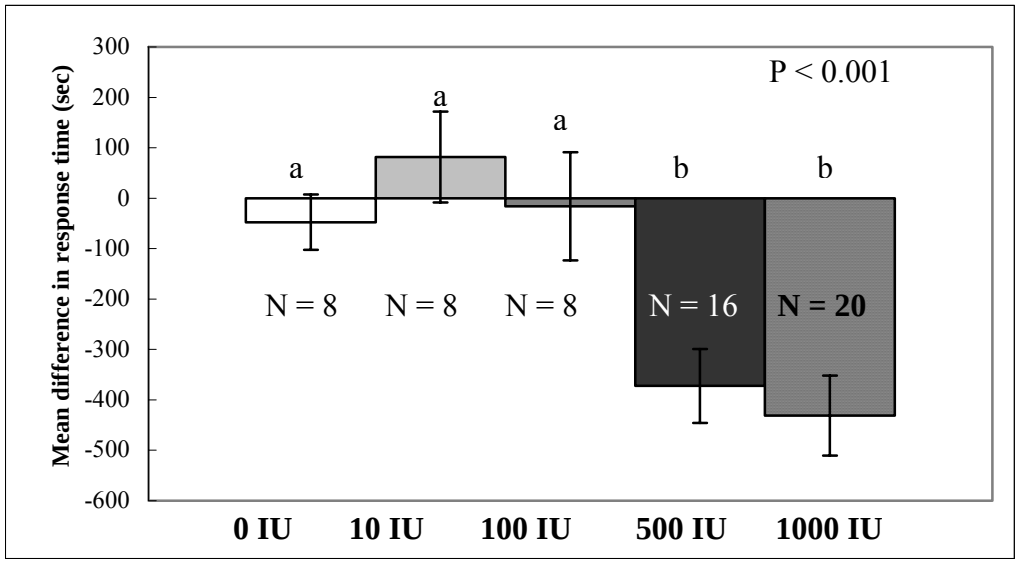


Fig. 4.1b. The difference in the time to respond to a conspecific signal before and after HCG treatment was compared among the five dose groups using a Kruskall Wallis test. Negative mean response times indicate that most females spent less time responding in the phonotaxis test after receiving HCG treatment. Reported values are mean ± s.e.

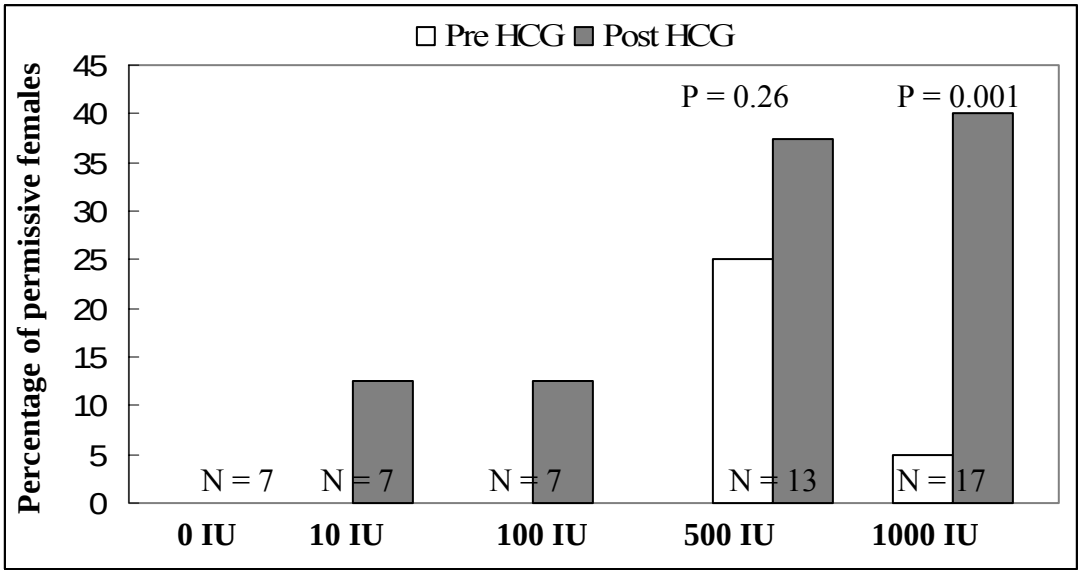


Fig. 4.2a. Responses to an artificial hybrid signal during the permissiveness phonotaxis test were compared before and after treatment with HCG. The probability of response was compared between pre and post HCG tests using Chi-Squared Goodness of Fit. Females that respond to white noise are removed from the analysis.

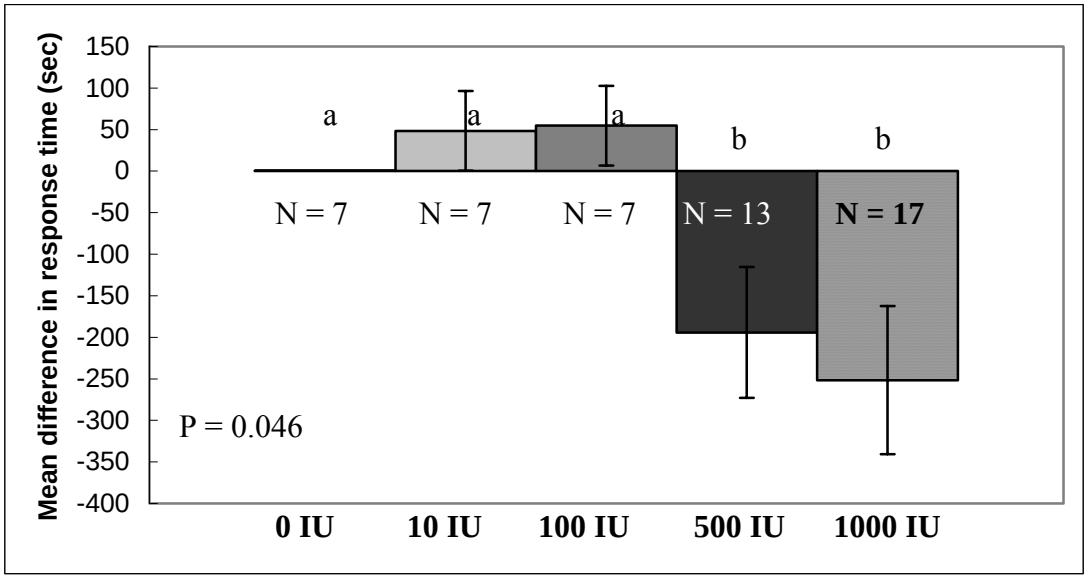


Fig. 4.2b. The difference in the time to respond to the artificial hybrid signal before and after HCG treatment was compared among the five dose groups using a Kruskall Wallis test. Negative mean response times indicate that most females spent less time responding in the phonotaxis test after receiving HCG treatment. Reported values are mean ± s.e.

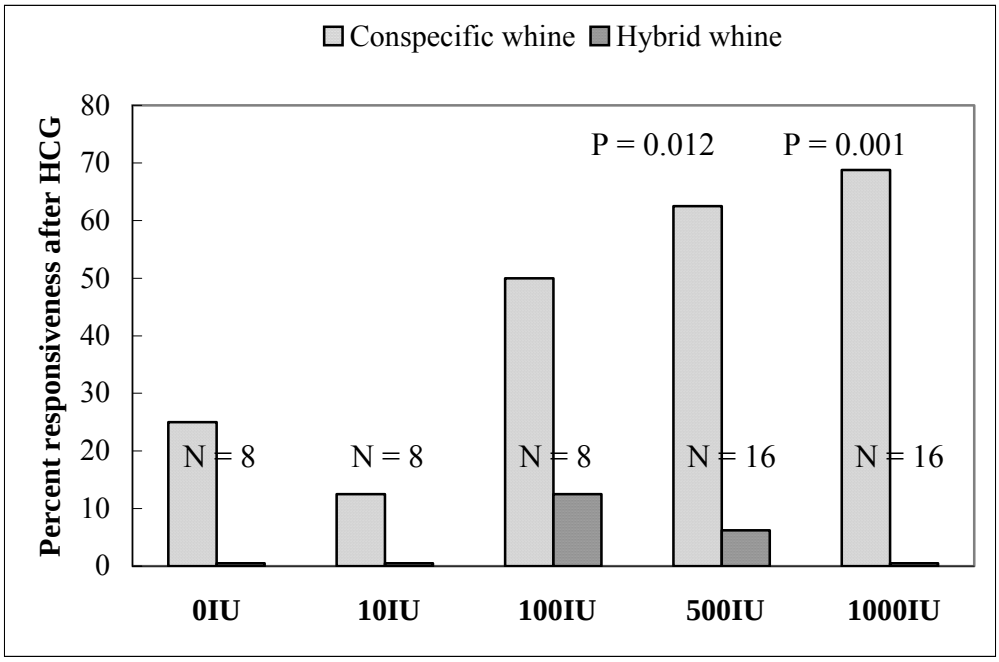


Fig. 4.3. The female's ability to discriminate the difference between the conspecific whine and the hybrid whine was compared at the level of the two highest doses because these are the groups in which responsiveness increased. A binomial test was used to examine whether the probability of a discriminatory response was significantly different from the probability of a random response.

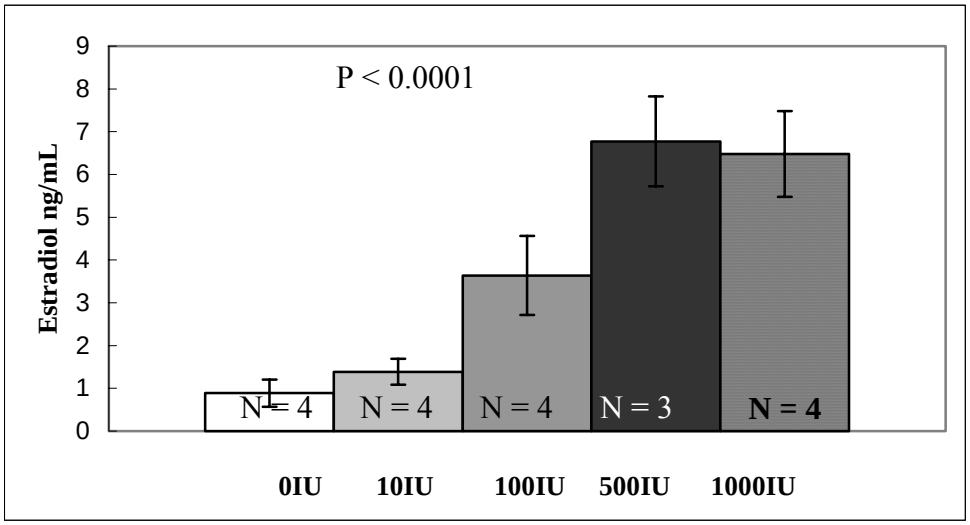


Fig. 4.4. Concentration of circulating estrogen increased in a dose-dependent manner. A Tukey's post hoc showed that a significant elevation in estrogen concentration occurred at the 500 IU dose and that there was no significant difference in estrogen concentration between 500 and 1000 IU. Differences in estrogen concentration were analyzed using one-way ANOVA. Reported values are mean ± s.e.

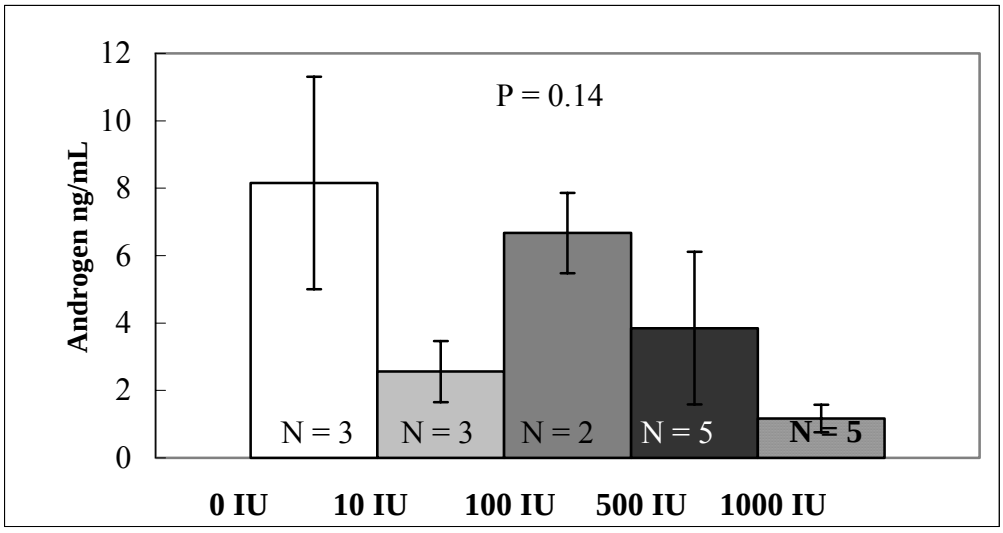


Fig. 4.5. There was no significant difference in the concentration of circulating androgens between the HCG doses. Differences in androgens concentration were analyzed using one-way ANOVA. Reported values are mean ± s.e.

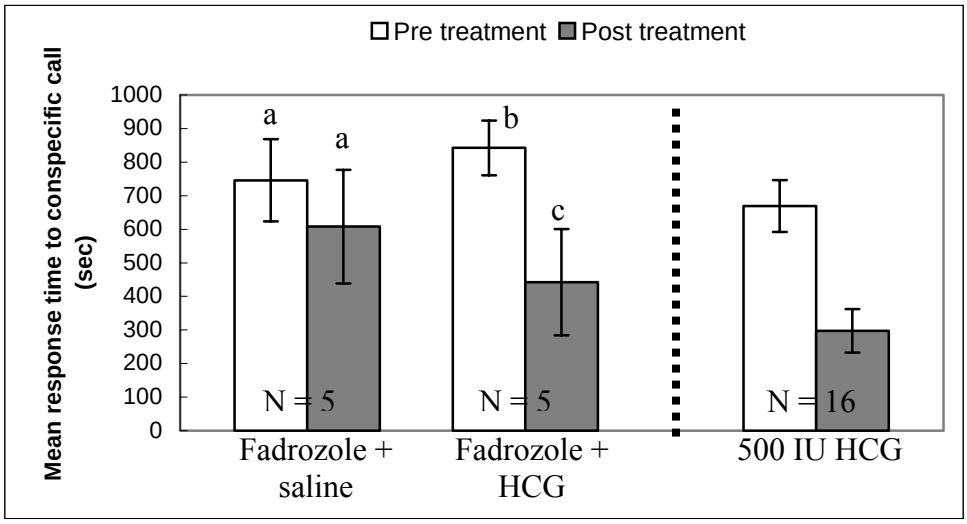


Fig.4.6. Time to respond to a conspecific mate signal during the receptivity test was compared between females treated with 50 μg of fadrozole for two days followed by administration of either 500 IU HCG or 0.9% saline. Differences in response time were analyzed using repeated measures mixed ANOVA. Response time for females injected with just 500 IU of HCG is also presented as a point of reference. A students t-test showed there was no difference in latency to respond between the fadrozole + HCG group and the 500 IU HCG group (see text). Reported values are mean ± s.e

Chapter 5

Auditory Responses in the Midbrain of a Female Anuran: The Role of Hormones

INTRODUCTION

During the breeding season, females in many taxa experience an elevation in peptide and steroid hormone levels (Nelson, 2000; Blaustein and Erskine, 2002 for review) and in most species, elevated hormone levels coincide with the expression of reproductive behavior (Wallen et al., 1984; Pierantoni et al., 1984; Tricas et al., 2000; Radder et al., 2001). In female anurans, the elevation in hormone levels induces receptivity toward male advertisement signals and also influences the range of mate signals to which females will respond (see chapter 3). It is possible that one mechanism for these behavioral changes is hormonal modulation of the central auditory system. For instance, when non-reproductive female midshipmen (*Porichthys notatus*) are treated with either testosterone or estrogen, the ability of the auditory system to encode the temporal pattern of the male's vocalization improves, which mirrors the auditory responses of reproductive females (Sisneros et al., 2004). In addition, studies using electrophysiology, immediate early genes and behavioral discrimination tasks demonstrate that hormones modulate sensory neurons in other taxa as well, including mammals (McFadden, 1998 for review; Bereiter and Barker, 1980), fish (Meyer et al., 1984; Keller et al.,1986; Zakon for review), and amphibians (Yovanof and Feng, 1983; Penna et al., 1992; Miranda and Wilczynski, 2004). In this study, we examine whether hormones influence the manner in which midbrain auditory neurons respond to mate signals in a female anuran.

The torus semicircularis (TS) is an auditory nucleus in the anuran midbrain. It is homologous to the mammalian inferior colliculus and is involved in selective phonotaxis of female anurans (Endepols et al., 2003). The TS is a major integrative structure within the anuran midbrain because it receives ascending auditory inputs and descending inputs as well as acting as an auditory-motor interface (Wilczynski, 1988). In two species of

anurans, the grass frog (*Rana temporaria)* and the fire-bellied toad (*Bombina bombina)*, it was found that there were a significantly higher proportion of spontaneously active neurons in the TS during the breeding season in relation to the winter months (Walkowiak, 1980). The seasonal variation in TS neuron activity coupled with the knowledge that gonadal hormone levels are elevated in many different anuran species during the breeding season (Licht et al., 1983; Pierantoni et al., 1984; Iela et al., 1986; Harvey et al.,1997) suggests that hormones may act as neuromodulators in the anuran auditory midbrain. Furthermore, cells within the TS concentrate sex steroids, specifically estrogen and dihydrotestosterone (Morrell et al., 1975; Kelley et al., 1978; Kelley, 1980; di Meglio et al., 1987) and there is some electrophysiological evidence that gonadal and peptide hormones modulate TS neurons in anurans (Yovanof and Feng, 1983; Penna et al., 1992; Miranda and Wilczynski, 2004). In this experiment, we treat non-reproductive female túngara frogs (*Physalaemus pustulosus*) with either a dose of human chorionic gonadotropin (HCG) or saline followed by either auditory stimulation with natural mate choruses or silence. HCG is a ligand for lutenizing hormone receptors. Therefore, it causes the production of steroids from the gonads (see chapter 4). We examine the influence of hormone levels on the responses of TS neurons to natural mate choruses to determine (1) whether HCG treatment influences auditory responses in the midbrain, (2) whether mate choruses influence auditory responses in the midbrain, and (3) the effect on auditory midbrain neurons when these two stimuli are paired (i.e. hormones + auditory stimulus).

There are a number of alternative patterns that could arise in this experiment (see fig. 5.1A-D for illustration of possible alternative patterns). First, it is possible that auditory response will be elevated only in females that receive both hormone and acoustic stimuli (fig. 5.1A). It also possible that the groups treated with either hormone + silence and saline + chorus will show equally increased auditory response, but the group receiving both stimuli may show an additive effect so that there will be a significantly greater auditory responses (fig. 5.1B). It may also be possible for auditory activity to be significantly elevated in the hormone + chorus group and marginally elevated in the

saline + chorus group in relation to the groups exposed to silence (fig. 5.1C). Finally, it may be that auditory response is equally low in the groups exposed to silence and equally elevated in the groups exposed to chorus (fig. 5.1D). Any of these patterns will reveal whether hormones influence auditory responses.

Induction of immediate early genes (IEG) represents evoked or immediate response within a neuron and can be used as a means of measuring neural activity. After membrane depolarization there is a pulse of increased IEG gene transcription that occurs within minutes after neuronal stimulation (Clayton, 2000). The expression of neuronal immediate early genes has been linked to the activation of second messenger systems (Jarvis, 2004). The benefit of using immediate early genes to measure neuron activity is that in most parts of the brain, electrophysiological activity and immediate early gene expression are co-induced by synaptic neurotransmitter release thereby providing an opportunity to simultaneously measure neuron activity in multiple brain areas (Jarvis, 2004). The limitations of using immediate early genes to measure neuron activity are that not all neurons respond to stimulation with the same suite of immediate early genes, therefore if a brain area lacks expression of a particular immediate early gene, it does not necessarily mean there was a lack of neuronal activation (Jarvis, 2004). Also, immediate early genes are only expressed during neuron excitation and not during neuron inhibition (Clayton, 2000).

We measured the expression of one immediate early gene, *egr-1* (early growth response gene 1), as our indicator of neuronal activity in the principal and laminar nuclei (PN and LN) of the TS. *Egr-1* expression is a common measure of neural activity in avian acoustic communication studies as a (Gentner et al., 2001; Sockman et al., 2002; Maney et al., 2003). One previous acoustic communication study has effectively used *egr-1* expression as a measure of neural activity in an anuran (Hoke et. al., 2004). This study showed that exposure to acoustic stimulation did effectively elevate *egr-1* expression in the auditory midbrain of male túngara frogs. We measure *egr-1* expression in both the principal nucleus (PN) and the laminar nucleus (LN) of the TS in the female túngara frog because these nuclei have distinct connections (Wilczynski, 1988). The

neurons in the PN receive the majority of ascending auditory neurons and send efferent connections to the thalamus. The LN is the major output nucleus of the TS and has extensive connections including premotor and motor areas (Endpols and Walkowiak, 2001). Consequently, it is thought that LN neurons are responsible for integration of auditory and motor systems (Endpols and Walkowiak, 2001). It has also been shown that neurons in the LN are targets for neuromodulatory neurons (Walkowiak and Luksch, 1994) and the LN neurons have been shown to be the specific site within the TS in which steroid hormones bind (Kelley et al., 1978; Kelley, 1980). We examine *egr-1* expression in both of these toral nuclei in order to determine whether hormonal treatment evokes differential neural activity in the auditory midbrain of female túngara frogs exposed to natural mate choruses.

MATERIALS AND METHODS

Female frogs were collected while in amplexus in Gamboa, Panama in July, 2004. All females released eggs and were then housed in the Smithsonian Tropical Research Institute laboratory for 15 days to allow for the dissipation of reproductive hormones (see chapter 2). Females were housed in 10 gallon aquaria with five frogs per aquarium, each aquaria contained water and a deep layer of leaf litter. A single toe was clipped on each frog to distinguish them from one another. Frogs were fed termites every other day until day 15. At 15 days post egg-laying, females were subcutaneously injected with either saline (N = 14) or 500 IU HCG (N = 14) in 50 µl volume. The 500 IU HCG dose has been shown to induce the production of physiological levels of estrogen and testosterone in actively breeding (i.e. amplexed) female túngara frogs (see chapter 4). After injection, females were placed in plastic bags with water and housed in sound attenuation boxes overnight. Approximately 24 hours later, half of the females in the saline and HCG groups were exposed to natural mate choruses and the other half received no acoustic stimulation totaling 4 treatment groups: saline + silence (N = 7), saline + chorus (N = 7), HCG + silence (N = 7), HCG + chorus (N = 7). Mate choruses were recorded by S. Rand in Gamboa, Panama. The mate choruses were broadcast with the peak amplitude at

88 dB (re 20μP) for 30 minutes. Females were rapidly sacrificed via decapitation immediately after acoustic exposure (or exposure to silence), their heads were then placed in Optimal Cutting Temperature (OCT) and flash frozen in liquid nitrogen. Tissue was shipped to University of Texas at Austin on dry ice and stored at – 80 ° C. Tissue was then sectioned at 16μm thickness and placed onto Superfrost Plus slides (Fisher Scientific, Santa Clara, CA) and stored at – 80 ° C until processed for in situ hybridization.

egr – 1 mRNA in situ hybridization

Plasmids containing *egr-1* DNA were obtained from K. Hoke and S. Burmeister. The plasmids were linearized by digestion with *EcoRV* and *BamHI* enzymes (InVitrogen, Carlsbad, CA). Linearized DNA was then purified using Qia quick Gel Extraction Kit (Qiagen, Valencia, Ca.) and concentration of linear DNA was measured using mass spectrophotometry. Linear DNA strands were then used as a template to assemble a complimentary radiolabelled (S35, Amersham, Piscataway, N.J.) RNA probes using T7 (sense) and SP6 (antisense) polymerase enzymes (Maxiscript kit, Ambion, Austin, TX). Radiolabelled probes were purified using NucAway spin columns (Ambion). The quality of the probe was assessed using gel electrophoresis and the quantity was measured using a scintillation count.

All tissue samples were simultaneously processed for *egr-1* in situ hybridization in order to avoid variation produced by the procedure. Tissue was fixed on the slide in 4% formaldehyde (diluted from 16% from Ted Pella, Redding, CA with 1x PBS, Ambion) then rinsed in 1x PBS and 0.1M triethanolamine (TEA; Sigma, St. Louis, Missouri). The tissue was rinsed in 0.05% acetic anhydride (Sigma) in 0.1M TEA to neutralize charges on the slide then rinsed in 2x SSC before dehydration in 50%, 75%, 95%, 100% and another 100% ethanol. Radiolabelled RNA probe was diluted to 2.5 million counts per minute in hybridization solution (Sigma) with 0.01M dithiothreitol (DTT; Sigma). Slides were then coverslipped and left overnight in mineral oil at approximately 65 ° C. The slides were dipped in two chloroform washes and 4x SSC in

order to remove excess mineral oil. Non-specifically bound probe was removed in a 2x SSC wash with 50% formamide and 500µl of 1M DTT at 65 ° C, followed by two 0.1x SSC washes with 500 µl 1M DTT at 65 ° C. Tissue was then dehydrated once again in ethanols. The slides were allowed to dry then dipped in 37 ° C Kodak NTB2 emulsion (VWR Scientific, Brisbane, CA), wrapped in light proof boxes and exposed in 4 ° C for 5 weeks. Tissue was developed with D19 developer (VWR) and Kodak fixer (VWR) then counterstained with cresyl violet and coverslipped with Permount (Fisher Scientific).

egr-1 Quantification and Analysis

The density of silver grains on the tissue hybridized with the sense probe was negligible. Background silver grain densities were measured on each section in areas with no cresyl-violet tissue staining and were also found to be negligible in all cases. The density of silver grains on the tissue hybridized with antisense probe was measured in two nuclei of the torus semicircularis (TS), the principal nucleus (PN) and the laminar nucleus (LN; see figure 5.2 for delineation of nuclei in the TS). Four tissue sections representing the caudal TS were chosen for each subject and were separate from each other by at least 32 µm. Five photographs were taken at 100 x magnification using systematic random sampling. Sections that were torn were not included in the quantification. We started sampling at the lateral most point of each nucleus and moved toward the midline in 150 µm increments. We created a digital picture of the cell area for each photograph as well as the silver grain area using Adobe Photoshop 7. Only silver grains over cells were included in the digital pictures. NIH Image J software was then used to calculate the area of each digital photograph covered by cells and the area covered by silver grains. The proportion of cell area covered by silver grain pixels was calculated and we calculated the mean silver grain area / cell area for each subject. A Kolmogorov-Smirnov test revealed that these data were normally distributed. We therefore analyzed the data using a two-way ANOVA. This analysis was done for both the laminar and the principal nuclei of the TS. All reported values are mean ± s.e.

RESULTS

In the laminar nucleus of the TS the main effect for acoustic treatment showed a nearly significant difference between the group exposed to mate choruses and the group exposed to silence (fig. 5.3; N = 23; DF = 1,19; F = 3.99; P = 0.06). In the laminar nucleus of the TS the main effect for hormone treatment showed a significant difference between the group treated with HCG and the group treated with saline (fig. 5.3; DF = 1,19; F = 6.76; P = 0.018). There was no significant interaction between hormone and acoustic treatment in the LN (DF = 1,19; F = 0.89; P = 0.357; fig. 5.3). The mean proportion of cell area covered by silver grains (both measured in pixels) was 0.0168 ± 0.0059 (N = 5; HCG / chorus), 0.0061 ± 0.00075 (N = 7; saline / chorus), 0.0079 ± 0.0026 (N = 6; HCG / silence) and, 0.0029 ± 0.0009 (n = 5; saline / silence).

In the principal nucleus of the TS the main effect for acoustic treatment showed no significant difference between the group exposed to mate choruses and the group exposed to silence (fig. 5.4; N = 25; DF = 1,21; F = 2.41; P = 0.136). In the principal nucleus of the TS the main effect for hormone treatment showed no significant difference between the group treated with HCG and the group treated with saline (fig. 5.4; DF = 1,21; F = 1.65; P = 0.213). There was no significant interaction between hormone and acoustic treatment in the PN (DF = 1,21; F = 0.174; P = 0.681). The mean proportion of cell area covered by silver grains (both measured in pixels) was 0.0162 ± 0.0058 (N = 7; HCG / chorus), 0.0099 ± 0.0013 (N = 6; saline / chorus), 0.0089 ± 0.0027 (N = 6; HCG / silence) and, 0.0057 ± 0.0019 (N = 5; saline / silence).

DISCUSSION

Hormonal treatment does influence neural responses to acoustic exposure in the laminar nucleus of the TS. There is a significant elevation in *egr-1* expression in the laminar nucleus in the groups receiving HCG injections, indicating that HCG increased neuron activity in the LN. There is a marginally significant elevation in *egr-1* expression in the laminar nucleus in the groups exposed to acoustic treatment, indicating that reception of mate signals also increased neuron activity in the LN. The pattern of *egr-1*

expression most closely fits the pattern shown in graph 5.1B, in which a small level of *egr-1* induction occurs equally in the hormone + silence and saline + chorus groups. The group that received both hormone + chorus, however, shows significantly greater *egr-1* response. Also, it is interesting that the pattern shows higher *egr-1* expression in the group treated with only HCG relative to saline + chorus and saline + silence. It is not clear why this pattern occurs. It may be due to increased spontaneous activity in the LN caused by hormone treatment. Hormones may elevate the baseline activity within this nucleus so that when sound is received by the nucleus, there is a sharp elevation in neural activity (fig. 5.3). Hormonal modulation of LN neurons is consistent with autoradiographic studies in *Xenopus laevis* that show estradiol and dihydrotestosterone concentrating cells in the laminar nucleus cells in the TS (Kelley et al., 1975; Kelley, 1980). Overall, the *egr-1* pattern in the LN indicates that this nucleus increases neural response to mate signals when hormones are elevated.

The pattern of *egr-1* expression in the PN suggests that this nucleus shows increased activity in the presence of both elevated hormones and acoustic treatment. However, the increase in response is not statistically different from the response in the control group, which may be due to the small sample size. Regardless, a clear trend suggests that the PN responds to acoustic treatment ($P = 0.136$). It is possible that the differential response to sound between the LN and PN is due to difference in the threshold for *egr-1* induction. It is also possible that the PN has more spontaneous activity that makes it more difficult to detect evoked activity. Consequently, it may require an increased sample size to detect *egr-1* differences in the PN.

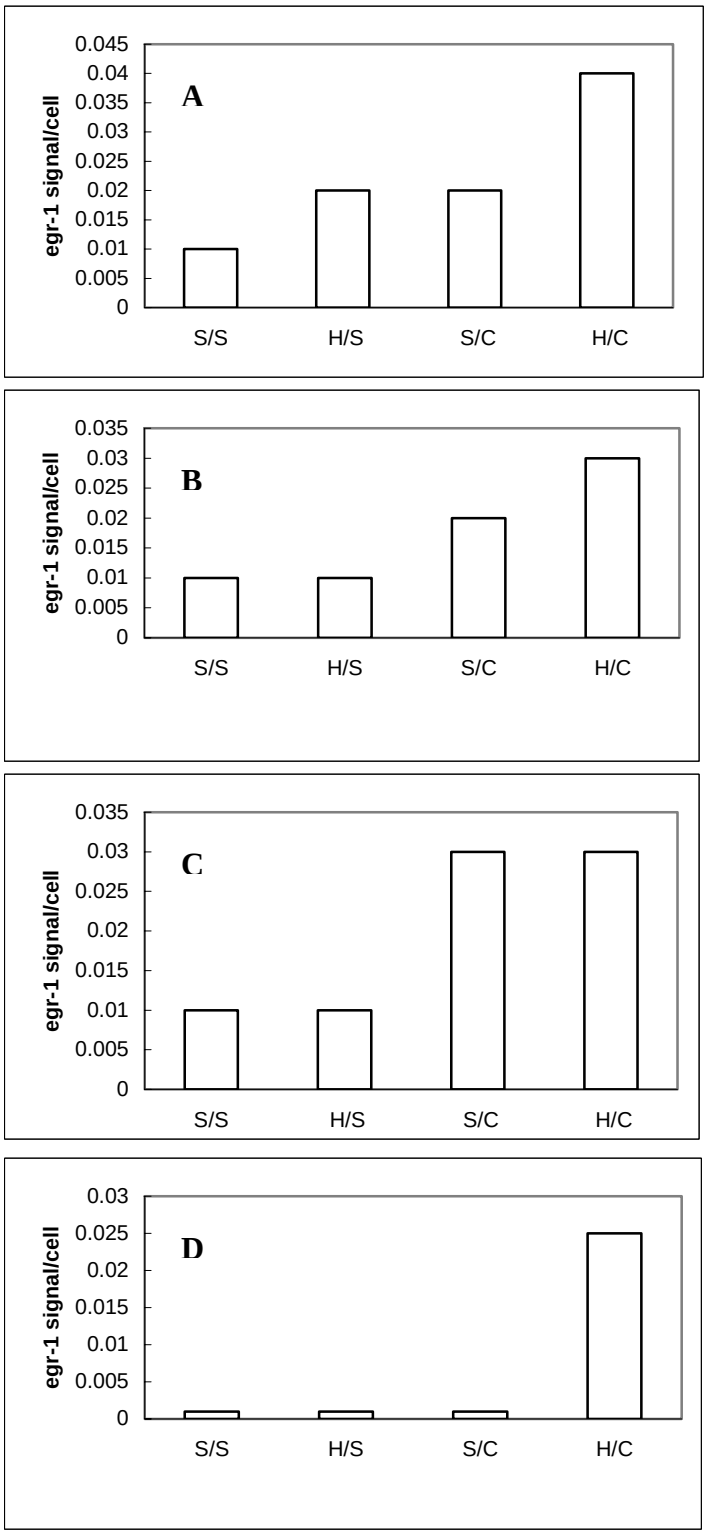
There data shows a tendency for PN neurons to elevate activity as a consequence of hormone treatment. Again, the increase in neural response is not statistically different from the response in the control group but the pattern does not allow us to conclude that hormones have no effect on neural responses in the PN ($P = 0.213$). It is clear that the response in the PN to these two treatments, hormones and sound, are not as strong as the responses in the LN. If it is to be concluded that hormones do influence some of the neural response to sound in the PN (fig. 5.4), then the difference between hormonal

modulation of LN and PN neurons may be explained by the presence of sex steroid receptors in the LN. It is possible, for instance, that hormones can act as direct neuromodulators on LN neurons whereas the PN receives indirect neuromodulatory input.

Our results show that activation of the hypothalamic-pituitary-gonadal axis using gonadotropin injections can modulate neural activity in at least one nucleus in the TS. We are unable to distinguish whether the gonadotropins themselves are able to modulate these neurons or whether increased production of gonadal steroids is required. For instance, our previous work has found that if HCG is administered while simultaneously inhibiting estrogen synthesis, receptive behaviors are still expressed in the female túngara frog (see chapter 3). No previous studies have reported the distribution of LH receptors in the brain of a female anuran. Recently, however, Yang and Kelley (2004) reported the receptor distribution of LH mRNA in the brain of male *X. laevis.* Their study did not find LH-r mRNA in any nuclei within the auditory neural pathway. It is more likely that modulation of neural activity in the LN occurred through the activity of steroids, especially because we know that steroid receptors exist there.

To our knowledge, this is the first report that neural modulation in the auditory midbrain of the amphibian via hormone administration occurs within the laminar nucleus. Our future studies will examine other brain areas that have been shown to express elevated *egr-1* levels in response to sound in the female túngara frog. These areas include superior olivary nucleus, striatum, suprachiasmatic nucleus, lateral hypothalamus, and posterior tuburculum (K. Hoke, pers. comm.). It is possible that hormones may influence neural responses to sound in these areas as well, especially in the striatum because hormone-concentrating cells have been reported there in *Rana esculenta* and *X. laevis* (diMeglio et al., 1987; Morrell et al., 1975). Multiple sites of hormone action within the auditory pathway may serve to ensure that females are able to synchronize the expression of receptive behavior with the appearance of male advertisement signals during the breeding season.

Figure 5.1. Graph A-D illustrates patterns showing that hormones can influence neural responses to acoustic exposure. Graph A shows clear elevated *egr-1* expression in the group exposed to hormones + acoustic stimuli. Graph B shows equally low *egr-1* expression in the groups treated with either hormones or acoustic stimulation alone, but an additive effect when the stimuli are paired. Graph C. shows greater *egr-1* induction in both groups exposed to chorus. Graph D shows a pattern that represents that hormones do not influence auditory response to sound.


A
egr-1 signal/cell
0.045
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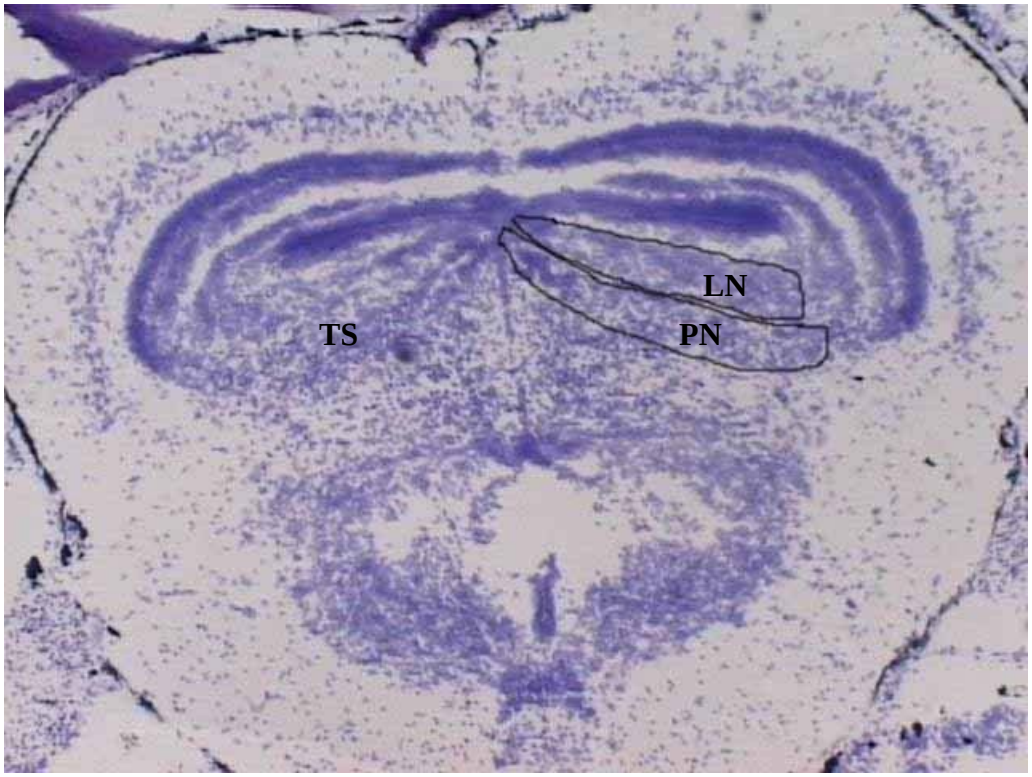

Figure 5.2. Photograph of the amphibian auditory midbrain taken at 4x magnification. The torus semicircularis (TS), seen here, is homologous to mammalian inferior colliculus. The lateral nucleus (LN) and the principal nucleus (PN) of the TS are delineated. Five pictures were taken in each nucleus starting at the most lateral point and moving medially in 150 μm increments.

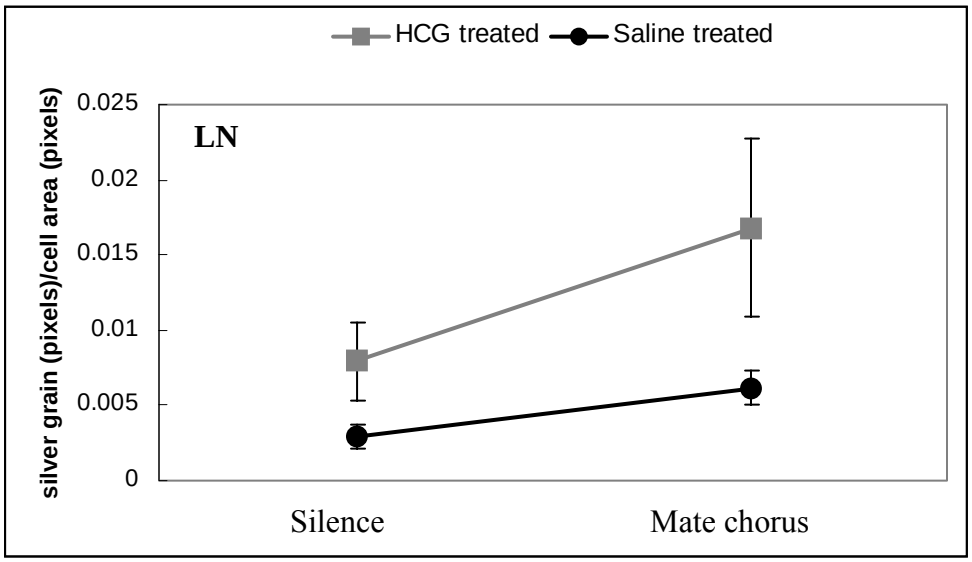

Fig. 5.3. This graph shows the pattern of *egr-1* expression in all the treatment groups within the laminar nucleus of the TS. A two-way ANOVA showed that hormone treatment had a significant effect on *egr-1* induction. Exposure to auditory stimulation also caused marginally significant elevations in *egr-1* induction. There is an elevation in *egr-1* induction between silence and chorus-exposure groups in both the saline and the hormone treated groups. The elevation in *egr-1* induction, however, shows a sharp elevation in the group treated with hormones.

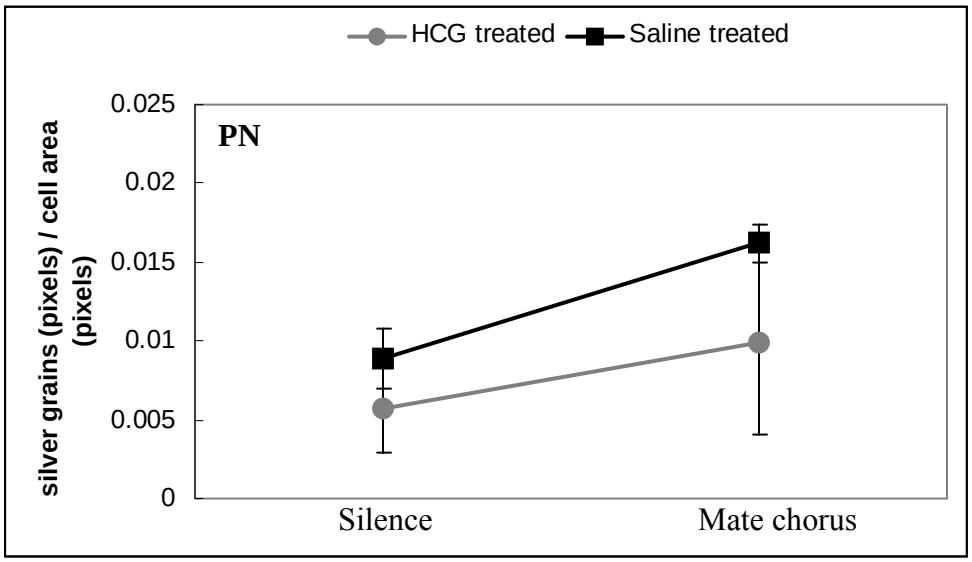

Figure 5.4. This graph shows the pattern of *egr-1* expression in all treatment groups within the principal nucleus of the TS. A two-way ANOVA showed that hormone treatment had no statistically significant effect on *egr-1* induction. Exposure to auditory stimulation caused a marginally significant elevation in *egr-1* induction.

Chapter 6

CONCLUSIONS

This research demonstrates that females show variation in which males they are willing to accept as mates and this variation can occur on a time scale as short as a single reproductive cycle. Furthermore, these behavioral experiments provide evidence that hormone changes can act as a mechanism for such changes in mate choice decisions. The results also show that hormones act as neuromodulators in a midbrain auditory nucleus, thereby influencing the way in which the female auditory system responds to male advertisement signals.

The significance of these findings is threefold. First, these results provide empirical support for Real's (1990) model of mate choice behavior, in which he predicts that females should decrease selectiveness in mate choice decisions as the search for a mate progresses and time constraints arise. An alternative model of mate choice behavior predicted just the opposite; that is, that females should become more selective as the search for a mate progresses (Crowley et al., 1991). Nonetheless, these results show that females will be flexible in their mate choice decisions and if this flexibility enhances the female's reproductive success, it would suggest that such variation in mate choice behavior is adaptive. Second, it is possible this variation in mate choice that occurs throughout a single reproductive cycle can affect the strength of directional selection on male mate signals. This indicates that variation in sexual selection can contribute to the variation in male advertisement signals that exist within a population. Finally, during the breeding season, female anurans are faced with the task of distinguishing conspecific males from a dense, mixed species chorus. They must also determine which males are the most suitable mates. These results suggest that neurons in this female's auditory system can adapt to the increased demands that occur during the breeding season through seasonal increases in hormone levels, which modulate the auditory midbrain's response to salient mate cues.

Summary of results

I found that females vary aspects of their mate choice behavior across three reproductive stages; unamplexed, amplexed and post-mated. Receptivity (response to a conspecific mate signal) and permissiveness (response to a signal that is less attractive than a conspecific signal) both increase during the amplexed stage, which is the stage closest to the point at which females will release eggs. An increase in permissiveness during this stage indicates that females will increase the range of mate signals they will accept as the time to oviposit approaches. Increased permissiveness that occurs during this stage is not associated with a decrease in the female's ability to discriminate between alternative mate signals. That is, discrimination is maintained during the amplexed stage. In the post-mated stage, females still responded to mate signals even though they had already released their eggs, however they responded indiscriminately. It is not clear why a female would respond at all during this stage unless it simply results from the gradual clearing of hormones that induced receptivity. It is not surprising, however, that the female in the post-mated stage show no preference for the whine-chuck at this time. The data in chapter 2 also show that it is difficult to disassociate changes in motivation to mate and changes in permissiveness. For instance, females that respond extremely quickly (in a matter of a few seconds) to a conspecific call are more likely to also display permissive mate choices. It is possible that there is a threshold in the female's receptive state at which she begins to become less choosy in her mate choices. In other words, there may be a receptivity "threshold" at which signals that were once unacceptable to the female now become acceptable.

I also describe the hormone profiles for three gonadal hormones, estrogen, progesterone and testosterone, during the same three reproductive stages in which mate choice behaviors were shown to fluctuate. It was possible that female túngara frogs did not conform to the classic hormone-behavior paradigm in which hormones levels are elevated and reproductive behaviors are expressed because they are able to constantly create and maintain oocytes (i.e. they have asynchronous oogenesis). Therefore, steroid hormones may have been expressed at a constant level throughout the breeding season so

hormones profiles of estrogen, progesterone and testosterone did not significantly fluctuate throughout the three reproductive stages in which behaviors fluctuated. This was not the case, however. These hormones did significantly fluctuate throughout the uanmplexed, amplexed and post-mated stages, thereby providing evidence that hormonal fluctuations could serve as a mechanism for flexibility in mate choice that occurs throughout these reproductive stages. Furthermore, in chapter 3, hormone levels in the post-mated stage were measured 10 days after the female released her eggs. The hormone pattern showed that levels of gonadal hormones in wild-caught females are naturally depressed 10 days after releasing eggs. This information was useful for further experiments (see chapter 5).

Although the data in chapter 3 was useful for determining whether it was possible for hormones to serve as a mechanism for fluctuations in mate choice, it did not provide experimental evidence for this hypothesis. In chapter 4, I experimentally manipulated the hypothalamic-pituitary-gonadal (HPG) axis using a gonadotropin (human chorionic gonadotropin; HCG). Because gonadotropins cause the gonads to produce steroid hormones, injections of gonadotropins, rather than just a single hormone such as estrogen, allowed me to approach the question of whether hormones contribute to flexible mate decisions from the broadest perspective by inducing simultaneous fluctuations in all pituitary and gonadal hormones in the HPG axis. The use of HCG in this study also raises that question of whether the activity of the gonadotropin itself could induce behavioral effects. Kelley (1982) and Wetzel and Kelley (1983) found that castrated male and female *X. laevis* that had been steroid primed were significantly more likely to express reproductive behavior after injection with gonadotropin. Therefore, the second experiment in chapter 4 examines whether HCG can induce behavioral change even when estrogen synthesis has been inhibited by fadrozole administration. Hormone assays verified that HCG influenced hormone concentrations in experiment 1 and that fadrozole decreased estrogen levels in experiment 2. The results showed that HCG administration caused the same flexibility in mate choice behavior as was reported in wild-caught females (chapter 2). That is, receptivity and permissiveness increased when hormones

were elevated due to HCG injection and a decrease in discrimination was not responsible for increased permissive mate choices. In addition, females continued to show receptive behaviors even after fadrozole + HCG administration, suggesting that estrogen is not necessary for inducing receptivity in the female túngara frog.

The behavioral experiments in the previous chapters established that females will vary their mate choices throughout a single reproductive cycle and such variation can be caused by fluctuations in hormone levels. One possible mechanism for hormonally induced variation in mate choice could be hormonal modulation of auditory responses in central auditory areas. I examined this possibility using three experimental groups and one control group; HCG + mate chorus, HCG + silence, saline + mate chorus, and saline + silence respectively. I examined immediate early gene (IEG) activity in each group to determine whether the central auditory nucleus, the torus semicircularis (TS; homolog to mammalian inferior colliculus), showed a response to sound or a response to hormone treatment or a response to both treatments combined. I examined two nuclei within the TS, the laminar nucleus (LN) and the principal nucleus (PN) because these nuclei have different cytoarchitecture and different connections. In general, the LN acts as an auditory/motor integrator whereas the PN specializes in receiving auditory inputs. The results show that IEG induction is elevated in response to acoustic treatment in the LN, although this elevation was not statistically significant ($P = 0.06$). However, the groups that received hormone treatment did show a statistically significant increase in IEG induction. Interestingly, there was in increase in IEG induction in the females treated with hormones in combination with no acoustic treatment. This suggests that hormones may increase the baseline level of activity of LN neurons. When salient acoustic cues are added to the hormone treatment, there is a dramatic rise in IEG induction. The pattern of IEG induction in the LN clearly shows that hormones modulate auditory response to sound in these neurons. The pattern of IEG induction in the PN is not as clear. There is a trend that suggests that IEG's are induced in response to sound in the PN ($P = 0.136$). It is possible that there is no significant increase in IEG expression in the PN because there is increased IEG induction, relative to the LN, in the group exposed to saline +

silence. This may be because there is an elevated level of baseline activity in the nucleus, such as increased spontaneous activation of neurons, which makes it difficult to detect auditory evoked activity. If this is the case, it is possible that an increase in sample size may reveal a different pattern. In addition, the pattern of IEG expression in the PN did not reveal a significant elevation in IEG induction as a consequence of hormone treatment. Again, there was a trend to suggest that PN neurons do express IEG in response to hormone treatment, but not a significant trend. It may be possible that hormones are not directly acting as neuromodulators on PN neurons but rather modulate the inputs into the PN. Overall, these results show that hormones modulate auditory response to sound in one nucleus of the TS, that is, the LN. It is not clear whether hormones change the sensitivity of neurons within the TS, but these results do indicate that there is hormone-dependent plasticity in the function of the neurons in the auditory midbrain of the female túngara frog.

Future directions

Each chapter's results raise interesting questions to be addressed in future studies. The results in chapter 2 raise questions related to the relationship of different aspects of mate choice behavior. The experiments in chapter 2 show that receptivity (i.e. motivation to mate) and permissiveness are associated so that changes in receptivity can influence the range of mate signals a female is willing to accept. It would be useful to try to disassociate these with one another so that a female's permissiveness can be changed without influencing her motivation to mate. I began to examine this question by conducting phonotaxis tests with only receptive females that already respond permissively. I then injected 500 IU of HCG and re-tested the female to determine if the threshold for her permissive response was lowered. That is, I examined whether she would now respond to an even less attractive signal. However, 500 IU may have been too high a dose to give to a female that already displays receptivity because the hormone treatment caused females to cease responding to both conspecific and hybrid calls. Future tests that examine this question would have to find the appropriate hormone dose

for a reproductively active female. In addition, this question could also be examined by looking at the extrinsic factors that cause an already receptive female to become less selective in her mate choices. Examples of such factors include predator density, mate density, and food availability.

Additional studies of hormone effect on mate choice could begin to reveal the exact hormonal mechanism for the results presented in chapter 4. Further hormonal manipulation studies may be able to reveal the role of each gonadal hormone in producing flexibility in female mate choice. Because the fadrozole injection study shows that estrogen is not necessary to induce HCG-related increases in receptivity, a study that administers estrogen and measured mate choice behavior may reveal more about the role of estrogen. For instance, it may be that estrogen acts in parallel with other factors such as progesterone. In addition, because the descriptive study of natural hormone fluctuations (see chapter 3) showed a dramatic elevation in progesterone concentration in amplexed females, it is possible that progesterone is the important factor in hormone-related changes in permissiveness. Another testable hypothesis is that activation of LH receptors is all that is needed to activate receptive behaviors and affect permissiveness. Injecting LH into ovariectomized females could test this. However, because it is still unclear what the exact hormonal mechanism is for such behavioral changes, it would be interesting to conduct phonotaxis tests and compare the physiology of females that show a high degree of permissive mate choices (i.e. respond to very unattractive mate signals) to females that do not show many permissive mate choices. The concentration of gonadal steroids could be compared between these two groups as well as immunoreactivity for a variety of peptides or catecholamines that may play a role in this behavior such as mesotocin, AVT or dopamine.

Although the IEG study clearly shows that the midbrain auditory nuclei are more responsive to mate signals when the HPG axis is stimulated, future studies can help to understand the reasons for this. Electrophysiological examination of the neurons after hormone administration could reveal whether hormones change the sensitivity of the neuron. In addition, it would be interesting to examine *egr-1* induction in other brain

areas thought to be important in female reproductive behavior. Also, other reductionistic experiments could reveal the mechanism by which hormones cause plasticity in these neurons. For instance, estrogen may be able to increase arborization of the neuron or ion channel function. Future experiments could reveal whether hormones can induce such changes in the auditory midbrain.

The experiments presented here demonstrate that female túngara frogs display plasticity in mate choice. It would be interesting to extend this work to areas in which this species has different constraints on mate choice behavior. The females in which mate choices were tested in this study were collected from Gamboa, Panama, an area in which female túngara frogs are not sympatric with other *Physalaemus* species. In addition, the behavioral tests in this study use an artificial hybrid call that hybridizes elements of a *P. pustulosus* call and a *P. enesefae* call. It would be interesting to conduct a similar set of experiments on female túngara frogs in areas where they are sympatric with *P. enesefae* males to determine if they are less likely to display such flexibility in mate choice behaviors.

This study shows that there is natural variation in female mate choice that can occur as a consequence of short-term physiological changes in the female. This variation in mate choice suggests that female preferences for a particular mate signal can be permissive, which indicates that there is flexibility in the types of mate signals males can produce and still attract a female. Consequently, this suggests that males can use a variety of signals to attract females, some of which may not exist in the species. This has significant implication for the evolution of mate choice because it suggests that variation in mate choice exists and this variation would allow potentially novel or divergent signals to be acceptable to females.

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