Center of Vocalization?

A Study of the Structure and Possible Dimorphism of the Anterior Preoptic Nucleus in *Hyla veriscolor*

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Abstract

Vocalization amongst anuran amphibians is often used to attract females of the same species for mating purposes. Females are not normally known to call. The anterior preoptic nucleus (APON) is considered to be the location in the brain responsible for setting off the neuronal circuit that coordinates male mating call behavior. The APON has access to both the cerebrospinal fluid and the blood, allowing for the reception of hormonal instruction on reproductive behavior. This study examined the APON in *Hyla versicolor* for possible structural sexual dimorphism. *Hyla versicolor* brains were prepared using a Luxol Fast Blue Staining Procedure. The analysis was performed using an image analyzer. The male preoptic area examined was found to be 2.6 times the size of the female areas. A trend could be seen from these results that male *Hyla versicolor* has a larger preoptic area then their female counterparts. These results, though not statistically significant, do support the theory that this area of the brain is involved with triggering the calling mechanism and may explain the absence of female calling behavior in this species.

Introduction

Vocalization among anuran amphibians is often used to attract members of the opposite sex for mating purposes. "Calling" is one way of assuring that mates of the appropriate species and sex respond. This mechanism works well because the majority of anurans can produce sounds, and further, most anurans are capable of hearing and recognizing sounds within a frequency range compatible with their own vocalizing abilities (McAlister 1969). Repeated tests have shown that gravid females of many species of frogs move toward males of their own species that are producing mating calls, or toward artificial sound that sufficiently resemble conspecific mating calls (Schmidt 1988, Gerhardt 1991, Gerhardt, Daniel, Perril and Scramm 1987).

The North American varieties of treefrogs of the anura Hylidae use calling as part of their repertoire of reproductive behaviors. This study will examine the mate calling center of the brain in one species in particular, *Hyla versicolor*.

During the treefrog breeding season, which for *Hyla versicolor* occurs during the nights of the spring (usually from late April until the end of June), males form aggregations known as choruses in an area of vegetation (Schwartz 1987, Gerhardt 1991, Hausfater, Gerhardt and Klump 1990). In *Hyla versicolor's* cousin, the green treefrog, three categories of behavior are commonly seen performed by the males (Gerhardt et al. 1987). These categories are calling, non-calling and satellite behaviors. In *Hyla versicolor*, calling behavior is seen only in males. The vocalizations are produced by the expulsion of air from the lungs which starts the free edges of the vocal cords vibrating (Holmes 1954). The laryngeal muscles may alternate the tension placed on the voc
al cords, changing the sound created (Holmes 1954). Males who have been calling can usually be identified by their inflated vocal pouches and bodies (Gerhardt et al. 1987).

Satellite behavior is seen in frogs that do not call, but remain in close proximity of a calling male. It was found that this behavior in green treefrogs (Gerhardt et al. 1987) was not always dependent on size and may be due to the cost of the energetic output needed for calling. Satellite behavior can be identified by a deflated vocal pouch and by a body with its head orientation in the direction of a calling male (Gerhardt et al. 1987). The non-calling behavior is similarly identified by a deflated pouch and body, but with no orientation towards a calling male (Gerhardt et al. 1987).

In *Hyla cinerea*, it was found that there were no dramatic differences in size between the calling and the satellite males

(Gerhardt et al. 1987). In addition, frogs were observed to switch strategies from night to night during the breeding season (Gerhardt et al. 1987).

Female response in *Hyla versicolor* to mating calls is movement toward the calling male. This female mating call phonotaxis is seen in many anurans and normally occurs only during a brief period between ovulation and the oviposition of eggs (Schmidt 1988). In response to this approach a calling or a satellite male will clasp onto the female with its forelegs (Gerhardt 1991, Holmes 1954). This hold will last until copulation is complete. At this time improperly clasped males or females may utter another call, the release call, and be set free (Gerhardt 1991).

As the male to female ratio of *Hyla versicolor* often tends to be large (Gerhardt 1991), vocalization is an important means of attracting a receptive female mate. Many studies have been performed on different phenotypic aspects of calling behavior in anuran amphibians. Temporal cues have been found to be important between *Hyla versicolor* and *Hyla chrysocelis* in order for males to be heard and to be distinguished from other conspecific and heterospecific calls (Moss and Simmons 1986, Rose, Brenowitz and Capranica 1985). Parasite infection and its connection to vocalization has been explored in *Hyla versicolor* also. No consistent relationship was found between the number and kinds of parasites harbored on the males and either the duration or the expenditure of their calls (Hausfater et al. 1990). Callers and non-callers, in a population of male American toads also showed no correlation between aerobic capacity and reproductive behavior, suggesting that patterns of behavior are due to some other physiological constraint (Taigen and Pough, 1985).

The site of one such constraint may be found in the preoptic area of the brain. The preoptic area is located in the anterior portion of the hypothalamus, contained within several cell clusters (Moore 1987). Moore (1987) describes the area as containing dendrites that project into the third ventricle, contacting the cerebrospinal fluid. Urano's review (1988) of Smoller's study (1965) of neurosecretory processes found that these dendrites can possess both secretory and sensory functions. Urano (1988) concludes that the neurons that contact CSF may be useful in monitoring hormonal signals and directing the neuronal circuitry to the proper response.

A study of seasonal changes in luteinizing hormone-releasing hormone (LH-RH) in the Japanese toad (Jokura and Urano 1984) found strongly immunoreactive LH-RH fibers projecting to the anterior preoptic nucleus. Further, in a golgi-electron microscopic study of

the APON, blood capillary contacting neurons were found (Urano 1984). These blood-capillary contacting neurons may integrate hormonal and neuronal signals much as the cerebrospinal-contacting neurons are proposed to do (Urano 1984). The APON, then, has several means of gathering information from the endocrine system.

This important neuroendocrine center is divisible into two halves, anterior and posterior (Urano 1988). The anterior part of the preoptic nucleus (APON) has been indicated as the center for triggering male mate calling behavior (Urano 1988). It was found that electrical stimulation can induce calling behaviors in both normal and castrated *Rana pipiens* (Wada and Gorbman 1977). The anterior nucleus, however, has been found to be highly influenced by the presence of sex steroids, especially androgens (Wada and Gorbman 1977, Schmidt 1983, Urano 1988, Schmidt 1989, Fujita, Jokura, Takami and Urano 1987, and Takami and Urano 1984c).

The goal of this study was to determine whether sexual dimorphism in the preoptic nucleus existed between males and females of this species and to quantify any such distinctions. The findings of this study were to provide evidence to account for calling behavior occurring only in males, and also to further investigate the androgen organization theory. This study was performed on a sample of *Hyla versicolor* obtained from their natural habitat.

Materials and Methods

This study took place through the University of Notre Dame Environmental Research Center (UNDERC), a field station in the Upper Peninsula of Michigan. The field component of the study took place from May 17 through July 21, 1993 at two sites on the UNDERC property: a bog site near the Housing Area, and at Bog Pot. Laboratory work took place at the student laboratory on the UNDERC property during May 17 through July 21, 1993. The remaining laboratory work and analysis was carried out at the University of Notre Dame, Notre Dame, Indiana, between September and November 1993.

The field work consisted of locating a population of *Hyla versicolor* at each of the two bog sites. Ten males and one female were found at the site near the housing area. The female escaped before the procedure began. Eight of the ten male *Hyla versicolor* had been injected with AVT in accordance with a fellow investigator's study. Because the half life of the AVT is short, this injection was not thought to interfere with this study.

Nine males and a suspected satellite male were caught at the Bog Pot site. All were injected with AVT. Two females were also found at this site. A third female was later discovered in the

Housing Area proper, on a ledge of Ward Hall.

The frogs were kept in plastic containers filled with several inches of bog water. A rock and some bog vegetation were placed in the bottom of the container. Frogs kept for over a 24 hour period were fed with live dragonflies.

The frogs were anesthetized with Benzocaine, then decapitated. The lower jaw was removed and the head was trimmed using scissors. The skull was scraped, immersed in Conway's fixative and refrigerated for 2-24 hours. The brain was then removed from the skull and reimmersed in fresh fixative for another 6-24 hours.

The preparation method for examing the anterior preoptic nucleus was the Luxol Fast Blue Procedure. The brains were immersed in a run of: 70% ethanol (EtOH), 80% EtOH, 95% EtOH, 100% Hemo-de, and paraffin saturated-Hemo-de solution for 12 hours each. Then the brains were immersed in melted paraffin in a water bath of 50-52° for two hours and transferred into fresh melted paraffin of the same temperature for another two hours. The brains were then placed in molds of melted paraffin and then cooled until the block was hardened (at least 24 hours).

The brains were sectioned on a manual microtome. Slides were flooded with subbing solution and warmed on a slide warmer (43°C). Sections were placed on the slide and dried. When fully dried, the slides were run through solutions of: distilled water, 70% EtOH, 95% EtOH, 100% EtOH and 100% EtOH, for two minutes each. Then the slides were placed in Blue Stain for Myelin (0.25g solvent blue 38 dissolved in 250ml of 95% EtOH for 18-24 hours at 56-60°C. Next the slides were run through solutions of: 70% EtOH, 50% EtOH, 25% EtOH, and distilled water (twice) for two minutes each. The slides were then placed in Differentiation Solution (0.25g lithium carbonate dissolved in 500ml distilled water) for 30 minutes. The slides were placed in two changes of 70% EtOH next, for one minute each. They were then rinsed in distilled water for five minutes and placed in Counterstain (1.25g Neutral Red dissolved in 250ml distilled water) for one to five minutes (averaging three minutes).

When the red stain was found to be too dark, the slide was placed in 70% EtOH until only the nuclei and Nissl substance were red. The slides were dried and then run through: 70% EtOH (for ten dips), 95% EtOH (2 minutes), 100% EtOH (2 minutes), 100% EtOH (2 minutes), Hemo-de (2 minutes), and Hemo-de (2 minutes). Finally, the slides were given four drops of Permount, covered with a coverslip and dried.

The sections were examined under a microscope to determine which ones best displayed the anterior preoptic nucleus. Four

brains were chosen, one male and two female. Sections of these brains were marked. An Image Analyzer, using the Image-1 program and an Olympus 4x with a Hamamatsu SIT was used to quantify the areas of the anterior preoptic nucleus for each frog.

Results

Three areas were found for the male frog sampled. When studied by the image analyzer, M7's preoptic nucleus had measurements of 577.472 mm^2, 296.739 mm^2, and 157.607 mm^2. All values had an error of +5% to -5%. The average area found for M7 was 343.939 mm^2.

Two female frogs' preoptic nucleus area were also measured. F3 had two measurements performed. The results were: 64.511 mm^2, and 43.780 mm^2. The average area of the preoptic nucleus for F3 was 54.1455 mm^2. F1 also had two measurements performed, yielding the values of 326.44 mm^2 and 64.5110 mm^2. All values had an error of +5% to -5%. The average area for F1 preoptic nucleus was 195.4755 mm^2. The average area for the females' preoptic nucleus was 129.99325 mm^2.

The large difference in male to female sex ratio was clearly seen during the capturing of the frogs. Few female frogs were found, and as a result, the findings of this study fail to have statistical significance. However, the data does tend to indicate certain trends. The average for the male preoptic nucleus was found to be approximately 2.6 times the size of the female preoptic nucleus. This observation supports the hypothesis that there would be dimorphism present in this structure.

Another difference observed between the sexes was that the male brains on the whole appeared larger in relation to their total body size as compared to the females. No great size difference was seen between the actual brains themselves, however.

A further observation was that the female brains tended to be more fragile than the males'. They appeared slightly more watery and tended to tear more easily.

Several difficulties were encountered during the course of this project. The first was the inaccessibility of a representative number of the *Hyla versicolor* population. The second problem faced was that sections were cut too thick, resulting in loss of tissue samples during the staining process. Thirdly, the preparation and measurements may have been affected by the development of the experimental skills of the researcher.

Discussion

Not only have several other studies found dimorphism to be present in other species of anuran amphibians, but research has

linked size difference in the preoptic nucleus to the presence of androgens.

A study of the subnuclear organization of the preoptic nucleus in the toad, *Bufo japonicus* (Takami et al. 1984b) determined that the anterior preoptic nucleus is a sex steroid-sensitive center which is capable of accumulating sex steroid hormones (Kelley, Morrell and Pfaff 1975, Morrell, Kelley and Pfaff 1975, and Kelley, Lieberburg, McEwen and Pfaff 1978). It was also found in a review by these investigators (Takami et al. 1984b) that internuclear implantation of testosterone into the APON enhanced male mating call behavior (Wada and Gorbman 1977). A study of the castration effects on the volume of the preoptic nucleus in the *Bufo japonicus* brain provided evidence for the theory that the activity of the neurons of this area are regulated by androgen as castration reduced the volume of the APON and testosterone implantation restored the volumes to or above normal levels (Fujita, Jokura, Takami and Urano 1987).

Because APON organization is dependent on androgen, "morphological changes in the amygdala-APON complex have been seen to precede physiological and behavioral changes during the breeding season of *Bufo japonicus*" (Takami and Urano 1984c, p. 256). When nissil-stained *Bufo japonicus* brains were examined, it was found that the nuclear volume of the APON area in hibernating males was larger than the volume found in post-breeding males (Takami and Urano 1984c).

Androgen regulation of the APON is also partially responsible for the sexual dimorphism seen in some species' APON (Takami and Urano 1984, Urano 1984, Fujita et al. 1987, Urano and Gorbman 1980, Schmidt 1989, and Schmidt 1983). A review by Urano (1988) of the neuroendocrine control of mating behavior highlighted several studies which found sexual dimorphism to occur in the anterior preoptic nucleus of amphibians, birds and mammals (Arnold 1980, Panzica, Vigletti-Panzica, Calacagni, Anselmetti, Schumacher and Balthazart 1987, Arimatsu, Seto and Amano 1981, Avnob and Greenough 1983, Gorski, Gordon, Shryne and Southam 1978, Matsumoto and Arai 1986 and Hannigan and Kelley 1981). A study of the neural correlates of frog calling (Schmidt 1983) found that female Northern leopard frogs possess complete mate calling circuits and that androgens are capable of masculinizing these calling circuits. Male and female green tree frogs have additionally been reported to have responded with mating-like calls to the presentation of conspecific tape recorded vocalizations after being implanted for some weeks with androgen pellets and then injected with a single dose of the neuropeptide arginine-vasotocin, also known as AVT (Penna, Capranica

and Somers 1992). When a quantitative analysis of the APON was performed on *Bufo japonicus*, the total cell number was found to 1.20-times greater in the male, which the authors (Takami et al. 1984a) identified as possibly being induced by the organizational effects of androgens. Thus, there is support for the theory that although the female has the neuronal capability to produce mating calls, in the absence of androgens they refrain from doing so.

In this study, the male preoptic nucleus was seen to be larger than that of the females, indicating the possibility that the size of the preoptic nucleus of *Hyla versicolor* may be under the control of similar mechanisms.

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