

**Effects of gonadal steroids on area of the
anterior preoptic area and pretrigeminal nucleus
in Green frogs, *Rana clamitans***

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ABSTRACT

Anuran mate-calling is a sexually dimorphic behavior believed to be controlled by specific regions of the brain. Some of these regions concentrate steroids, and have larger volume in males than in females in certain species. The anterior preoptic area (APOA) and pretrigeminal nucleus (PTN) were studied in *Rana clamitans* to ascertain if any sexual dimorphisms in cross-sectional area in these regions exist. Male frogs were also castrated to determine effects of testicular steroid removal on brain area. One female subject was available for PTN analysis, and had a cross-sectional area lower than all of the untreated males. A trend was spotted in the PTN for castrated frogs to have lower area than untreated frogs, implying the possibility of activational effects of gonadal steroids in this region in males. In the APOA, no females were available for analysis. Area of the single castrated frog APOA appeared to show no effects of treatment. Our results suggest that there exist multiple mechanisms by which androgens regulate mate-calling centers of the anuran brain. Further study with greater numbers of subjects is needed for clearer analysis of possible sexual dimorphisms and hormonal effects.

INTRODUCTION

Many species of vertebrates have been shown to have behavior, physiology, and anatomy that is sex-specific; that is, following either a distinct male pattern, or a distinct female pattern. These differences occur during sexual differentiation, and are largely due to the presence (or absence) of certain gonadal steroid hormones (Goy and McEwen, 1980).

Mate calling is a sexually dimorphic behavior in anurans; males exhibit calling behavior while females do not. Calling behavior also appears to be under the hormonal control of steroids. Castration has been shown to abolish calling in Ranids (Schmidt, 1966b), while calling is reinstated in castrated *Xenopus laevis* by administration of testosterone and dihydrotestosterone (Wetzel and Kelley, 1983). Specific brain regions are believed to participate in a neural pathway for calling and other vocal behaviors. Calling circuits in *Rana pipiens* can be triggered by electrical stimulation of the anterior preoptic nucleus (Schmidt, 1984). Other studies confirm that both the anterior preoptic area (APOA) and the pretrigeminal nucleus (PTN) both play roles in neuronal control of calling (Schmidt, 1973, 1974; Wetzel et al., 1985).

Both the APOA and the PTN accumulate steroids in certain species of anurans. *X. laevis* APOA and PTN accumulate testosterone (Kelley et al., 1975; Kelley, 1980), and *R. pipiens* APOA accumulates testosterone (Kelley et al., 1978). This suggests that perhaps steroid accumulation in the PTN is species specific. There are also instances of anurans in which some sexual dimorphism exists in both of these regions. The APOA of *Bufo japonicus* is significantly larger in males than in females (Takami and Urano, 1984). Using succinic dehydrogenase (SDH) staining in *Bufo americanus*, the PTN of male toads has greater numbers of large, darkly stained cells than do females, implying a higher metabolic rate for males in this region (Schmidt, 1982).

This study is an attempt to determine if any such sexual dimorphisms exist in the Green frog (*Rana clamitans*). The cross-sectional areas of the PTN and APOA were compared between male and female *R. clamitans*. Furthermore, the effects of castration on APOA and PTN area in males was observed. This study also was to allow us to determine if a correlation exists between abolished calling behavior in castrated males and reduction of area in the regions of the *R. clamitans* brain discussed above.

MATERIALS AND METHODS

ANIMALS

All male frogs were collected at the University of Notre Dame Environmental Research Center in Gogebic Co., MI. The female frog was collected on the middle branch of the Ontonagon River in Ontonagon Co., WI. Collection and

Effects of Gonadal Steroids

sacrifice of frogs was performed during the months of June and July of 1991, during the normal breeding season for *Rana clamitans*. Frogs were toe clipped for identification after capture. The animals were fed dragonflies 3 times/week. All animals were housed in chicken wire-covered wooden cages approximately 1 yd.³ in dimension. The cages were bedded with moistened peat moss. The frogs were subject to the ambient light:darkness ratio, as well as temperature.

SURGICAL PROCEDURE

Two groups of male frogs received surgical treatment. One group received gonadectomies. This group was anesthetized with a 10% benzocaine solution prior to surgery. A small incision was made in the pelvic region of the frog, about 1.5 cm in length. The gonads were removed, and the incisions were stitched closed. The other group received sham operations. In these animals, incisions were made in the preceding fashion and closed without removal of the gonads.

HISTOLOGY

All animals received benzocaine anesthetic before brain harvesting. After being anesthetized, animals were sacrificed by decapitation. Brain and skull from each animal was allowed to sit in fixative for two hours following decapitation. The fixative used was a 1:1 solution of 100 ml .133 M paraformaldehyde, and 100 ml 0.1 M phosphate buffer (comprised of 30.5 ml of 0.2 M dibasic sodium phosphate and 19.5 ml of 0.2 M monobasic sodium phosphate, diluted to 100 ml with distilled water). Following the initial fixation period, the brains were evacuated from the cranium, the dura mater was removed, and brains were returned to fresh fixative for 12 hours. Brains were then immersed in the following solutions successively for 6 hours each:

- 1) 70% EtOH
- 2) 80% EtOH
- 3) 95% EtOH
- 4) 100% EtOH
- 5) 100% chloroform
- 6) 1:1 mixture of chloroform and paraffin

After this sequence, brains were placed in paraffin at a temperature of 50-52° C. Paraffin was changed 3 times at 30 minute intervals. At the end of the last interval, the brains were embedded in a block of paraffin and allowed to cool. The paraffin-brain blocks were dissected into transverse sections 15 µm in width by a microtome. These sections were then mounted on chrome alum-gelatin subbed slides and then stained with cresyl violet acetate.

Both aqueous and buffered cresyl violet acetate solutions were used. 6.22 x 10⁻⁴ M aqueous cresyl violet was made. For the buffered cresyl violet solution,

Effects of Gonadal Steroids

100 ml of buffer (94 ml of 0.1 M acetic acid; 6 ml of 0.1 M sodium acetate) was added to 6 ml of the aqueous cresyl violet solution. Slides were immersed in the following solutions in succession for the time marked in parentheses:

- 1) Xylene (3 minutes)
- 2) Xylene (2 minutes)
- 3) 100% EtOH (2 minutes)
- 4) 95% EtOH (2 minutes)
- 5) 70% EtOH (2 minutes)
- 6) Distilled Water (2 minutes)
- 7) Buffered Cresyl Violet (10 minutes)
- 8) Aqueous Cresyl Violet (1 minute)
- 9) 70% EtOH (10 dips)
- 10) 95% EtOH (2 minutes)
- 11) 100% EtOH (2 minutes)
- 12) 100% EtOH (2 minutes)
- 13) Xylene (2 minutes)
- 14) Xylene (2 minutes)

The amount of time spent in the aqueous cresyl violet solution was varied from 1-2 minutes to adjust the darkness of the staining as needed.

GENERAL PROCEDURE

The untreated males and the female frog were sacrificed, and their brains embedded in paraffin upon capture. Gonadectomized and sham-operated frogs were housed for two weeks after surgery before sacrifice and harvesting of brains. While the frogs were housed, they were allowed to sit in an antibiotic solution of 1 g penicillin and 1 g streptomycin per liter of water. They received this treatment in two hour sessions, three times per week until sacrificed.

After slides were created, they were then observed using computer image analysis equipment. The mean cross-sectional area for left and right regions of the pretrigeminal nucleus were found, as well as the cross-sectional area of the preoptic area (see figures 1-3). Differences in area were only able to be analyzed statistically between some of the treatment groups due to insufficient subjects. In the single case where analysis was possible, the Mann-Whitney U-test was used.

RESULTS

Insufficient amounts of data were recorded for female *R. clamitans* due to lack of captured females. This stemmed from the fact that frogs were captured at

Effects of Gonadal Steroids

FIGURE 2

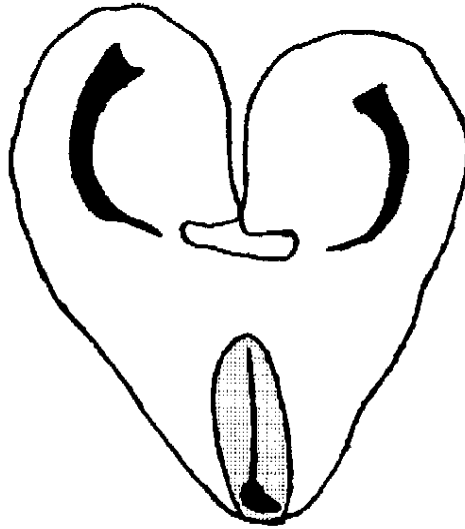


Figure 2: Cross-section of the forebrain of *Rana clamitans*. Blackened regions represent ventricles. The shaded region represents the preoptic area.

FIGURE 3

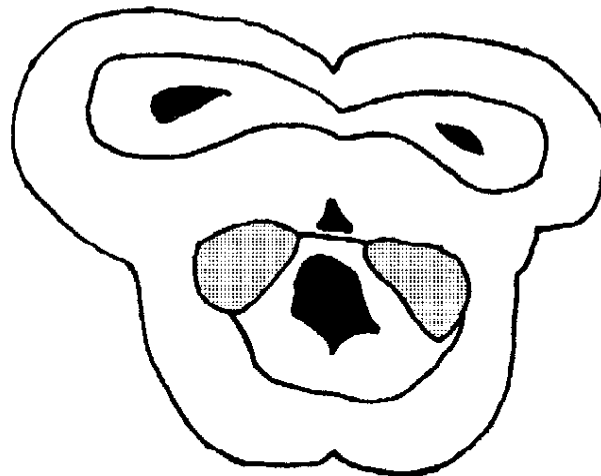


Figure 3: Cross-section of the midbrain of *Rana clamitans*. Blackened regions represent ventricles. The shaded regions represent the pretrigeminal nuclei, both left and right.

FIGURE 1

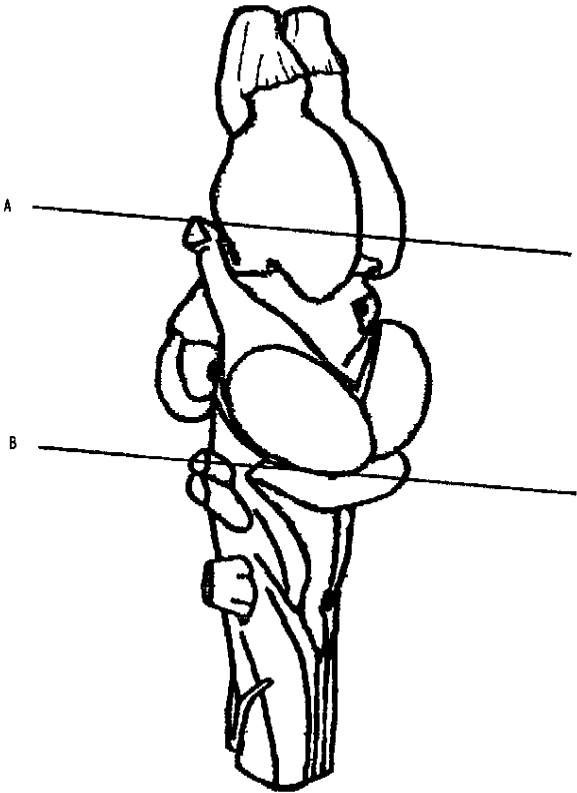


Figure 1: Dorsolateral view of the brain of a frog. Transect A represents where sections containing the preoptic area were taken for analysis of area. Transect B represents the region where sections were taken for area analysis of the pretrigeminal nucleus. (From Torrey, T.W. : *Morphogenesis of the Vertebrates*. 2nd edition. John Wiley and Sons, 1962)

Effects of Gonadal Steroids

night using the sound from the male mate call to locate frogs. Female frogs, who do not call, were only found when they happened to be in the vicinity of a calling male when he was captured. The single female that was captured was only able to contribute data for the PTN, as damage to brain sections containing the APOA prevented analysis of that area.

Mortality during surgery and tissue damage contributed to reduced data as well. For the castration surgery, survival rate was 29% (4 of 14). Of those four, only two were able to contribute to PTN results, and one added to APOA results. In the sham-operation, survival rates were higher, at 83% (5 of 6), but tissue damage afforded similar results to the gonadectomized males (1 PTN, 2 APOA).

APOA

For the APOA (see table 1), untreated males (mean area: 0.222 ± 0.070 mm²) and sham-operated males (mean area: 0.202 ± 0.091 mm²) were grouped together for analysis, as all of the values for the sham-operated males fell within 95% confidence intervals of the untreated males (± 2 standard deviations of the mean). All control males together had a mean area for the APOA of 0.215 ± 0.050 mm². Data from only one animal was available for the gonadectomized males (0.279 mm²). Because only one result exists for the group of gonadectomized males, no comparison between groups was available.

PTN

In this case, untreated males were considered separately from sham-operated males (see table 2). Untreated males had a mean PTN area of 0.259 ± 0.010 mm². Castrated males, with a mean area of 0.152 ± 0.037 mm², were compared to untreated males by the Mann-Whitney U-test ($p=0.06$) due to low number of subjects. The remaining groups only had one subject each; sham-operated males (mean area = 0.162 mm²) and females (mean area = 0.176 mm²). Due to this fact, no further analysis was available between groups for PTN area.

DISCUSSION

Different areas of *R. clamitans* brain involved with control of mating calling may have different responses to changes in testosterone levels. The cross-sectional area of the PTN appears to be larger in males than in females. Also observed was a trend for castration to reduce the area of PTN to approximate female levels. Castration appeared to have no effect on APOA area. These results suggest that the PTN and APOA differ in their ability to be activated on a short-term basis by steroids.

The APOA is the center of the anuran mate-calling system, which in turn

Effects of Gonadal Steroids

TABLE 1
Comparison of cross-sectional area of
anterior preoptic area of Green frogs

Group	n	Area (mm ²) ^a
Control Males ^b	6	0.215 ± 0.050
Gonadectomized Males	1	0.279 ^c

^a Data presented are means ± standard error of the means.

^b This group represents 4 untreated males and 2 sham-operated males (within ± 2 S.D. of the mean of the untreated males).

^c No standard error was available due to insufficient subjects.

TABLE 2
Comparison of cross-sectional area of
pretrigeminal nucleus of Green frogs

Group	n	Area (mm ²) ^a
Untreated Males	4	0.259 ± 0.010 ^b
Sham-operated Males	1	0.162 ^c
Gonadectomized Males	2	0.152 ± 0.037
Untreated Females	1	0.176 ^c

^a Data presented are means ± standard error of the means.

^b Compared to gonadectomized males by Mann-Whitney U Test;
p=0.06

^c No standard error was available due to insufficient subjects.

Effects of Gonadal Steroids

initiates calling by means of the PTN and the laryngeal motor neurons. *R. pipiens* brainstem exhibits activation of calling circuits after electrical stimulation of the APOA (Schmidt, 1984). Implantation of testosterone into the APOA stimulates calling in castrated male frogs (Wada and Gorbman, 1977), demonstrating the incitatory effects of androgens on the APOA and mate-calling behavior.

As discussed earlier, Takami and Urano (1984) found that a sexual dimorphism exists in the APOA of both post-breeding *B. japonicus* (captured in March) and hibernating toads (captured in October). The increased size of the APOA in males is believed to be due to increased cell numbers in that region (Takami et al., 1984), which in turn, may be due to the organizational effects of gonadal steroids. *B. japonicus* males also exhibit differences in APOA volume between post-breeding and hibernating groups (no seasonal differences were found in female toads). Takami and Urano reported that these seasonal changes are believed to be the result of hypertrophy on neurons in the region. Due to the fact that our study implies that castration and the subsequent removal of gonadal steroids has no effect on APOA area in male *R. clamitans*, it may be the case that if seasonal changes exist in male APOA area, that they are the product of some factor other than gonadal steroids. Another possibility is that gonadal steroids are the cause of seasonal changes, but that the difference in time frame of hormonal reduction (7 months in *B. japonicus* vs. 2 weeks in *R. clamitans*) did not allow for any reduction in neuronal size to occur.

Seasonal changes in male APOA and the dearth of a similar change in females implies that activation of this area during breeding season is crucial only to male reproductive behavior. It seems that the activation of the APOA is caused by some factor specific to males, be it either a hormonal or structural factor that accommodates neuronal hypertrophy in that region.

There appeared in our results, however, a trend for PTN area of gonadectomized males to be lower than that of untreated males. If this trend holds, it is possible that the PTN of *R. clamitans* accumulates testosterone, and that PTN area is regulated by it as well. The results from the single female and sham-operated male fall within confidence limits of the mean area of the PTN for castrated males, which may (if validated) support our earlier conclusion.

A reduced value in the PTN area of the sham-operated males could possibly be due to animals being stressed by surgery and/or captivity. Studies performed with the rough skinned newt (*Taricha granulosa*) correlate stress and increased corticosterone levels with lowered plasma androgen levels (Moore and Zoeller, 1985). Licht et al. (1983) also showed that male *Rana catesbiana* held in collecting sacks after capture show reduced plasma gonadotropin and androgen levels, and increased plasma corticosterone levels. In order to test this hypothesis, and monitor the efficacy of our control, measurements of plasma hormone levels for each of the groups involved would be necessary, in addition to making all conscious efforts available to reduce stress during captivity and

Effects of Gonadal Steroids

surgical procedures.

If it is true that PTN area is reduced to female levels by removal of gonadal hormones, it is possible that hormone treatment of females would lead to masculinization of the PTN area. Schmidt (1982) reports unverified data supporting the claim that SDH staining in female *B. americanus* can be masculinized (i.e., greater number and size of stained cells) by androgen treatment. Verified data of this kind would suggest that PTN area may be under the activational (short-term) control of steroids, and perhaps seasonally variable as well. In addition, if PTN neurons are reduced in size two weeks after castration, this may negate the previously stated hypothesis that a reduction in neuron size did not have sufficient time to manifest itself in *R. clamitans* APOA.

Mating calling behavior differs in its ability to be elicited in female anurans. Female *X. laevis* cannot be induced to mate call, even after androgen treatment (Hannigan and Kelley, 1986), while female *Hyla cinerea* will give the mate call after testicular and pituitary implantations (Schmidt, 1966a). These findings imply that regardless of hormonal constraints, the neural circuitry required for calling may exist in females of some species and be absent or reduced in others. Data from Wetzel et al. (1985) indicate that projections from APOA to PTN are reduced, and projections from nerve IX-X to the PTN may be missing in *X. laevis* females, which could be the cause of inability to produce mate calls in females of the species. The activation of the anuran PTN (increase in size and metabolic rate), and both the augmented structure and activation during breeding season of the APOA may be additional factors in the neural pathway of mate-calling that are crucial to the ability to produce the call.

The anuran mate-calling mechanisms show many similarities to mechanisms controlling singing behavior in some species of birds. Male birds sing predominantly, and singing can be reduced in zebra finches by castration and then restored with androgen treatment (Arnold, 1975). Like the sexual dimorphism in the APOA of the Japanese toad, parts of the songbird brain in the main song-control circuit are larger in males than in females (Nottebohm and Arnold, 1976). In adult zebra finches, some of these areas increase in volume after androgen therapy, while others do not (Arnold, 1980), implying that regions of the songbird brain differ in their ability to be activated by steroid hormones. The similarities between anurans and birds, along with our own results, suggest that this difference may exist in *R. clamitans* as well.

Our study leads us to believe that brain regions of the anuran mate-calling system are affected in two separate ways by gonadal steroids. This may be due to structural differences in the APOA and the PTN that facilitate different neuronal responses when exposed to androgens. The mechanisms which cause this to occur may be crucial in production and modulation of mate-calling behavior. Information about how these different responses arise may give insight into similar questions concerning gonadal steroids in other vertebrates.

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Effects of Gonadal Steroids

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