Frequency Manipulation of Mating Calls of *Hyla versicolor* with Arginine Vasotocin

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Abstract

The effects of AVT, arginine vasotocin, on mating call production has been studied thoroughly. In many frogs hormones have some effect on the rate of mating calls (Salthe 1974). Other research has shown that certain hormones can initiate calling behavior in some species of Ranids (Boyd 1992). These studies, however, have not investigated whether these hormones have any effect on the frequency of the call itself. In this experiment, the effects of AVT on both rate and frequency of the mating call of Hyla versicolor was investigated.

Male frogs were captured in the field and injected with 100um of AVT or an equal amount of Amphibian Ringer's solution during the mating season. Recording of these frogs were taken and analyzed using the ILS sound analysis system. The changes in frequencies were noted along with the increase in rate of calling by the male frogs.

The rate of calling in AVT injected frogs was significantly higher than the saline treated frogs. There is also an increase in amplitude of the lower frequency between 900-1000 Hz over time in the AVT injected individuals. For the saline frogs, there is no increase in the same 900-1000 hz region which indicated that the overall call is slightly lowered upon injection of AVT. The data support that injection of AVT into male H. versicolor increases the rate of calling and lowers the frequency of the call over time.

Introduction

Demographics of H. Versicolor

The greater gray tree frog or Hyla versicolor inhabits a vast range comprising the entire eastern half of the United States from the Great Lakes to the Gulf of Mexico. This frog can be found concentrated in mixed forests and temporary wetlands of the northern part of the States while its cousin Hyla chrysocelis or the lesser gray frog is found in the southern portion of the States (Tyning 1990).

Hyla versicolor has large rounded toe disks which allow this frog to climb trees. The coloration of this frog is mostly gray with varying patterns covering the body. The adults are two inches to two and a half inches in length and have a very dry, warty appearance. The males can be distinguished from the females by the presence of a black or gray throat with orange coloration while the females have a white throat. Also, males are capable of vocalization while the females are not. It is this characteristic that this experiment will analyze in depth.

Mate Calling in H. Versicolor

The vocalizations, especially the mating calls, are apparent at night in the early spring or summer when the temperature rises above 20^{o} C. The mating calls are usually produced in chorus with other males. The calls are divided up into three different types: the advertisement, courtship, and aggressive call. This experiment focused on the advertisement call which is characterized by a loud, prolonged trill (Tyning1990). This trill which last several seconds is repeated several times a minute, and is used as a beacon for receptive females. The calling males are also flanked by numerous satellite males which do not call. Field observations demonstrate that occasionally satellite males have a higher percentage of reproductive success than the calling male (Tyning1990). In Hyla versicolor, however, these satellite males take over the singing perch of the calling male (Tyning 1990).

The ability to produce mating calls is limited to cyclic breeding males and is influence by hormones (Salthe 1974). Researchers have discovered that injection of Rana pituitaries with chorionic gonadatropin will bring Hyla cinerea males into calling condition, and pituitary injection will increase the frequency of mating calling in Rana pipiens (Schmidt 1966).

For many frogs, mate calling is the most prevalent reproductive behavior. The reproductive success of an individual depends on the ability to evoke the proper calling behavior. In Hyla Versicolor, the mating call consists of two major frequencies at 1000Hz and 1300 Hz. The dominant frequency is at 1300Hz where the frog invests most of its energy. The secondary frequency is at 1000Hz which could be a side product of the initial frequency or an independent indicator to the frog. The females are primed to respond to these specific frequencies and deviations from them may result in lower reproductive success for the male (Gerhart 1987). Females are also able to discriminate subtle changes in call frequency and temporal properties (Schwartz 1987). They also prefer heterospecific calls, complex calls consisting of several frequencies, rather than single frequency calls (Schwartz 1987.)

The analysis of the mate calling can be done in several ways. Traditional methods include sonogram analysis which compares changes in the presence of dominant frequencies. In this experiment, power spectra are used which indicate what frequencies the organism is investing its energy. This is a better method of analysis than sonograms due to the fact that the power spectra demonstrates exactly where the animal is investing its energy. This provides information on both energetic as well as frequency analysis.

Arginine Vasotocin effects on Mate Calls

Arginine vasotocin is a small neuropeptide isolated from the neurohypophyses of nonmamalian vertebrates which controls hydromineral balance (Acher 1985). This neuropeptide is very similar to arginine vasopressin (AVP) in humans. AVT controls reproductive behavior in some amphibians. Its site of actions has been determined to be in the brain by injecting small amounts of the chemical in order to activate clasping behaviors in T. granulosa (Moore and Miller 1983). Also in T. granulosa the concentrations of AVT seem to vary seasonally in correlation to sexual behaviors (Moore 1987). AVT may act at several sites and be aided or influenced by the presence of Gonadotropin and other hormones like prolactin. These neuropeptides could control reproductive behavior by modulating behaviorally important neurons in the sensory pathway, the central nervous system, and the motor pathway (Moore 1987).

This evidence of AVT's influence on sexual behavior in amphibians indicates some correlation between AVT and the reproductive calling of amphibians. This experiment investigated the probable influence of AVT on the mating call of Hyla versicolor, specifically on the rate of calling and modification of call frequency.

Materials and Methods

In order to assess the behavioral aspects and locations of *Hyla versicolor*, observations and recordings of mate calling were made. The frogs, which were found at the same location on the University of Notre Dame Environmental Research Center, were studied for two nights, June 1 and 2. The frogs were recorded and observed in the field using a Sanyo tape recorder and parabolic ear. Also, time, temperature, and weather conditions were noted for each evening of observation.

On June 4, 1993 the preliminary trial was conducted in which twenty male frogs were captured and placed into wire cages. Ten frogs received a 100um injection of AVT in the abdominal cavity. The remaining ten frogs received an injection of Amphibian Ringer's solution which acted as the control. The frogs were placed in two separate cages and observed and recorded at 30 minute intervals for a total of 90 minutes.

On June 6, 1993, the full experiment was conducted in the field. For this experiment, plastic buckets were used as holding containers for the frogs. Twenty male frogs were captured and injected with either 100um AVT or an equal amount of Amphibian Ringer's solution in the abdominal cavity. The frogs were placed into the individual buckets and recorded periodically for 90 minutes at thirty minute intervals. For the duration of the experiment, the rate of calling was counted for each individual on the same interval. Also during this trial temperature and time were recorded for further notation (Table 1).

Analysis of the calls was done at the University of Notre Dame using the ILS sound analysis system installed on an 486 IBM compatible computer. Playback of the tapes were done using a Marantz tape recorder. Examples of calls consisting of non injected frogs, AVT injected frogs, and Saline injected frogs were analyzed with the system using a power spectrum analysis (Figure 1 and Figure 2). For the AVT injected frogs, further investigation of time was introduced by including both the 30, and 60 minute time trials of the calls for both regular and individuals in buckets.

The graphs of the power spectrum provided by the program were analyzed by graphically measuring the highest peaks of the power spectra and the frequency of the specific peak. The secondary peak was also examined in this fashion. These values were complied for each replication of the experiment and analyzed by means of the Tukey test for statistical significance which supported the statistical relativity of the data. Mean values for amplitude and frequency for all groups of frogs were found using Systat 5.0 (Table 2).

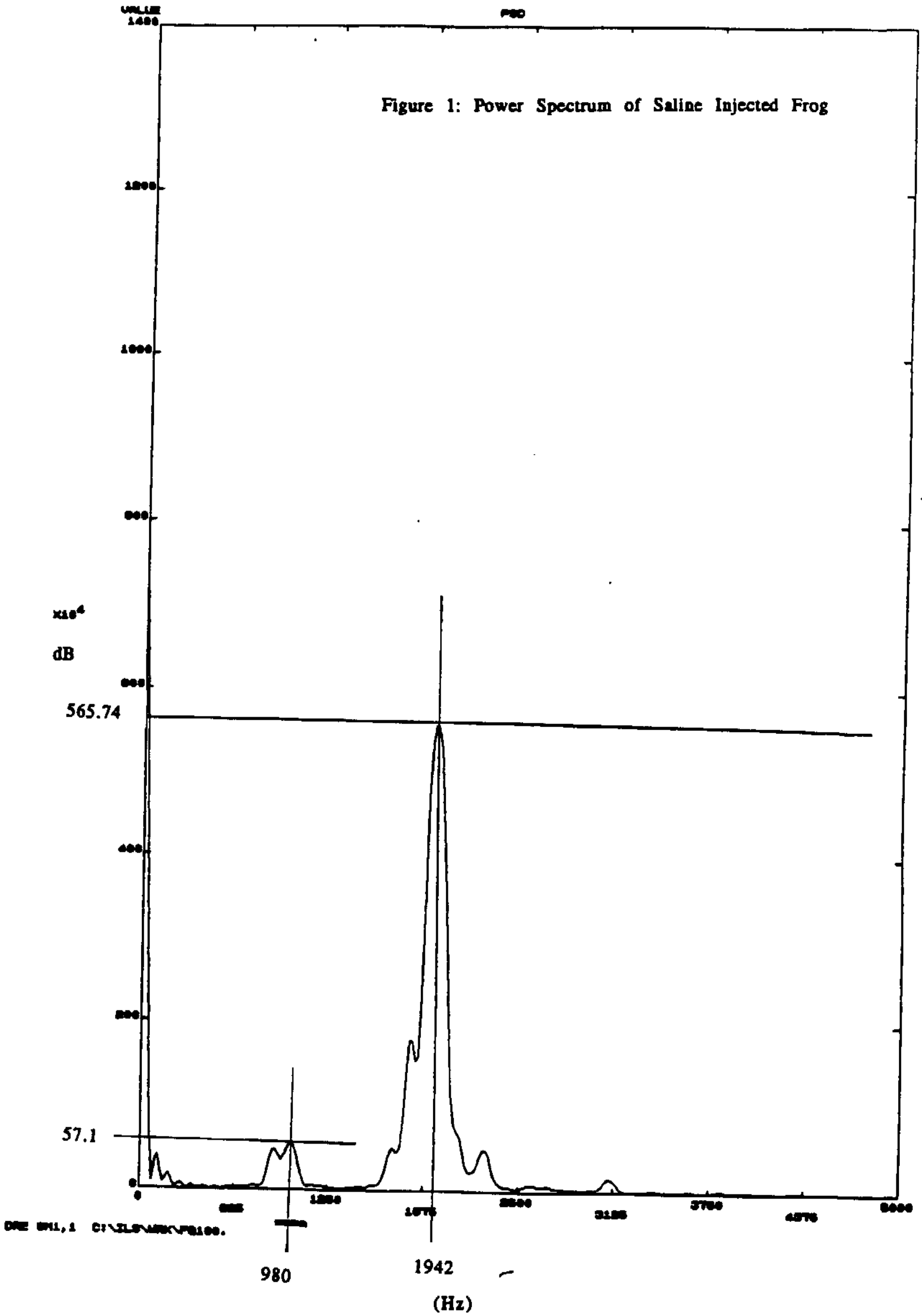


Figure 1: Power Spectrum of Saline Injected Frog

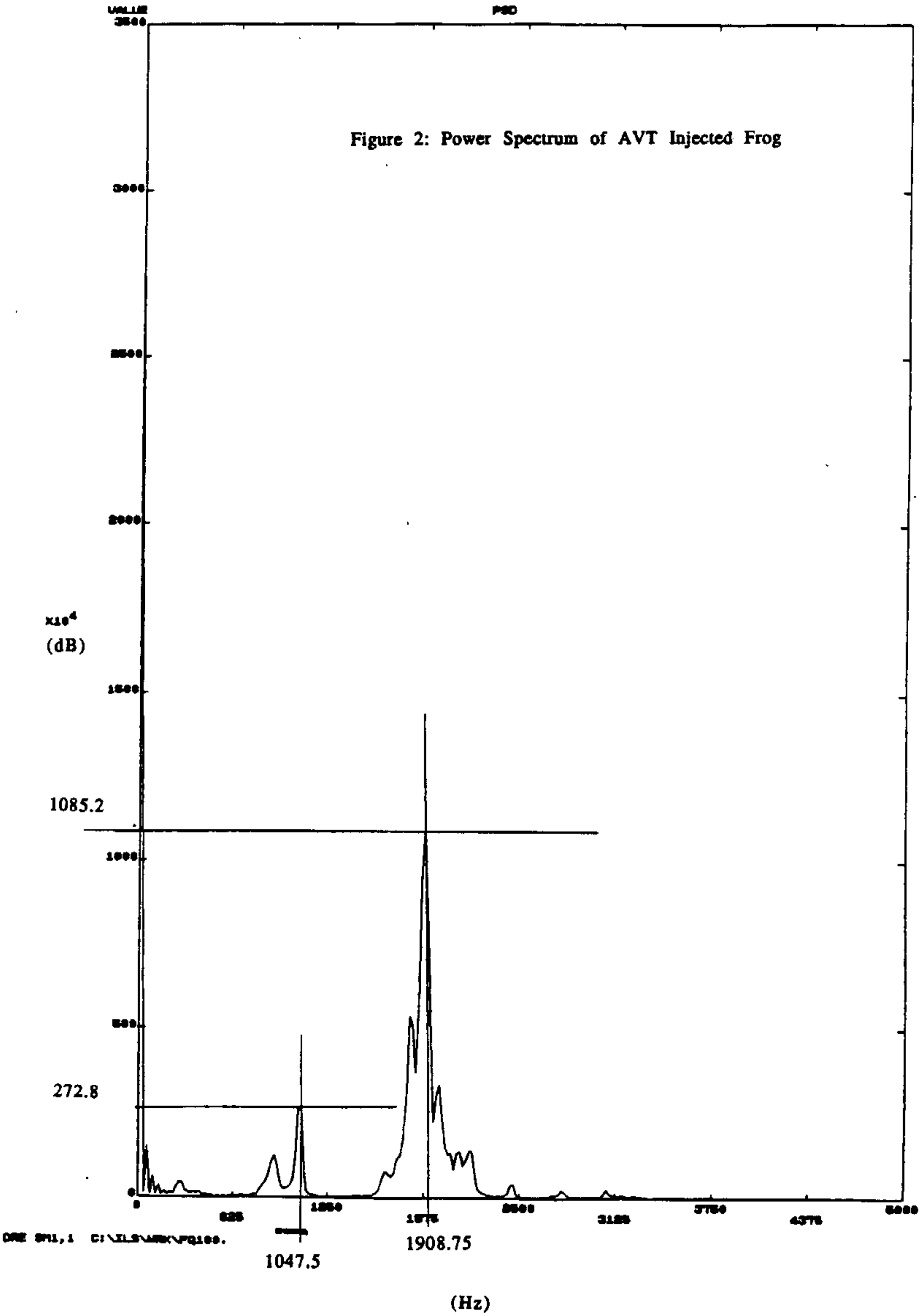
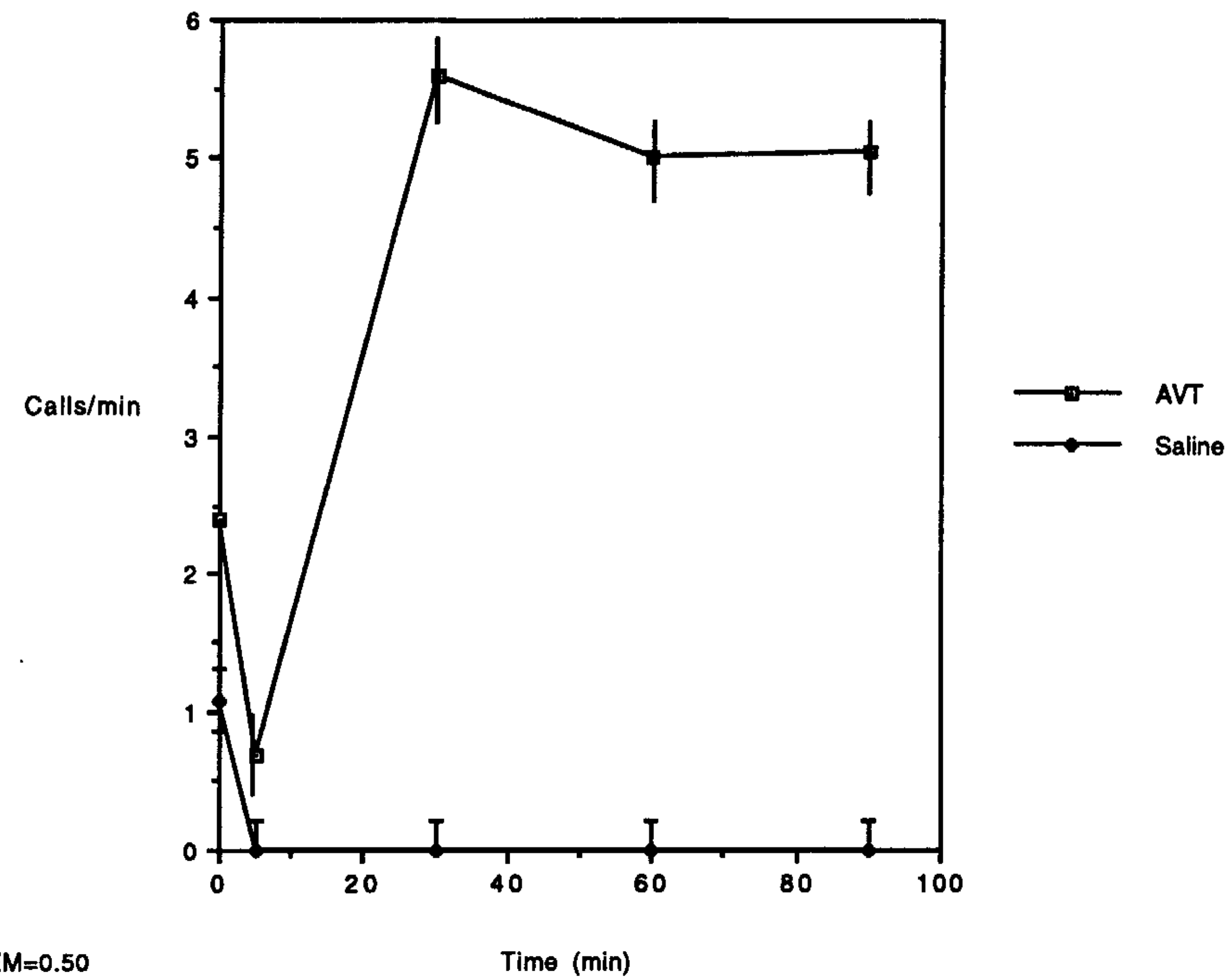


Figure 2: Power Spectrum of AVT Injected Frog

Figure 3: Effects of AVT on Calling Rate



SEM=0.50

Time	Precipitation	Temperature	Lightintensity
30 minute	0mm	18°C	No moon
60 minute	0mm	17°C	No moon
90 minute	0mm	17°C	No moon

Table 1: Weather conditions of Experiment on July 6, 1993

Frog Type	Primary Frequency Displacement (dB) x 10^4 SEM=151.876	Secondary Frequency Displacement (dB) x 10^4 SEM=104.163	Primary Frequency (Hz) SEM= 31.555	Secondary Frequency (Hz) SEM= 14.824
Saline	934.163	74.810	1981.071	1018.571
Saline Bucket	2126.096	117.692 [a]	1894.286	1020.500
AVT	471.600	204.553	2072.083	1057.083
AVT at 30 min	1434.978	461.800	1770.00	1313.750
AVT at 60 min	1896.77	1300.533 [b]	1905.000	1025.00

Table 2: Effects of AVT on Frequency of Mating Calls in H. versicolor

1. [a] t-test $p < 0.14$

2. [b] t-test $p < 0.14$

Results

The AVT injected frogs had a higher rate of calling versus the saline injected frogs. In Figure 3, the mean data of all calling replications showed a sharp increase in the rate of calling in the AVT injected frogs after a five minute lag period. This increase in calling peaked at 5.6 calls/ min at 30 minutes and then leveled off to 5 calls/min after 60 minutes. For the saline injected frogs, a complete decrease in calling was noted which resulted in a cessation of all calling after five minutes.

Overall, the power spectra indicated that there was an increase in amplitude in the lower secondary frequency around 1000Hz in the experimental frogs (Table 2). This increase in amplitude was noted over a time between 30 and 60 minutes. The saline injected frogs show little or no increase in amplitude relative to frequency during this time frame. The AVT injected frogs at 60 minutes did indeed have a lower frequency than the saline injected frogs or the AVT injected frogs at 30 minutes.

Further analysis of the power spectra indicated that the primary frequency did not change in either amplitude or frequency. The mean frequency of the primary frequencies was around 1900Hz. The displacements were also relatively constant for this frequency in both AVT injected frogs and salined injected frogs (Table 2).

Discussion

Significance of AVT

Overall, studies have focused on the influence of external stimuli on the nervous control of the sound-producing apparatus (Schneider 1974). This experiment examined the effects of hormonal changes on the mating call of Hyla versicolor.

The analysis of the physiological aspects of mating calling began with a study of the behavior patterns of the male frogs. The males typically begin calling in early spring and finish calling in late summer. The daily calling phase for the mating call begins at twilight when the air temperature exceeds the lower threshold for calling. The daily cycle of the mating call is not only dependent on temperature, but also on light intensity (Schneider 1974). Temperature affects calling in that it must be above the lower threshold of 20^{o} C if calling activity is to take place (Schneider 1974). Not only do temperature and light intensity play a role in calling behavior, but so does atmospheric pressure and precipitation levels (Blankenhorn 1972). If a low pressure system or heavy precipitation occurs during the day, the frogs usually call in the evening (Garton 1975).

The central nervous system plays a major role in the production of mating calls. Electrical stimulation of the brain provides information regarding the function and location of centers involved in calling. So far, research concentrated on stimulation of the preoptic region of the brain (Schneider 1974). Electrical stimulation of this area of the brain particularly the anterior preoptic and magnocellular nuclei produces a behavioral pattern termed "readiness to call" (Schneider 1974). Further stimulation of the anterior preoptic region produces the actual calling in males.

A considerable amount of experimentation has been done on the effects of AVT on the releasing call of bullfrogs, R. catesbeiana. AVT reduces the number of releasing calls in female bullfrogs (Boyd 1992). Also, AVT stimulates mate calling in Hyla cinera (Penna and Capranica 1984) and mate calling phonotaxis in B. americanus (Schmidt 1985). AVT, however, may work in conjunction with gonadal steroid hormones because of the role of these hormone in maintaining the neural receptors of AVT (Boyd and Moore 1991). These interactions are seen in the influence of steroid-AVT interactions in reproductive behavior especially mate calling in amphibians (Zoeller and Moore 1982 and Schmidt 1985).

Although many other gonadal steroid hormones do have some influence on reproductive behaviors, attempts to evoke reproductive behaviors by injecting testosterone and a variety of other steroid hormones into male amphibians are unsuccessful (Blair 1946, Moore and Muller 1977). Other experiments indicate that there is another testicular factor other that testosterone which influences sexual behaviors in R. pipens (Palka and Gorbman 1973). This second factor has yet to be confirmed, but this has lead to further investigation. In the case of Hyla versicolor, no studies have been done on the effects of arginine vasotocin or other hormones on mating calls.

Further evidence which supports the influence of AVT on sexual behaviors in amphibians comes from studies in which AVT is suppressed by an AVP antagonist (Moore and Miller 1983). AVT injected into female R. pipens in order to demonstrate that the females become more sexually receptive which is apparent by the reduction of releasing calls (Diakow and Raimondi 1981). In male newts, AVT injections increase their responsiveness to female sexual stimuli. This presents evidence that neuropeptides like AVT can influence the propensity to respond to external stimuli by acting on neurons in the sensory afferent pathway or central neurons which integrate sensory input (Moore 1987).

Calling Rate Increase with AVT

The experiment demonstrated the effects of AVT on calling rate. From Figure 3, there is an increase in rate of calling in the AVT injected frogs while the saline injected frogs cease to call after some time. The importance of the increase in rate is substantial due to the effects of both fatigue and selective pressure.

Since the hormone increased the calling rate of the frogs, the vocal cords could be lengthened by fatigue. This lengthening of the vocal cords result in a change in the frequency of the call itself. This hypothesis could explain why the calls of some frogs degraded and changed frequency after injection with AVT. Along with this, selective pressure can be critical for frog survival. The increase in calling exposes the frog to predators which can have a profound effect on the reproductive success of the individual.

An increase in calling rate, however, can be beneficial. Since aerobic metabolism is directly related to call rate, an increase in call rate could indicate a healthy male is calling (Taigen and Wells 1985). This is definitely a selective advantage for the male frog since female would more likely come.

Frequency Changes with AVT

AVT influences the mating call characteristics in H. Versicolor. The power spectra, which shows the relative energy input of an organism into a given frequency, indicate that AVT does lower the frequency of the mating call over time.

Initially, the primary frequency at 1900Hz remained relatively stable and continued to dominate the call even after injection with AVT. The amplitude of this frequency remained the dominant frequency throughout time. As shown in Table 3, there was a gradual but significant increase in the amplitude of the lower, secondary frequencies at 1000Hz of the AVT injected frogs. This indicated an overall increase in the strength of the lower frequencies over time. The net result was a decrease in the frequency of the call in general.

Frequency Changes with Saline

The saline injected frogs, both in the cages and buckets, did not demonstrate any increase in the lower range of 1000Hz. The frequencies themselves for both AVT and saline injected frogs were consistent with each other, and the use of buckets did not distort the call in any significant manner. This can be seen by comparing the major frequencies of the non-injected frogs with the saline injected frogs in buckets (Table 2). The AVT injected frogs in buckets did not vary from the AVT injected frogs in cages with regards to primary frequencies (Table 2).

Comparison of AVT and Saline injected frogs

There was a distinct difference between the amplitudes of the lower, secondary frequency of the AVT injected frogs at 60 minutes and all saline frogs (Table 2). It also showed significant differences in amplitude between AVT injected frogs in buckets at 30 minutes which indicated that the lower frequencies increased in strength over time. At the secondary peak, the amplitude increased compared to the amplitude in the saline frogs.

Experiments performed by Gerhart showed that attenuation of the amplitude of the frequency by 20 dB or, more critically, the decrease in the amplitude of a frequency by 10 dB rendered synthetic calls ineffective in H. cinera (Gerhart 1974). Therefore, the shifts in amplitude due to AVT could have a profound impact on the reproductive success of males injected with AVT.

This change in frequency may be due to the hormones effect on certain vocal cords or laryngeal muscles of the frogs which takes time to manifest. Another explanation could be that since the hormone increases calling rate of the frogs, the vocal cords could be lengthened by fatigue. Schmidt noted that the larynx and the thoracic muscles had a profound impact on the amplitude and frequency of calls in R. pipiens (Schmidt 1965). He also hypothesized that the central nervous system controlled some aspects of the laryngeal muscle. Boyd has shown that AVT does have an effect on the releasing calls in R. catesbiena by means of the neural system (Boyd 1992). This hypothesis could explain why the calls of some frogs degraded after the experiment and the overall increase in the amplitude of the lower, secondary frequencies over time.

The hypothesis that the decrease in call frequency was due to fatigue, was supported not only by the degradation of several calls, but also the inability to induce calling in males 12 hours after the experiment. Total loosening or exhaustion of the vocal cords could cause this cessation in post-experimental calling. Schmidt noted that sufficient air pressure is needed in the thoracic cavity to produce a call even with proper neural stimulation (Schmidt 1965). Complete thoracic muscle or laryngeal muscle fatigue may not allow for the creation of enough pressure for a call.

Other Factors affecting Frequency

Harmonics play an important role in calling frequencies (Gerhardt, 1988). Frogs are able to use harmonics as key frequencies in determining the identify and relative fitness of individuals (Gerhart 1988). The use of buckets, however, did not seem to affect the major frequencies of the calls. The power spectra showed no significant deviations of calling frequencies between individuals in cages or in buckets (Table 2). Also, field calls of non-injected frogs did not differ from the calls of saline injected frogs in buckets which indicated that the buckets had no effect of the major frequencies (Table 2).

The calling characteristics of frogs can, however, be affected by temperature, light intensity, and weather conditions (Garton 1975). Below the critical temperature, 20^{o} C, the characteristics of frog calling change, most notably frequency and rate (Garton 1975). At these temperatures, both the frequency and rate decrease relative to temperature drops. Also, weather conditions and light intensity seem to influence the rate and frequency of calling in many species of frogs. The choruses occurred at night following warm rains or when storms were imminent. This indicates that a decrease in barometric pressure has an effect on calling (Garton 1975). The conditions for the experiment were kept as consistent as possible, but these variables may have contributed to a decrease in frequency of the call.

Conclusions

The importance of this experiment is two-fold. First, this experiment supports earlier evidence that AVT does have an effect on calling behavior in frogs, specifically in rate calling. It can be deduced from this experiment that AVT does initiate calling in H. versicolor just as it does in other species of frogs (Boyd 1992). The effects of this increase in rate could result in a modification of the frequencies produced by these frogs after an injection with AVT. This could be due to either direct lengthening of vocal cords by the hormone or indirectly as a result of fatigue.

The significance of these findings is a demonstration that AVT does modify calling behavior in H. versicolor which could have dramatic consequences in the effectiveness of communication. Although an animal's signal may be complex, behavioral studies often demonstrate that only a few, relatively simple features of a signal are required for recognition by a conspecific (Marler 1966). Female frogs have shown preference for calls that are most conspicuous to them. They choose calls that have a higher call rate and attained the specific frequency that they are attuned to (Gerhart 1987). In this case, modification of the mating call by shifting the attuned frequencies could decrease the effectiveness of an individual's reproductive success. On the other hand, an increase in rate has shown to be beneficial for the attraction of females.

Gerhart also noticed that in very dense choruses, background noise may be so great that calling is an ineffective strategy for any male that cannot call loudly. His experiments indicate that the calls of the individual must be at least as intense as the background chorus in order for the female to locate him (Gerhart 1987). The power spectra indicated a gradual shift towards the lower secondary frequencies which means a shift in energy investment by the frog. This may be detrimental for the male since energy is being shifted towards another peak rather than being focused at one specific frequency. If the females are unable to hear the males calls, any changes in frequency for uniqueness could be counterproductive.

Since reproductive success is an important measure of an organism's success, any advantage or disadvantage is critical. The investment of an organism's energy in reproduction is a perilous balance between many forces. For Hyla versicolor, the male investment in calling behavior is very specific, and any changes in these behaviors influence the males' reproductive success. In H. versicolor AVT's effects on calling behavior by directly increasing the calling rate, and indirectly modifying the amplitude of the frequency peaks can have drastic effects on the male's ability to attract a mate. Even though the biochemical interactions of AVT have yet to be fully disclosed, the impact and possible ramifications of this neuropeptide on behavior is clear.

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