SEXUAL CONDITIONING IN THE DYEING POISON DART FROG

(DENDROBATES TINCTORIUS)

A Dissertation
Presented to
The Academic Faculty

by

Diann E. Gaalema

In Partial Fulfillment
of the Requirements for the Degree
DOCTOR of PHILOSOPHY in the
School of PSYCHOLOGY

Georgia Institute of Technology
MAY, 2010

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Approved by:

Dr. Marcus J. Marr, Advisor
School of Psychology
Georgia Institute of Technology

Dr. Terry L. Maple
School of Psychology
Georgia Institute of Technology

Dr. Paul M. Corballis
School of Psychology
Georgia Institute of Technology

Dr. Mollie A. Bloomsmith
Yerkes National Primate Research Center
Emory University

Dr. Tara S. Stoinski
Primatology Department
Zoo Atlanta

Date Approved: [December 4th, 2009]

ACKNOWLEDGEMENTS

I would like to thank Robert Hill from the Atlanta Botanical Garden for his invaluable help in conducting all aspects of this research. Also, thank you to my advisor Jack Marr. Even though he was rather forced into taking me on as a graduate student he has done a fantastic job. I would not be the researcher I am today, and likely would not have finished graduate school, without his guidance and encouragement.

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UCS	Unconditional stimulus
UCR	Unconditional response
CS	Conditional stimulus
CR	Conditional response

SUMMARY

Amphibian populations worldwide are currently in decline. One approach to preventing extinction of some of the affected species is to create assurance colonies. These sustainable captive populations might some day be used to reestablish wild populations. One issue with creating assurance colonies is successful breeding; often difficulties arise when attempting to breed exotic animals in captivity. Sexual conditioning, a form of Pavlovian conditioning, has been shown to improve breeding behavior. In this project the efficacy of sexual conditioning to improve breeding behavior in the dyeing dart frog (Dendrobates tinctorius) was tested. The frogs were trained with a stimulus that was either predictive of or independent of exposure to a member of the opposite sex. The group trained with the predictive stimulus showed shorter latencies to a variety of breeding behaviors and produced more eggs than the control groups. The sexual conditioning procedure also increased expression of various breeding behaviors allowing for careful examination of calls and visual signaling within this species.

CHAPTER 1

INTRODUCTION

Currently, world-wide, amphibian populations are in crisis. A large number of species are suffering catastrophic declines and widespread extinctions (Norris, 2007). As this decline continues researchers are scrambling for ways to preserve these species. While many species are suffering declines, some groups of animals seem to be at a greater risk than others. Declines are associated with species that inhabit moderate or high elevations and those that have aquatic larvae (Lips, Burrowes, Mendelson, and Parra-Olea, 2005). One oft-cited proximate cause of amphibian species decline is the presence of a chytrid fungus (*Batrachochytrium dendrobatidis*) which kills many of the amphibians it infects.

While explanations for the cause of this decline may differ (e.g. Wake, 2007) there is little doubt that an extinction wave is occurring and something must be done, and soon. One suggested tactic is to collect animals from the wild before they go extinct and breed them in captivity, which is currently the only recourse for some species that have already disappeared from the wild (Lips, Burrowes, Mendelson, and Parra-Olea, 2005). The goal of collecting these species would be to one day reestablish populations in the wild, once the causes for the decline can be dealt with. This approach would require the creation of captive breeding facilities where assurance colonies of these animals could be created and maintained (Gagliardo, Crump, Griffith, Mendelson, Ross, and Zippel, 2008). This tactic is particularly appropriate for extinctions caused by chytid fungus. Fungus infections spread relatively predictably across the landscape, allowing for populations to be removed from an area before the fungus moves in. Indeed, *ex situ* breeding programs are already being incorporated into current attempts to preserve amphibians from affected areas. However, some species have proved difficult to maintain and others are not breeding well in captivity.

Breeding of exotic animals in captivity is often problematic (e.g. Zhang, Swaisgood, and Zhang, 2004). During breeding attempts, animals may show inappropriate aggression, a lack of proper breeding behavior, or breeding attempts may prove unsuccessful due to infertility or unviable offspring (Augustus, Casavant, Troxel, Rieches, and Bercovitch, 2006; Bishop, Haigh, Marshall, and Tocher, 2009; Daleum, Creel and Hall, 2006; Munkwitz, Turner, Kershner, Farabaugh, and Heath, 2005; Swaisgood, Dickman, White, 2006; Zhang, Swaisgood, and Zhang, 2004). Consequently, practices that can potentially address these issues would prove quite valuable in captive breeding. In fact, some assisted reproductive technologies are being perfected to be used on frogs due to difficulties in captive breeding (Browne, Clulow, Mahony, and Clark, 1998). For example, some approaches that have been attempted recently are the cryopreservation of frog sperm and supplementing males and females with hormones. However, the use of purely behavioral techniques is much rarer. One approach that has not yet been attempted with amphibians is sexual conditioning.

Sexual Conditioning

Sexual conditioning, a form of Pavlovian conditioning, has been shown to improve breeding outcomes on a variety of measures. Reductions in inappropriate aggression, and increases in breeding behavior and reproductive success have been demonstrated from this procedures, the same outcomes that would be needed to address known issues when attempting to breed exotics in captivity (Domjan and Hollis, 1988; Hollis, Cadieux, and Colbert, 1989; Domjan, Blesbois, and Williams, 1998; Adkins-Regan and MacKillop, 2003). In this method, a stimulus that is initially ineffective at eliciting the target behavior (the conditional stimulus or CS) is repeatedly paired with exposure to a potential sexual partner (the unconditional stimulus or UCS) which will produce an unconditional response (UR) in the subject. After repeated pairings the animals are exposed to the CS and given a longer period of time to interact. Presumably, repeated pairings of the UCS and CS will elicit a conditional response (CR) during CS presentation which will result in improved breeding behavior (Pfaus, Kippin and Centeno, 2001).

Behavioral Changes in Sexual Conditioning

One of the most common outcomes seen in sexual conditioning is an increase in behaviors associated with breeding and a decrease in non-breeding related behaviors. For example, in an experiment using blue gouramis, sexual conditioning was shown to have positive effects on several aspects of behavior associated with reproduction (Hollis, 1984). Signaled presentation of a rival allowed males to defend their territory more aggressively than control males. Also, having signaled aggressive encounters increased males' chances to win not only the immediate fight but also fights in the future (Hollis, Dumas, Singh, and Fackelman, 1995). Sexual conditioning can also convey advantages in male-male competition for female access (Gutiérrez and Domjan, 1996). In quail, when two males are given access to a female, the male who previously had signaled encounters will copulate with the female sooner than the male who did not have that history of pairings.

Additionally, sexual conditioning can have direct effects on breeding behaviors. Males in groups where access to a mate was reliably signaled showed more courtship behavior and less aggressive behavior towards females than males in the control group (Domjan and Hollis, 1988; Hollis, Cadieux, and Colbert, 1989; Hollis, Martin, Cadieux, and Colbert, 1984). Interestingly, sexual conditioning has also been shown to be effective in both sexes. Female quail with reliably signaled encounters had positive changes in their breeding behavior as compared to control females (Gutiérrez and Domjan, 1997). However, differences can arise in how the conditional behavior is emitted: while male quail tend to approach a conditional stimulus, females will respond to the stimulus by an increase in squatting behavior (a sexually receptive behavior).

Sexual conditioning has also been shown to improve breeding behavior in other ways. One common outcome is a reduction in latency to copulation or ejaculation (e.g. Domjan, Lyons, North and Bruell, 1986; De Jonge, Oldenburger, Louwerse and Van De Poll, 1992). Sexual conditioning has also been used to improve sexual performance of rats that had previously failed to copulate successfully (Cutmore and Zamble, 1988).

Sexual conditioning has also been demonstrated in studies conducted for other reasons. For example Van Kempen (1997) attempted to show that food-calling behavior in roosters might also have a reproductive component. In an unintended result, roosters performed more reproductive behaviors in rooms where they had been exposed to females.

Conditioning of sexual behavior has also been examined in male and female humans. In one experiment, male sexual behavior was conditioned by presenting semi-nude pictures of women either alone, or paired with an erotic video (Lalumiére and

Quinsey, 1998). Males in the paired group showed a 10% relative increase in arousal to the pictures. In a similar experiment females were exposed to pairings of an amber light with erotic video clips (Letourneau and O'Donohue, 1997). Subjects reported increased feelings of arousal when exposed to the light as compared to when they were not. However, the validity of many of the earlier human sexual conditioning studies has been questioned (O'Donohue and Plaud, 1994) as these studies do not adhere to the requirements of a properly controlled classical conditioning study.

Reproductive Success

Sexual conditioning has also been shown to have direct reproductive benefits. For example, predictive signals have been shown to increase successful ejaculations and the amount and quality of sperm released by male rats, as well as the probability of fertilization of eggs in quail (Cutmore and Zamble, 1988; Domjan, Blesbois, and Williams, 1998). In fact sexual conditioning can increase reproductive success in quail independent of which sex is being conditioned (Adkins-Regan and MacKillop, 2003). Additionally, male blue gouramis who had been trained with pairing of a signal with access to a female spawned with females sooner, clasped females more often, and produced more young than control group males (Hollis, Pharr, Dumas, Britton, and Field, 1997; Hollis, 1990).

Neutral Stimuli as Signals

Several experiments have successfully used "arbitrary" stimuli such as flashing lights in sexual conditioning procedures (e.g. Hollis, Pharr, Dumas, Britton, and Field, 1997; Hollis, 1990). Why would such a sensitivity to pairings of arbitrary stimuli and sexual opportunity occur? The ability to use signals that predict a female's presence would give any polygynous male an advantage (Hollis, 1990). Additionally, the signal that proves predictive may vary faster than the genes could adjust to keep up with it. Therefore it might be better to have some flexibility in what can be used as a signal. However, most likely, the effectiveness of a particular stimulus for predicting sexual opportunities is a combination of genetic dispositions and history of pairings with past opportunities (Pfaus, Kippin and Centeno, 2001).

Conditional Approach

One common outcome of sexual conditioning procedures is conditional approach of stimuli involved in the procedure. As pairing trials progress subjects will begin to approach the CS or UCS during CS presentation. This behavior appears to develop rapidly, independent of species used (Domjan, Lyons, North and Bruell, 1986). Conditional approach can occur in quail under a wide variety of CSs, chamber sizes, UCS lengths, and CS-UCS intervals (Domjan, Lyons, North and Bruell, 1986; Crawford and Domjan, 1993). Under all of these different conditions, the most reliable indicator of sexual conditioning is conditional approach of the CS (Pfaus, Kippin, and Centeno, 2001).

Another interesting characteristic of sexual conditioning in this species is the propensity to elicit sign-tracking (approach CS) instead of goal-tracking (approach UCS) behavior (Burns and Domjan, 1996). This can be tested by placing the UCS access gate further away from the CS. Additionally, unlike with a food UCS, increasing the distance between a CS and a sexual UCS or inserting a delay between CS and UC presentation does not shift behavior from sign-tracking to goal-tracking.

Non-Neutral Stimuli

While many sexual conditioning studies have been conducted with initially neutral stimuli (e.g. Hollis, 1984) other studies have used CSs that are arguably anything but neutral. Many of the experiments conducted with Japanese quail used a conditional stimulus that mimicked a female quail (Holloway and Domjan, 1993a). While the results of other experiments suggested that sexual conditioning can occur with a large range of stimuli (Domjan, O'Vary and Greene, 1988), conditioning may proceed more rapidly and be more resistant to extinction when the CS shares characteristics with the UCS (Cusato and Domjan, 1998; Domjan, Cusato, and Krause, 2004). Indeed, using species-specific stimuli appears to produce conditioning that is more resistant to blocking and increases in the CS-UCS interval (Domjan, Cusato and Krause, 2004). In fact Domjan and his colleagues argue that learning in the lab with arbitrary CSs may only occur because of the artificial rates of co-occurrence that can be applied in a controlled setting. However, one could also argue that using a CS that shares characteristics with the UCS is not Pavlovian conditioning at all. Pavlovian conditioning by definition requires the use of a stimulus that is initially neutral in regard to the behavior being elicited.

If the question of neutrality is put aside, three types of cues seem to be of importance in sexual conditioning: local, species-typical, and contextual (Domjan, 1994). Each of these cues can facilitate the others, allowing sexual conditioning to proceed over a wide variety of situations. Whereas initially only species-specific cues facilitate sexual behavior, through sexual conditioning, local and even contextual cues can come to elicit similar effects.

Species-specific cues seem to facilitate sexual conditioning (Domjan, 1994). Such a result would appear to fit well with the behavior system model of learning (Timberlake, 1993; Timberlake, 1999). Learning is presumed to have evolved to support alterations, based on experience, of an already functioning system. These alterations would allow for a closer fit of behavior to the current environment. To illustrate, an outcome of learning would depend on several starting factors. Each factor has its own set of predispositions and constraints, which might be imagined as dips and hills. When a learning situation occurs these systems are in a sense combined, or overlaid and the system "settles" at an output that satisfies all causal sequences. In terms of sexual conditioning a non-neutral stimulus might be imagined to have a lower dip than a neutral stimuli, allowing behavior to settle there more easily.

Establishing Operations and History Effects

Sexual conditioning, like other forms of Pavlovian conditioning, is sensitive to establishing operations. For example, Holloway and Domjan (1993b) found that sexual conditioning is also a function of sexual motivation. When subjects were kept at a longer photo period (to simulate the breeding season) sexual motivation increased, allowing greater sexual conditioning.

Sexual conditioning is also susceptible to satiation effects. Sexual satiation reduced responding to a sexually conditional stimulus (resulting in almost no copulations); although, conditioned approach of the CS remained strong (Hilliard and Domjan, 1995). Consequently, when motivation for sexual behavior is diminished (by changing the photoperiod to represent the non-breeding season) even conditional approach is diminished (Holloway and Domjan, 1993b). Conditional approach behavior could later be reestablished by lengthening the photoperiod or administering testosterone. Satiation effects were also shown to reduce approach to a second-order sexually conditional stimulus (Crawford and Domjan, 1995). However, in general, it appears that satiation affects copulatory activity more than search behavior in sexual conditioning (Hilliard, Domjan, Nguyen, and Cusato, 1998).

Individual history of an organism also has important effects on sexual conditioning procedures. For example, male rats with some sexual experience respond more rapidly to the procedure than animals that are sexually naïve (De Jonge, Oldenburger, Louwerse, and Van De Poll, 1992), a finding that has also been replicated with quail (Holloway and Domjan, 1993a).

Flexibility in Methodology

One extraordinary characteristic of sexual conditioning is its effectiveness even with large variations in methodological variables; the methodology appears to be remarkably flexible. For example, male quail will approach a light that predicts visual exposure to a female (Crawford and Domjan, 1993) with no appreciable differences in behavior even when the UCS differs in length from 30-240 seconds. Conditioning can occur under a wide variety of situations. Changes in breeding behavior have been obtained using white or red lights, large or small chambers, and a CS-UCS interval of 10 or 30 seconds (Domjan, Lyons, North and Bruell, 1986). The conditional stimuli successfully used for sexual conditioning has also varied widely. Stimuli used have included: wooden blocks (Burns and Domjan, 1996), terry-cloth figures (Akins, 2000), a mounted female quail head (Domjan, 1997), different sounds (Gutiérrez and Domjan, 1996), visual access to a female (Hilliard and Domjan, 1995), red and green lights (Crawford and Domjan, 1993), contexts (different cages) (Adkins-Regan and MacKillop, 2003), odors (Kippin, et al., 1998) and even a stuffed toy dog (Domjan, O'Vary and Greene, 1988).

Sexual conditioning experiments have also varied in the number of pairing trials. One experiment with blue gouramis involved a UCS of visual access, one pairing trial per day for 18 days, and a testing trial lasting 6 days (Hollis, Pharr, Dumas, Britton and Field, 1997). In some research with quail, comparatively, conditioning has been achieved using as little as a single pairing trial, with copulation as the UCS and a testing period lasting only two minutes (Hilliard, Nguyen and Domjan, 1997). Other experiments have specifically explored the requirements in terms of timing and number of pairings necessary for successful sexual conditioning (Zamble, Mitchell and Findlay, 1986). For example, for the rat, the ideal CS-UCS interval is between eight and sixteen minutes and less than ten trials are necessary for reliable conditioning.

Sexual Conditioning as Pavlovian Conditioning

Sexual conditioning also shows many of the same characteristics as other types of Pavlovian conditioning. Extinction has been demonstrated, conditional responses do decrease with unpaired trials (Domjan, Lyons, North, and Bruell, 1986). Also, second-order conditioning and conditioned inhibition have been demonstrated in quail and rats (Crawford and Domjan, 1995; Crawford and Domjan, 1996; Zamble, Hadad, Mitchell and Cutmore, 1985). In second-order conditioning a stimulus that has acquired a conditional response is paired with a second, neutral stimulus. As a result the second stimulus will also produce a conditional response, even though it has never been presented with the unconditional stimulus. Conditional inhibition was demonstrated by training discrimination between stimuli that were predictive of the presence or absence of a female quail (Crawford and Domjan, 1996). Later, during testing, the addition of the CS- was shown to inhibit sexual behavior in the presence of the CS+.

Sexual conditioning resembles other types of classical conditioning in other ways as well. For example, generally, conditional responses are acquired more readily in a delay procedure where the CS presentation precedes UCS presentation, but with some overlap of the two, as compared to a trace procedure with a delay between the end of the CS presentation and the beginning of the UCS presentation. Sexual conditioning is no exception, conditional responses are acquired when the UCS immediately follows the CS but are not acquired with ten or twenty minute trace intervals (Hilliard, Domjan, Nguyen, and Cusato, 1998).

Operant Effects

Within many Pavlovian procedures, operant effects may also be at work. It is often difficult to clearly define certain procedures as either operant or classical conditioning (Schwartz and Gamzu, 1977). The process of auto-shaping is particularly illustrative of this concept. Initially a bird can be induced into pecking a lighted key purely by pairing the light with the presentation of food, no behavior is required. However, once the pecking is established, it is commonly used as behavioral measure of operant procedures. Where exactly does the classical conditioning end and the operant conditioning begin?

While sexual conditioning seems likely to include elements of operant conditioning (e.g. approach of the CS is “reinforced” with access to a mate) the sexual conditioning procedure appears to be resistant to omission contingencies (Crawford and Domjan, 1993). In an omission contingency test, approach to the CS causes the UCS not to be presented. It is assumed that if operant conditioning is an important part of the procedure the omission contingency will either eliminate or retard acquisition of conditioning. In this experiment, subjects exposed to an omission contingency were not slower to acquire a conditional response than yoked control subjects. These results imply strong Pavlovian control over conditional responses in sexual behavior.

Conditional Preference

Sexual conditioning can also have the effect of influencing preference for particular stimuli. For example, sexual conditioning has been shown to influence sexual preference. In one experiment, a conditional stimulus (almond odor) was paired with receptive females (Kippin et al., 1998). Later male rats were shown to prefer (by first ejaculation) females that were marked with the almond odor. Additionally, hamsters have been shown to prefer a colored compartment where they had experienced sexual encounters (Meisel and Joppa, 1994). However, it is impossible to untangle the operant and Pavlovian effects of these particular procedures.

The reinforcing properties of other stimuli can also be influenced by sexual conditioning. In one experiment lights or white noise were paired with the presentation or absence of a sexually receptive female (Everitt, Fray, Kostarczyk, Taylor and Stacey, 1987). Later the lights or noise were used as a reinforcing stimulus for lever-pressing. When the CSs were present, lever pressing increased and when they were omitted, response frequency decreased. This interesting approach allowed for the measurement of motivational effects while separating out the reflexive behaviors that are usually used as outcome measures.

Physiological Correlates of the Conditioning Procedure

The physiological mechanisms that accompany the increased reproductive success seen in sexual conditioning have also been examined. For example, Graham and Desjardins (1980) found that rats that had been conditioned produced luteinizing hormones and testosterone in response to the conditional stimulus. In this species, release of hormones may have been one of the physiological correlates of the conditioning procedure. Having already secreted these hormones before the female was present would possibly have reproductive benefits for the rat as he is already chemically “prepared” for the mating opportunity.

Sexual conditioning has been shown to be effective in a wide range of species, from fruit flies to humans, and as such would be expected to have similar effects in a frog population (Woodson, 2002). However, historically there have been difficulties in demonstrating learning in frogs, so any attempt to produce conditioning in a frog should be done with cognizance of the potential issues (Zavala, 1968).

Classical Conditioning in Amphibians

Research with learning in frogs has varied in its success. Initially there was great difficulty with classically conditioning frogs (Zavala, 1968). Many stimuli that were considered neutral to other organisms (lights, buzzers, tones, etc.) may have actually been aversive to frog species, inhibiting movement and making conditioning of those stimuli with shock difficult. However, by using bubbles in water as a CS, a level of responding at about 30% after 75 trials could be obtained. While not particularly impressive, this result was much more successful than earlier conditioning attempts.

More successful results were obtained when attempting to condition movements of the nictitating membrane in frogs (Goldstein, Spies, and Sepinwall, 1964). For this study the UCS consisted of a touch to the cornea and the CS a touch to the snout. Three days of 25 trials per day produced reliable CRs and extinction and relearning were prompt (about three sessions and one session respectively). Other studies replicated the success of conditioning the nictitating membrane reflex (Glanzman and Schmidt, 1979). Subjects in these studies showed normal characteristics within the areas of habituation, generalization, and spontaneous recovery, simple vertebrate models appropriate for study of basic learning principles.

Positive results have also been obtained when conditioning less "active" behaviors in amphibians. Daneri, Papini and Muzio (2007) demonstrated classical conditioning in common toads using fluid immersion. Immersion in a hypertonic solution was preceded by the presence of a different fluid by either 30 or 180 seconds. Toads with a reliably predictive signal showed changes in behavior during both the CS and the UCS presentation (as measured by increased heart rate). Additionally, the members of the experimental group were able to, over the four trials, prevent the hypertonic solution from even being absorbed.

Classical conditioning has also been used to study odor detection in salamanders. In one study, tiger salamanders exposed to parings of common odors and electric shocks (Dorries, White and Kauer, 1997). The salamanders showed signs of conditioning, measured by physiological response (skin potential responses), to three of the four odors. The salamander's ability to detect camphor is now in question. Conditioning has also been used to attempt to find flavor aversion learning in amphibians (Paradis and Cabanac, 2004). The experimenters in this case did not find flavor-aversion learning in this taxa although whether the CS or the UCS used in the experiment were actually salient to the amphibians is unknown.

Another interesting application of classical conditioning in amphibians has been the study of social learning in frogs. Ferrari and Chivers (2008) paired odor of an unknown predator (tiger salamander) with behavioral responses from tadpoles (of a different species) that were familiar with tiger salamanders as predators. The conditioning was successful, subjects showed a substantial decrease in activity (normal tadpole anti-predator response) the next time they were exposed to salamander odor.

The study of learning in frogs has not been restricted to classical conditioning, although there are fewer reports on operant conditioning. In one interesting example, fire-bellied toads were trained to run complex mazes to a criterion of three errors or fewer (Brattstrom, 1990). The critical elements in this experiment were finding an anuran that crawled rather than hopped (so as not to escape the maze) and using water as a reinforcer for the slightly dehydrated subjects. It appears that, given the correct stimuli and procedures, learning can be demonstrated with frogs in a variety of situations.

Dyeing Dart Frogs

The subjects used in this study were *Dendrobates tinctorius*, also known as the dyeing dart frog, members of the poison dart frog family. The poison dart frogs are a group of fascinating and often physically striking species. Members of this group show a great diversity in parental care, coloration, and toxicity. In fact the exact phylogeny of this group, and how many times these various characters have developed, is still under contention (Vences, et al., 2003; Vences, et al., 2000). Of all the unique characteristics of Dendrobatoidae, one of the most interesting (especially for display purposes) is their bright and varied coloration. The striking coloration of this group is suggested to serve as aposematic warning signals, as many of the species are highly toxic (Lötters, Jungfer, Henkel and Schmidt, 2007). The toxins themselves are of interest, as most of them seem to be derived from alkaloids obtained by the frogs though their diets (often ants). However, despite a long persisting assumption that the bright coloration serves as a warning for the toxic members of this group, no research has been conducted to directly test it. Only indirect evidence can be used to support this idea. For example, the toxins are not only deadly, but also apparently taste very bad, some non-toxic species in this group have similar coloration to the toxic members (Batesian mimicry), and many of these species are diurnal, a dangerous proposition for a brightly colored animal without defenses.

The Dendrobatoidae are also very interesting in how they differ from other frogs in their reproductive behavior (Lötters, Jungfer, Henkel and Schmidt, 2007). This group tends to lay fewer eggs, but have increased parental care (either by both sexes or by the male) as compared to other anurans. Additionally, amplexus (clasping of the female by the male during copulation), which is typically found in frogs, is either absent or has been modified in this group. Instead, for many of these species, the female simply lays the eggs and then the male fertilizes them.

The dyeing dart frogs are one of the toxic members of the group, although living in captivity most likely separates them from the naturally occurring alkaloids and robs them of much of their toxicity (Lötters, Jungfer, Henkel and Schmidt, 2007). These frogs are fairly large for dart frogs (34-60 mm) and are brightly colored, with patterns varying greatly between individuals. In fact, this high diversity in coloration had proved deceptive. *Dendrobates azureus* had been described as a separate species from *D. tinctorius* based in part on their extreme differences in coloration. However, based on recent genetic evidence, the two groups have now been combined.

The large size and bright coloration of dyeing dart frogs make them ideal for display, and as a result they are a fairly common dart frog in both commercial and private collections. Due to their popularity, more information on husbandry, breeding, and even enrichment are available for this species than for many other types of frogs (Pfeiffer, 2003; Hurme, et al. 2003). However, many specifics of their behavior are still unknown. Descriptions of the breeding behavior of related species may help clarify which behaviors might be important in this species as well.

Breeding Behavior in Related Species

Calls

Dendrobates tinctorius is part of a taxa labeled the tinctorius group. The species of this group, those most closely related to *D. tinctorius*, include *Dendrobates auratus*, *Dendrobates leucomelas*, and *Dendrobates truncatus* (Summers and Earn, 1999). This group also includes *D. azureus*, as it is just a sub-species of tinctorius.

Objective descriptions of calls exist for several related species of dendrobatid. Most of the calls in the tinctorius group are of the type described as a "buzz call", classified by a single note of 20-400ms, consisting of similar pulses with or without slight frequency modulation (Lötters, Reichle, and Jungfer, 2003). Both the call of *D. auratus*, described as a soft buzzing noise of approximately two seconds in duration (Dunn, 1941) and the call of *D. truncatus*, described as a quiet, low, buzzing noise (Wilkinson, 2004), fall under this classification. However, due to lack of objective measurements, the *D.*

truncatus call is not officially classified under the system proposed by Lötters, Reichle, and Jungfer (2003). Additionally, there is some debate as to the classification of the *D. truncatus* call, it has also been described elsewhere as a trill call (Zimmerman and Zimmerman, 1988). The *D. leucomelas* call is the exception to the group, a relatively loud call characterized as a trill (Lötters, Reichle, and Jungfer, 2003). The *D. tinctorius* call was also not classified under this system due to lack of objective measurements. However, the call has been subjectively described as a soft buzz and should most likely be categorized with the calls given by *D. truncatus* and *D. auratus*.

Other Breeding Behavior

Courtship in *D. auratus* includes elaborate tactile interactions between partners, with the female taking the more active role (Wells, 1978; 1977). Calling males are approached by females who initiate tactile interactions (Dunn, 1941). Early events in courtship may include snout-touching and touching of the male's back by the female. Sometimes the female may climb on top of the male with heads aligned and touch him with her front feet, or she may climb on him facing the opposite direction and drum on his back with her hind feet. Members of both sexes have been observed to face each other while moving their front feet up and down. In *D. leucomelas* the male leads the female while the female strokes and nudges him and climbs on his back (Summers, 1992). The male calls intermittently during courtship and females emit more tactile behavior than males. The behavior of these two species can be compared to the related outgroup *Dendrobates histrionicus* where tactile interactions are few, and the few that occur are initiated by the male.

Little is known about the breeding behavior of *D. truncatus*. The female may take the more active role in courtship as interactions have included the description: "if a male is successful in attracting a female …" (Wilkinson, 2004). Husbandry guidelines exist for this species (Londoño and Tovar, 2008) including descriptions of the conditions necessary to produce breeding in this species and notes about egg and tadpole development. However, there are few descriptions of the behavior involved in breeding situations (Lötters, Jungfer, Hinkel, and Schmidt, 2007). However, it has been suggested that the breeding behavior of *D. truncatus* is very similar to that of *D. auratus* and *D. tinctorius* (Zimmerman and Zimmerman, 1988).

The breeding behavior for the dyeing dart frog has not been fully described, and in ethograms devised for this species categories such as "courtship behavior" often lack objective descriptions (Hurme, et al. 2003). However, some aspects of courtship in this species have been described (Lötters, Jungfer, Henkel and Schmidt, 2007). Only the males call and the calls may serve to lead the female to a proper egg-laying site. The female is reported to initiate courtship by rubbing or stroking the male and jumping on his back (Polder, 1974, as reported in Wells, 1978; Wells, 1977). The female appears to take an active part in courtship, and females have been reported to touch a male's back while being led to the appropriate site. Generally the breeding behavior in this species is described as being very similar to that of *D. auratus*.

Visual Signaling

A variety of types of visual signaling during courtship has been reported in some frog species (Hödl and Amézquita, 2001). These can include elaborate visual displays such as running and jumping, extension and waving of the back legs, or even inflating of the body. Several examples of visual signaling may be particularly relevant to the subject species. These include: “toe-trembling” (twitching or vibrating the toe without moving the leg), “limb-shaking” (rapid up and down movements of a fore or hind foot), “body-lowering” (pressing either the anterior portion of the body or the whole body against the substrate), and “upright posture” (extending arms and raising the anterior part of the body, used as a posture or combined with walking).

Visual signaling has been reported in *D. tinctorius* and in other related species, although it has not been well described. For example, in *Colostethus trinitatis* males turn black and jump up and down in front of females (Wells, 1977). In *Dendrobates histrionicus* (a species in a sister group to the tinctorius group) several types of visual signaling have been reported (Silverstone, 1973). This includes “shaking” (brief kicking or trembling of fore or hind limbs), “bowing” (raising and lowering the anterior part of the body), “crouching” (flattening entire body against the substrate), and “circling” (female rotates whole body while next to male). In some dendrobatids, such as *Colostethus inguinalis*, visual interactions may be very simple (Wells, 1980). Females may crouch in front of males to initiate courtship and males circle females before leading them off to an oviposition site. While toe-trembling and limb-shaking have been reported in the *tinctorius* group, tactile interactions have historically been emphasized (Lötters, Jungfer, Hinkel, and Schmidt, 2007).

Of historical note, this species is known as the dyeing dart frog as it was reported that indigenous tribes would pluck the green feathers off of young parrots then rub the bare skin with secretions from this frog (Lötters, Jungfer, Henkel & Schmidt, 2007). The feathers then supposedly would grow back red or yellow. However, no one has actually proved this claim and it is considered unlikely.

The dyeing dart frog already has several ties to the amphibian crisis. These dart frogs are known to be susceptible to the chytrid fungus. In fact *Dendrobates tinctorius* have been used as an outcome measure to see if other amphibians are indeed carriers of the fungus (Daszak, et al., 2004). The relatively high availability for these frogs (as compared to other exotic species) makes them ideal for experiments regarding these issues. Infected tissue from these frogs has even been used to help improve chytrid identification techniques (Van Ells, et al., 2003). In this project it was hoped that contributions would be made by this species not through their death but through their breeding.

Several characteristics of this breed make these frogs good candidates for sexual conditioning. Dyeing dart frogs are diurnal, fairly terrestrial, and relatively active (Lötters, Jungfer, Henkel and Schmidt, 2007). Also both sexes appear to play an active part in courtship, allowing for the potential of conditioning of both sexes and subsequently, more positive outcomes.

In this project the efficacy of sexual conditioning to improve breeding behavior in the dyeing dart frog (*Dendrobates tinctorius*) was tested. The frogs were trained with a stimulus that was either predictive of or independent of exposure to a member of the opposite sex. It was expected that the group trained with the predictive stimulus would show shorter latencies to a variety of breeding behaviors and produced more eggs than the control groups.

CHAPTER 2

METHODS

Subjects

Subjects were 38 dyeing dart frogs (*Dendrobates tinctorius*), 19 males and 19 females, housed at the Atlanta Botanical Garden in Atlanta, Georgia. Subjects were paired based on sex and population morph and then randomly assigned into three groups: a basic control group, and active control group and an experimental group. Both control groups contained six pairs of frogs and the experimental group contained seven pairs. Frogs were housed in conditions known to promote breeding behavior (personal communication, Hill). They were kept at a photoperiod of 12:12, fed fruit flies three times per week and misted twice daily. Frog pairs were housed, one pair per tank, in ten gallon aquariums with metal mesh lids. The tanks were lined with a charcoal mix layer topped with sphagnum moss and included live plants and coconut breeding huts. Each hut contained a glass Petri dish, which has proven to be a suitable site for egg laying in this species (Pfeiffer, 2003). All frogs were given at least five days to habituate to the new tanks before training started.

Materials

Each ten gallon tank contained a divider separating halves of the tank (Figure 1). The divider was opaque and could be raised through a slit in the lid. Green LED lights were installed on the exterior of the tank, one on each side of the divider.

Figure 1: Tank configuration and an example of conditional approach behavior.

Conditional Stimuli

Historically a wide range of stimuli have been used as CSs in sexual conditioning, from odors to a stuffed dog (Kippin, et al., 1998; Domjan, O'Vary and Greene, 1988). For this study a flashing green light was used. Lights have been shown to facilitate sexual behaviors in both fish and birds (Hollis, 1990; Domjan, 1994) and also have the advantage of being localized, which could prove a problem if auditory stimuli were employed. Visual access to the conspecific and species-specific calls were also considered as possible CSs but were rejected. Such stimuli would not be initially neutral to the target response. In an attempt to create a CS that this species would be sensitive to, the frequency of the flashing light was set to occur at a rate that approximated the speed of behaviors emitted by this species. The limb-shaking, which is often shown during male-female interactions, occurs at a maximum rate of approximately two shakes per second (personal observation). Toe-trembling, which is correlated with feeding as well as male-female interactions, occurs at a maximum rate of approximately five vibrations per second (personal observation). The light was set to be within this range, at a rate of three flashes per second.

Unconditional Stimuli

The UCS used in sexual conditioning has also varied in past research, although it tends to come in one of two forms: visual access to the member of the opposite sex or full access with opportunities for copulation (e.g. Holloway and Domjan, 1993a; Crawford, Holloway and Domjan, 1993). It does appear that visual access to a member of the opposite sex without copulation can serve effectively for conditioning (Zamble, et al., 1985), although copulatory access can increase effectiveness of the procedure (Crawford, Holloway and Domjan, 1993). However, initial pilot study data suggest that visual access alone may produce aggressive rather than breeding behavior (data not shown). It is possible that tactile or olfactory cues are required for identification of sex between individuals in this species. Olfactory cues have been shown to be important for breeding behavior in other frog species (Oldham, 1967). As the reproductive procedure in this species might be dependent on cues other than visual, full access to the conspecific during the UCS was employed.

Procedure

Methods for sexual conditioning have varied widely in the number of trials, the characteristics of the CS and UCS and the lengths of CS and UCS periods (Pfaus, Kippin, and Centeno, 2001). The current procedure falls within the bounds of prior successful experiments conducted with other species.

In this procedure, both sexes were exposed to the protocol. Past experiments have shown that both males and females of other species will respond to the sexual conditioning procedure (Adkins-Regan and MacKillop, 2003). In all three groups, pairs of animals were housed with one animal on each side of the tanks. In the experimental and active control groups, each pair was exposed to five training trials per day for five days. CS presentations were 90 seconds in length and UCS presentations were two minutes long. All trials were a minimum of two minutes apart. The times chosen were within ranges of past successful sexual conditioning procedures (Pfaus, Kippin, and Centeno, 2001; Hollis, 1990).

Trials were conducted in accordance with Rescorla's (1967) suggested experimental and control procedures for classical conditioning. For the experimental group, inter-trial intervals were randomly generated with a range of two to ten minutes. For the active control group, two random number generators were employed, using the same time ranges, one for presentations of the CS and the other for presentations of the UCS. This exposed both groups to both sets of stimuli for the same amount of time while separating the groups by the predictive value of the signal. The CS for the active control group must not be predictive of the UCS but it must also not be inhibitive, otherwise the differences obtained may be inflated by comparing a CS+ with a CS- (e.g. Crawford and Domjan, 1996; Cutmore and Zamble, 1988). In this procedure, in the active control group, the CS may precede, succeed, or overlap with the UCS. The relationship between the stimuli is completely independent. This procedure equates many of the factors between the two groups, preventing, among other things, misinterpretation of results due to pseudo-conditioning (May, 1949).

After the five sets of training sessions had elapsed, testing trials were conducted. During the testing trial the procedure for the active control and experimental groups were the same. The CS was presented for 90 seconds after which the barrier was raised allowing interaction between the pairs. The first hour of the testing trial was videotaped and the divider remained raised for five days. The tank was checked for eggs twice a day for five days. Testing for the basic control group pairs was the same as for the other groups except there was no presentation of the CS.

Scoring

Focal-group sampling observation methods were employed (Altmann, 1974). Due to the small number in the group (two), their proximity to each other, and general restriction of location, both animals could be observed simultaneously; continuous focal observations of both animals were conducted without issue. Trials were scored on the amount and type of behavior exhibited by both sexes, as well as the location of the subjects within the tank. For the testing trials, the latency to exhibition of various behaviors, the frequency of various behaviors, the latency to egg production, and the number of eggs produced were recorded for each pair.

The behaviors of interest were either identified during a pilot study or taken from descriptions of breeding behavior of related species. Behaviors were chosen based on the ability of the behaviors to be easily defined, differentiated, and potentially relevant to breeding behavior in this species. These behaviors are listed and described in Appendix A.

Location within the tank during training and testing trials was recorded, as location within the testing area is a common measure of sexual conditioning. For example, the amount of time spent in the part of the cage containing the CS is a common measure for inferring conditional approach behavior (Hilliard and Domjan, 1995) and across largely varying experimental conditions the most reliable indicator of sexual conditioning has been approach of the CS (Domjan, Lyons, North and Bruell, 1986). As such, which part of the tank is being occupied by the subjects during both the CS and the UCS are important variables of interest. For this study each half of the tank was divided into eight equal parts, the horizontal plane was divided into four sections and the vertical plane into two (as these animals do spend a significant amount of time in the top half of their enclosure). Time spent in the eighth of the tank containing the CS was recorded.

Breeding Behaviors

As the breeding behavior of this species is not well defined (Lötters, Jungfer, Henkel and Schmidt, 2007), behavior during breeding trials was carefully examined to determine whether behaviors shown by similar species were present in this species as well. Of particular interest were tactile interactions (suggested to be important in this species, Wells, 1978; 1977; Lötters, et al., 2007)) and visual signaling (suggested to be important in related species, Silverstone, 1973).

Another potential outcome of interest is the relationship between the behaviors being observed in this study and egg laying. By tracking which behaviors vary together and how they vary in relation to the different experimental conditions and reproductive outcomes, more could be learned about this species' breeding behavior. Behaviors occurring during the first 30 minutes of the testing trial were tracked to see if any of them correlated with eventual egg laying (predictive success did not improve by adding the additional 30 minutes, data not shown).

Call Recordings

Objective descriptions of the calls of this species do not currently exist in the literature (Lötters, Jungfer, Henkel and Schmidt, 2007). Calls were recorded using a FR-2LE recorder with a basic OADE mod using a CK32 microphone head and a HM1000 body (Figure 2). Calls were recorded at 48 kHz 24-bit and filtered and analyzed using RavenLite and Audacity software. Calls were recorded during USC periods during the training or testing trials at temperatures of approximately 24º C. Frogs for which calls had not been obtained during the experiment (generally animals in the control groups) were exposed to the experimental methodology after the study was completed in an attempt to elicit calls. For each call the individual and population morph were recorded.

Figure 2: Recording of frog calls.

Data Analysis

Data collected during this experiment largely did not conform to the assumptions necessary for parametric statistics, such as normally distributed data and equal variance and covariance between groups. As such, non-parametric statistics were used. For comparisons during training trials Mann-Whitney U, Wilcoxon signed ranks and trend tests were employed. Kruskal-Wallis tests were used to examine differences in testing trial data between the three groups. Pre-planned comparisons of testing data were also analyzed using Mann-Whitney U tests. Correlations between expression of behaviors and reproductive success and group membership were computed as point-biserial correlations. Behaviors were categorized as occurring either early in the testing session (within eight minutes), late in the session (within 30 minutes) or not at all, and then correlated with whether egg laying occurred in that pair. Statistics were calculated either by hand or with SPSS version 17.0.

Differences between locations within the tank, observed breeding behaviors and reproductive success during testing trials as well as the change in locations and behaviors within subjects were examined. It was expected that, as trials progressed, the subjects in the experimental group would spend more time near the CS and show more breeding related behaviors during CS presentation than the active control group. Additionally, it was expected that during the testing trials the experimental group would show lower latencies to breeding behavior, exhibit more appropriate breeding behaviors and lay more eggs, and lay them sooner, than either of the control groups.

CHAPTER 3

RESULTS

Training Sessions

Behavioral Differences between Groups

Of all the behaviors tracked during training, the biggest difference between the experimental and active control groups was in calling behavior. The number of calls produced during the CS differed between the two groups with the experimental group producing significantly more calls during the CS period than the active control group (Mann-Whitney U, $U = 6$, $N = 12$, $p = .03$, one-tailed) (Figure 3). Additionally, the number of calls produced during the UCS increased over time in the experimental group, but not in the active control group (trend test, $z = 2.33$, $p < .01$) (Figure 4).

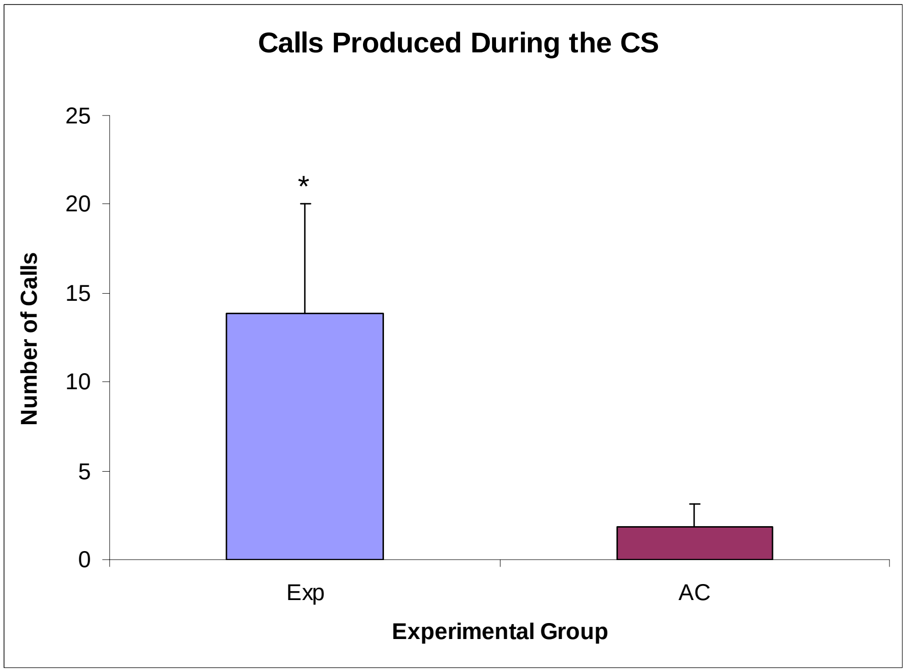

Figure 3: Calls produced during CS presentation in training trials in the active control and experimental groups. * denotes a difference significant at $p \leq .05$.

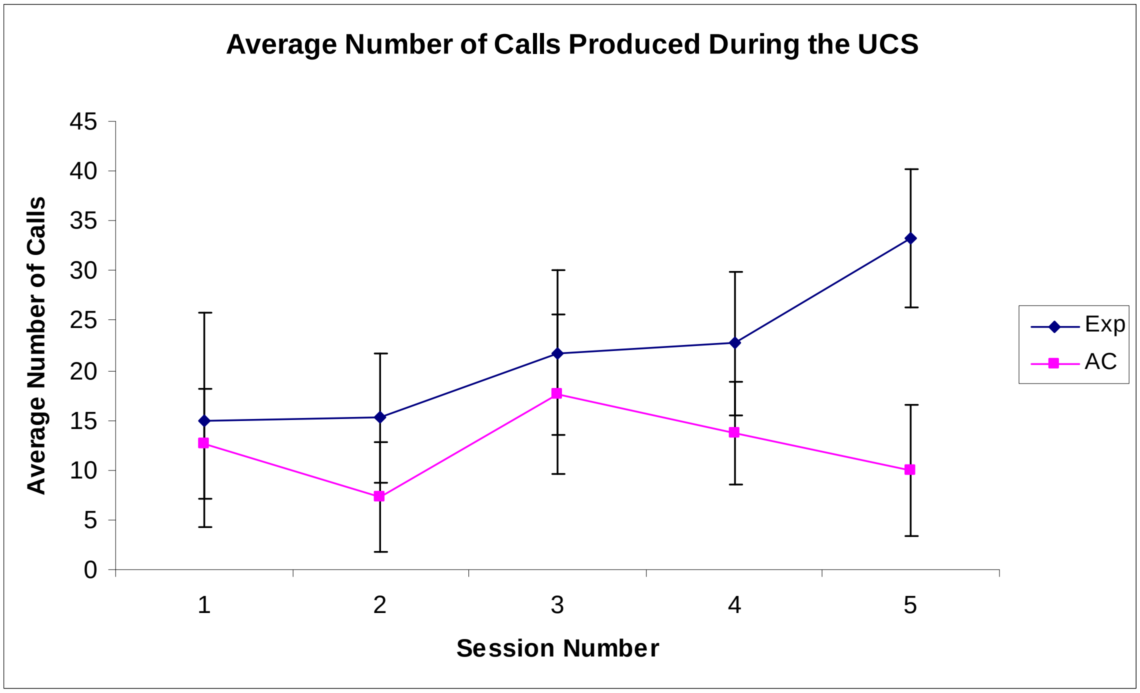

Figure 4: Average number of calls produced during the UCS period of the training trials in the active control and experimental groups. * denotes a difference significant at $p \leq .05$.

Location Differences between Groups

Conditional approach was observed in this experiment (Figure 1): the amount of time spent in the presence of the CS did differ between groups with the experimental group spending significantly more time near the CS during CS presentation (Mann-Whitney U, U = 9, N = 13, p=.05, one-tailed) (Figure 5). However, differences in the mean percentage of time spent near the CS were not nearly as impressive as in other studies (exp mean 60% vs. ac group 39%). Conditional approach becomes even less clear if all trials where a CS time of 1:30 were recorded are removed. A score of 1:30 implies that the animal was in that sector during the entire CS presentation time, negating the possibility of approach. If these times are removed, differences are no longer significant (Mann-Whitney U, U=12, N = 13, p=.115, one-tailed) and percent time spent in the CS area becomes even less impressive for the experimental group (exp group 40% vs. ac group 23%).

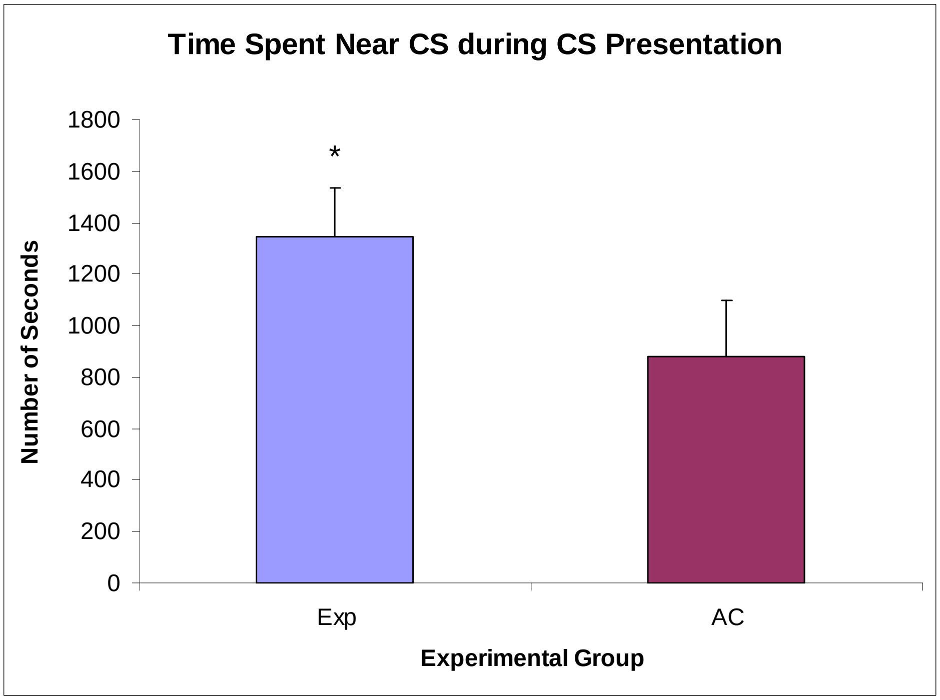

Figure 5: Time spent in the CS area by group. * denotes a difference significant at $p \leq .05$.

This measure may have been confounded by individuals starting in the CS area and not moving for the entire time period or individuals moving back and forth along the length of the barrier rather than remaining in the CS area. One alternative way to measure conditional approach (especially if confounded with goal-tracking) would be to compare the number of times the CS area was entered rather than the total amount of time spent in that area. However, this metric does not reveal a significant difference (Mann Whitney U, $U = 9$, $N = 11$ $p = .16$). Indeed, a difference can only be seen when comparing the number of trials where an individual entered the CS area at all during the CS period. This may start to differentiate between individuals moving into the CS area, and those that entered the area multiple times in the context of, perhaps, excitatory conditioning. Of the trials where subjects moved into the CS area, the experimental group entered the CS area significantly more times during the CS period than did the active control group (Mann Whitney U, U = 9.5 , N= 13, p=.05, one-tailed) (Figure 6). CS approach values may also be confounded with behavioral differences between the sexes. Independent of experimental group, females entered the CS area more often than males (Mann-Whitney U, U = 25, N = 22, p = .019, two-tailed) (Figure 7).

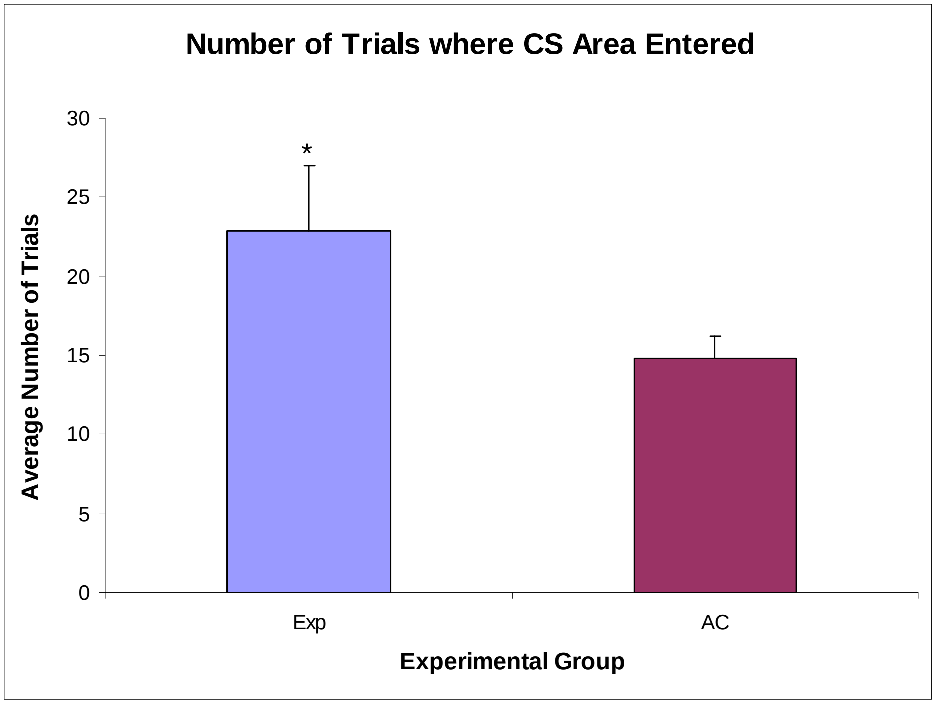

Figure 6: Average number of trials by pair where the CS area was entered during CS presentation for the active control and experimental groups. * denotes a difference significant at p ≤ .05.

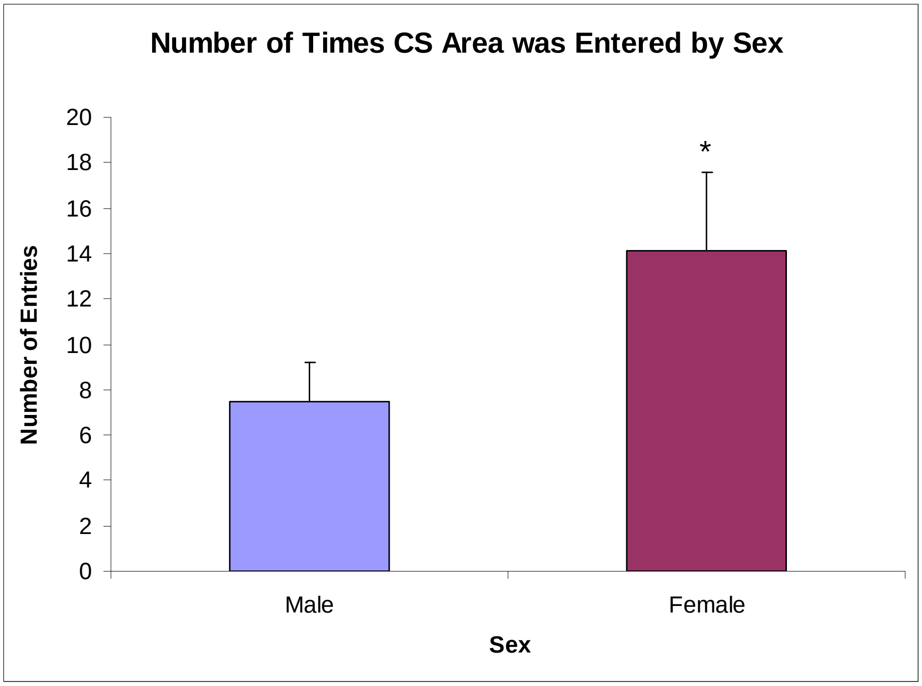

Figure 7: Average number of times the CS area was entered by males and females. * denotes a difference significant at $p \leq .05$.

Differences between Early and Late Trials in the Experimental Group

It is possible that having five trials within a day might be too many, that later trials would be less effective. To check for diminishing returns latency to contact between frogs and number of trials where contact occurred were compared between the 1st and 5th trials of the sessions for the experimental pairs. Neither latency to contact (Wilcoxon signed ranks test, $z = -1.183$, $df = 1$, $p = .237$) nor number of trials where contact occurred (Wilcoxon signed ranks test, $z = -1$, $df = 1$, $p = .317$) differed between the first and last trials for the experimental group.

Testing Sessions

Amount of Time Spent Engaged in Breeding Behaviors

There was no significant difference in number of calls produced during the testing period (Kruskal-Wallis, chi-square = .737, df = 2, p = .69). However, differences in the time pairs spent within one length of each other were marginally significant (Kruskal-Wallis, chi-square = 5.128, df = 2, p = .077) (Figure 8). Preplanned comparisons showed a significant difference between the experimental and active control group and no difference between the two control groups, with the experimental group producing the most calls. While the pattern of data suggests a difference between the experimental and control groups, the difference was not significant (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 3, N = 12, p = .009; exp vs. con (one-tailed), U = 12, N = 13, p = .11; ac vs. con (two-tailed), U = 12, N = 11, p = .662).

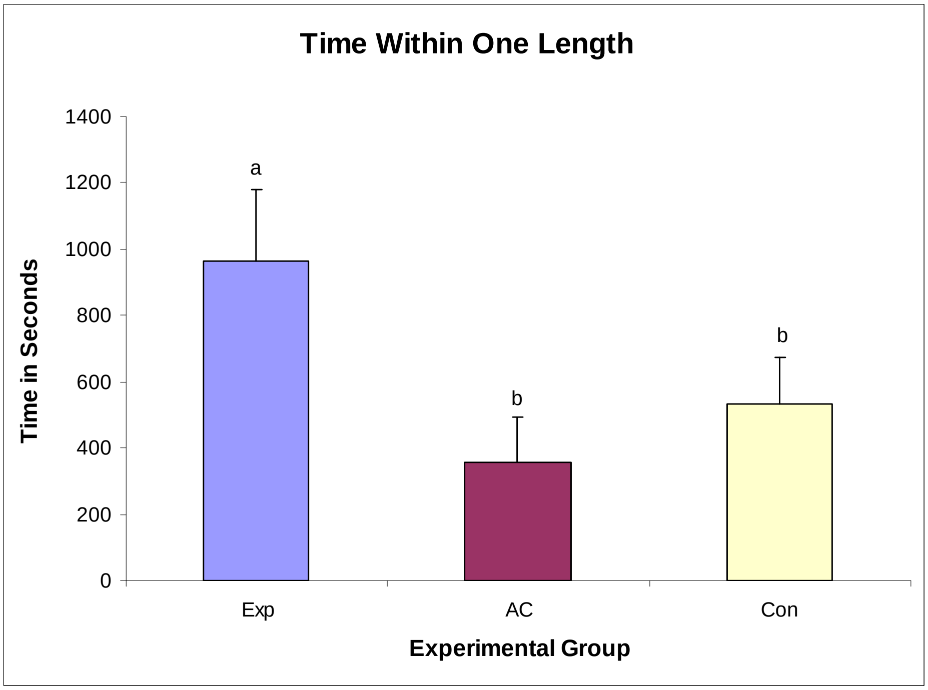

Figure 8: Time pairs spent within one frog length of each other during testing. Within each behavior, having different letters denotes a significant difference between the groups at $p \leq .05$, except for between the experimental and control group where $p \leq .10$.

Latencies to Breeding Behaviors

Differences in latencies to exhibit different breeding behaviors during testing were one of the most consistent differences between the groups (Figure 9). For example, latency to first contact was significantly different between the groups (Kruskal-Wallis, chi-square = 13.071, df = 2, p = .001) with preplanned comparisons demonstrating significant differences between all three groups with the latencies being the shortest for the experimental group and longest for the control group (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 0, N = 11, p = .002; exp vs. con (one-tailed), U = 0, N = 12, p = .001; ac vs. con (two-tailed), U = 3, N = 11, p = .030).

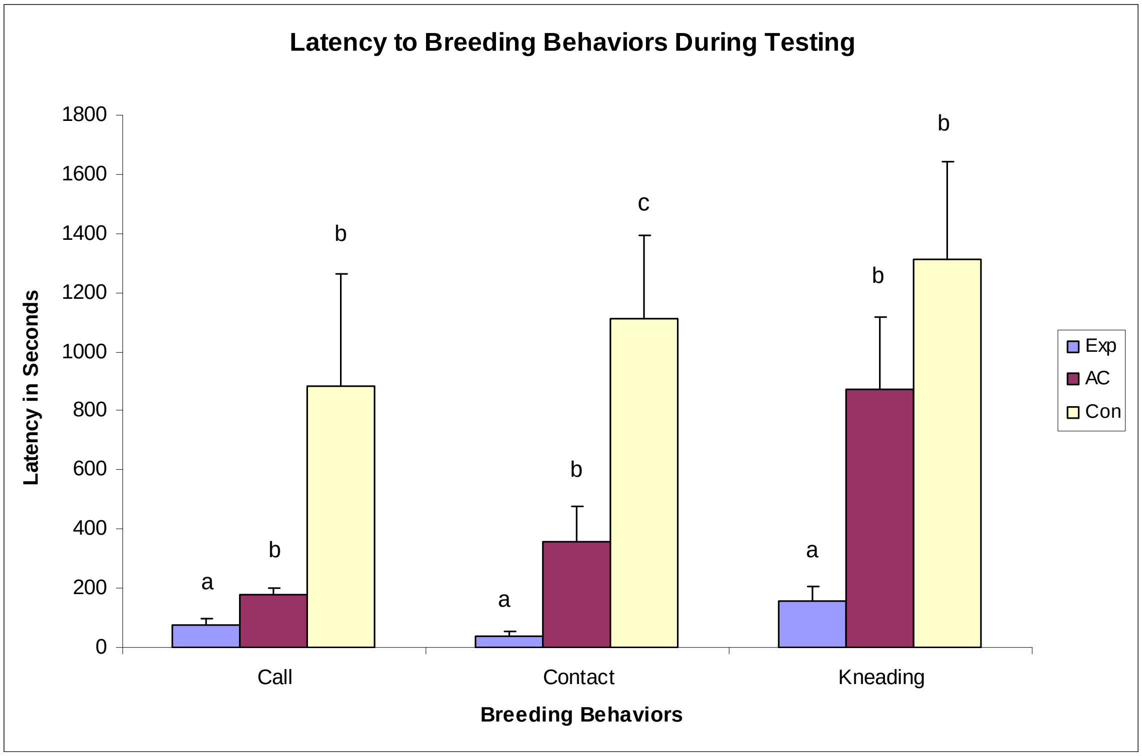

Figure 9: Differences in latencies in behavior over the three groups. Within each behavior, having the same letter denotes no significant difference between the groups. P = .05, except for call where p = .10.

Differences in sex-specific behaviors are also apparent. Differences in latency to first call were suggested by the data, although they were not significant (Kruskal-Wallis, chi-square = 5.357, df = 2, p = .069) with a marginally significant difference only between the experimental group and the two control groups with the experimental group having the shorter latencies (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 0, N = 5, p = .10; exp vs. con (one-tailed), U = 0, N = 5, p = .10; ac vs. con (two-tailed), U = 0, N = 4, p = .333) (Figure 9). The latency to kneading behavior was significantly different between groups (Kruskal-Wallis, chi-square = 9.36, df = 2, p= .009) with significant differences only between the experimental group and the two control groups with the experimental group having shorter latencies (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 1, N = 10, p = .080; exp vs. con (one-tailed), U = 0, N = 10, p = .004; ac vs. con (two-tailed), U = 7, N = 10, p = .310) (Figure 9). Latency to male mount showed a slightly different pattern (Kruskal-Wallis, chi-square = 7.017, df = 2, p = .030) with a significant difference only between the control group and the other two groups with the control group having the longest latencies (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 5, N = 9, p = .381; exp vs. con (one-tailed), U = 3, N = 11, p = .015; ac vs. con (two-tailed), U = 0, N = 8, p = .036).

Differences were also apparent in the latency for the two sexes to approach each other (Figure 10). The latency to male pursuit of the female differs (Kruskal-Wallis, chi-square = 14.235, df = 2, p = .001) with significant differences between all three groups where the experimental group having the lowest latencies and the control group the highest (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 0, N = 11, p = .002; exp vs. con (one-tailed), U = 0, N = 12, p = .001; ac vs. con (two-tailed), U = 0, N = 11, p =

.004). Latency to pursuit by female also differs (Kruskal-Wallis, chi-square = 12.74, df = 2, p = .002) with significant differences only between the experimental group and the two control groups with lower latencies in the experimental group (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 0, N = 11, p = .002; exp vs. con (one-tailed), U = 0, N = 12, p = .001; ac vs. con (two-tailed), U = 4, N = 11, p = .052).

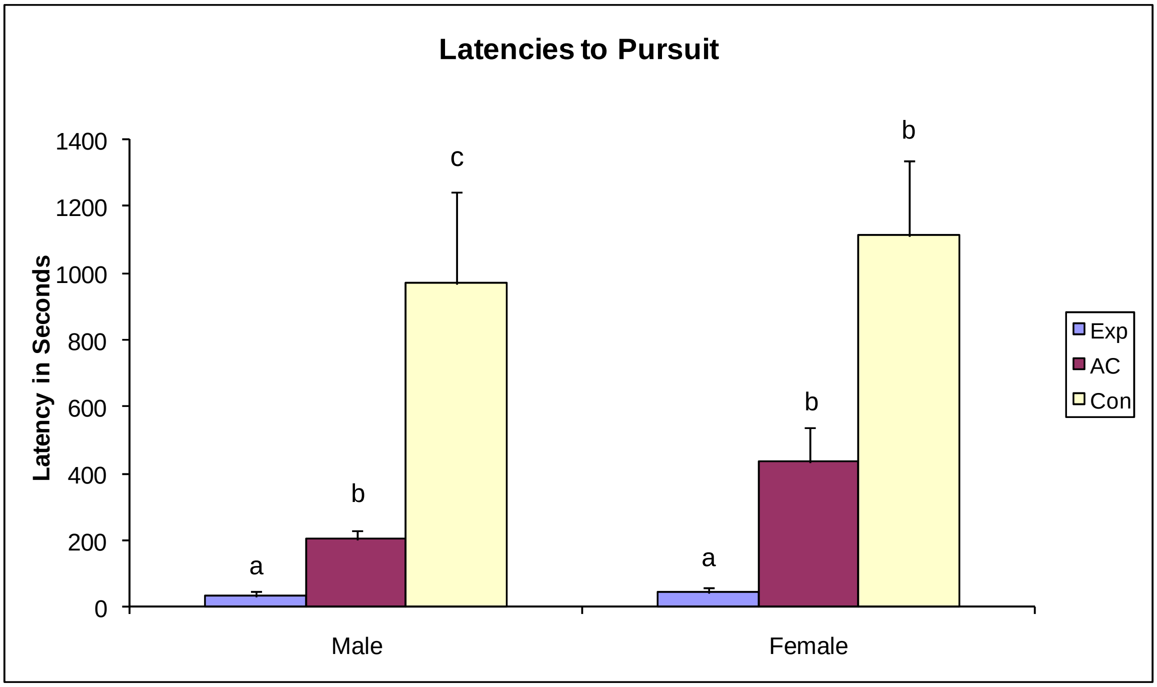

Figure 10: Male and female latencies to pursuit. Having different letters denotes a significant difference between the groups at $p \leq .05$.

Only the experimental group had differences in pursuit that were grouped significantly in a non-random manner (Figure 11) (Chi-square: exp: chi-square value: 5.444, p = .020, ac: chi-square value: 2.667, p = .264, con: chi-square value: 4.667, p = .097). All experimental groups had latency to male and female pursuits that were within one minute of each other. Of additional interest, where male and female latencies to pursuit differ, females were more likely to take longer to pursue than the males (chi-square test, chi-square = 4.455, p = .035).

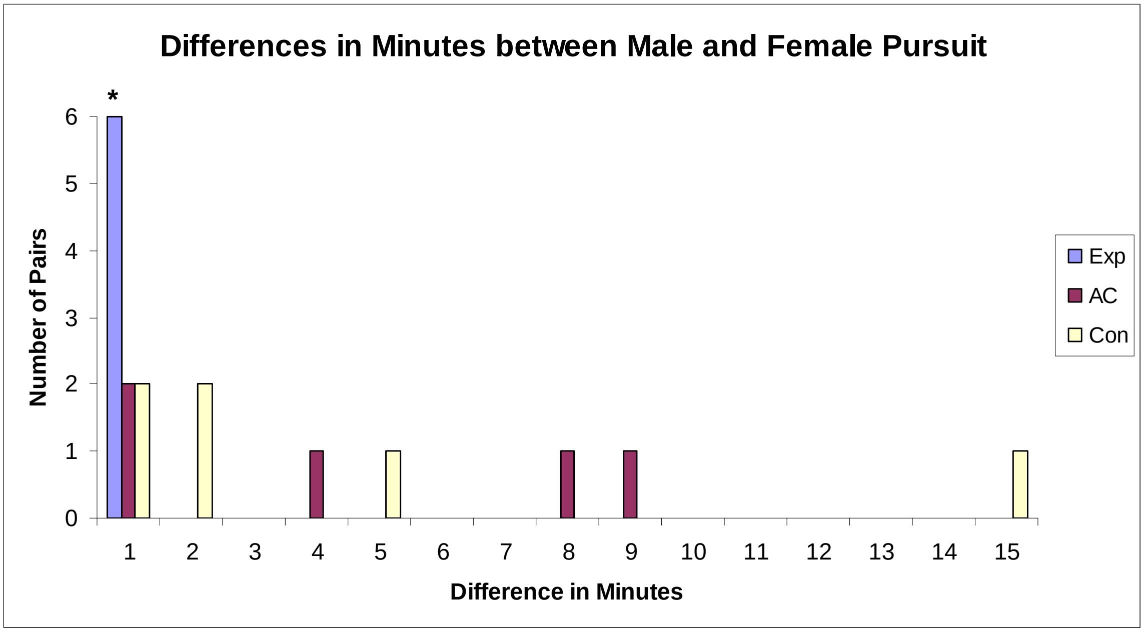

Figure 11: Symmetry of pursuit behavior. * denotes distribution is not uniform p < .05.

Egg Production

In the experimental group, four pairs produced eggs (average: 4 eggs). In the control group only one pair produced eggs (2 eggs) and in the active control group only one pair produced eggs (2 eggs). The number of eggs produced in the three groups were significantly different (Kruskal-Wallis, chi-square = 5.987, df = 2, p = .05) with the experimental group producing significantly more eggs than the two control groups (Mann-Whitney U tests: exp vs. ac (one-tailed), U = 8.5, N = 13, p = .036; exp vs. con (one-tailed), U = 8.5, N = 13, p = .036; ac vs. con (two-tailed), U = 18, N = 12, p = 1.0) (Figure 12). While most eggs produced during the experiment were infertile, the only fertile eggs produced were from experimental pairs. There was little variability in latency to egg production, 67% were found on the morning of the third day. The other two sets were found on the evening of the second day and the evening of the fourth day and were both laid by experimental pairs.

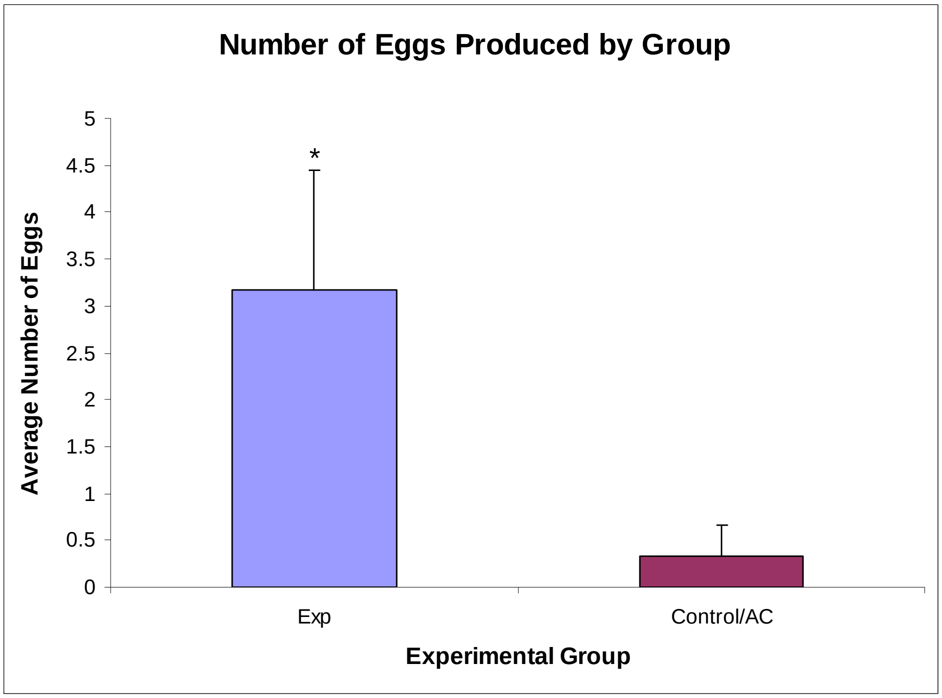

Figure 12: Number of eggs produced by each group. * denotes a difference significant at $p \leq .05$.

Behaviors Predictive of Egg-Laying

Several behaviors were uncorrelated with egg-laying, these included: latency to first call, number of calls, latency to male mount, symmetry (male and female latencies to pursuit differing by less than one minute), and time spent by male in breeding hut. One behavior, female in breeding hut, was negatively correlated with egg-laying. Several other behaviors were significantly correlated with egg laying, including: latency to first contact (Pearson correlation, .525, N = 18, p = .025), latency to pursuit by male (Pearson correlation, .461, N = 18, p = .054), latency to pursuit by female (Pearson correlation,

.474, N = 18, p = .047), latency to kneading (Pearson correlation, .572, N = 18, p = .013), and time within one length (Pearson correlation, .664, N = 18, p = .003).

Correlations of Behaviors with Group Membership

Some behaviors were correlated significantly with group membership. These included: latency to first contact (Pearson correlation, .709, N = 18, p = .001), latency to pursuit by male (Pearson correlation, .719, N = 18, p = .001), latency to pursuit by female (Pearson correlation, .818, N = 18, p = .000), latency to kneading (Pearson correlation, .672, N = 18, p = .002), and symmetry in pursuit (Pearson correlation, .485, N = 18, p = .041). The correlation for time within one length was only marginally significant (Pearson correlation, .454, N = 18, p = .058).

Behaviors Elicited by the Procedure

Breeding Behaviors

The following behaviors that have been reported in other related species (Wells, 1978; 1977; Dunn, 1941) were observed in this species during the sexual conditioning procedures: strokes, toe-trembling, limb-shaking, upright posture (Figure 13), and body-lowering (seen in females only). Like in *D. auratus* the behavior of moving both front feet up and down was seen, but only in the females. This behavior may have particular relevance to breeding in this species and has been labeled as “kneading” (see Figure 14).

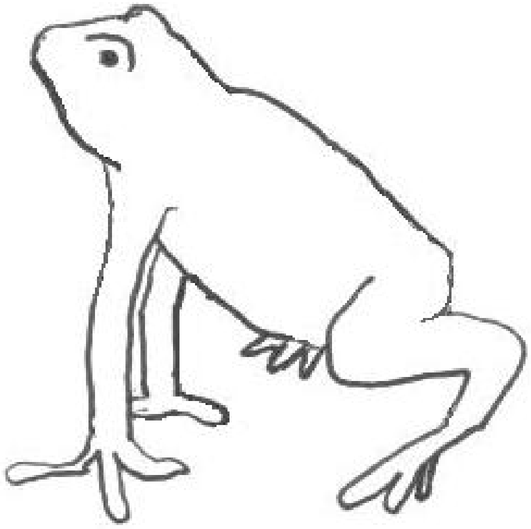

Figure 13: Example of upright posture in a male. Note the over-extended arms.

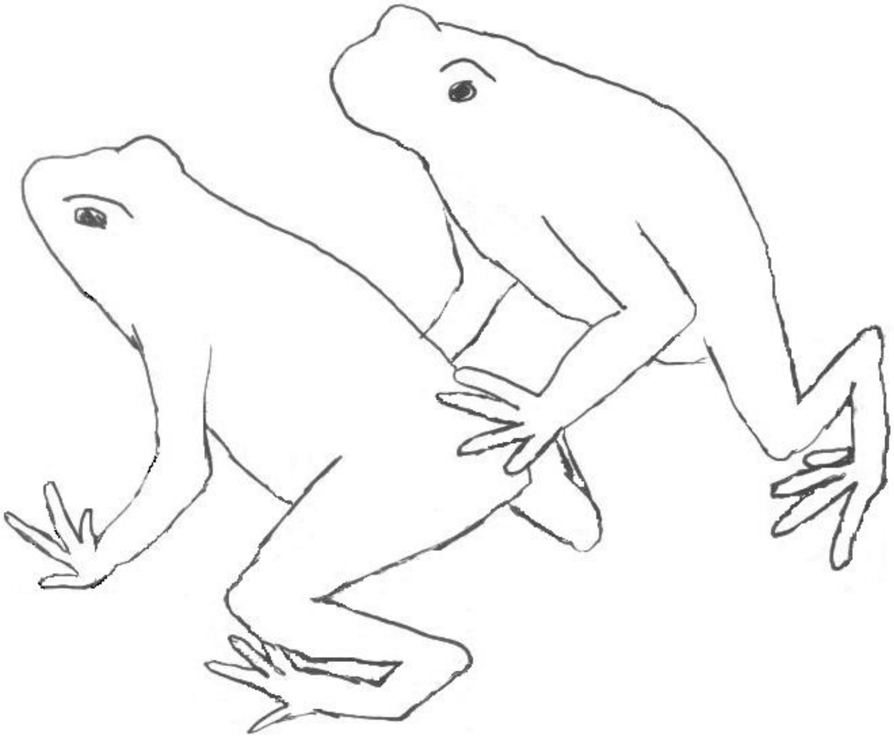

Figure 14: Example of kneading behavior. The female raises and lowers her front feet in a rhythmic manner, often on the back of the male.

Sample 60 second courtship interaction:

04 – Female, with upright posture, approaches male.
10 – Male orients toward female, assumes upright posture, performs limb-shake with front foot.
19 – Female strokes male's front leg, male calls.
20 – Female limb-shakes front foot and kneads substrate next to male.
21 – Male limb-shakes front foot and moves, with upright posture, away from female.
27 – Male limb-shakes front foot.
33 – Male moves towards female, strokes her back leg, and calls.
37 – Female orients towards male and limb-shakes with front foot.
40 – Female limb-shakes front foot.
43 – Male moves away from female.
48 – Male limb-shakes front foot and moves towards female.
52 – Females body-lowers.
53 – Male strokes female's front foot and calls.
58 – Female kneads on ground and male's back leg.
60 – Female kneads male's back.

Expressions of these behaviors showed some regularity. Both sexes pursued each other and both sexes initiated tactile contact, although most appear to be initiated by the female. Both sexes exhibited upright posture, but only females were observed to body-lower. Males were observed to jump on the female's back, but this did not seem to be a breeding related behavior. The kneading behavior (only performed by females), while performed both near and on the male frog, usually progressed to being performed on the male's back. Limb-shakes were performed more frequently with the front feet, but were observed with the back feet as well.

Calling Behavior

Calls were successfully recorded from six males. Four males that had not called during the experiment were exposed to the experimental procedure in an attempt to elicit calling. Calls were obtained from two of those four males. Overall, calls were obtained from six individuals, two from each of three different populations (Inferalanis, Azureus, and Cobalt). Basic quantitative characteristics of the calls were obtained for all six males (Table 1). Objective representations (oscillograms and audiospectrograms) of the calls were obtained using RavenLite and Audacity software.

Table 1: Characteristics of individual *Dendrobates tinctorius* calls.

Population	Specimen	Average Refresh Rate (sec)	Minimum Refresh Rate (sec)	Call Duration (ms)	Peak Frequency (Hz)	Secondary Frequency (Hz)
Inferalanis	Brad	8.92	7.09	1168	2204	2707
	Charles	16.85	9.86	1057	2861	2296
Cobalt	Woad	5.06	2.56	815	2530	2492
	Mystery	11.81	6.02	765	2452	2223
Azureus	Chachi	12.15	7.06	1096	2435	3175
	Cerulean	14.94	12.76	720	2156	2726
	Average	11.62	7.56	937	2440	2603

Qualitatively, the call of this species sounds like a faint “burr” noise. Objectively, the calls are very broad in frequency range (approximately 2000-3000hz), and generally bimodal, with little or no frequency modulation within clicks or calls (Figures 15 and 16). Each call is approximately one second in length and consists of a series of individual clicks, each click being approximately 8-10 ms long (Figure 17). Time between calls and peak frequencies differ by individual, but average about 12 seconds and 2400hz respectively (See Table 1). Another interesting individual difference is in the distribution of clicks within calls. While some individuals have an uninterrupted pattern of clicks within a call (Figure 18) other individuals often have “choppy” calls which have gaps in the click sequence (Figure 19). Additionally, individuals may emit clicks preceding or following the main call (Figure 20).

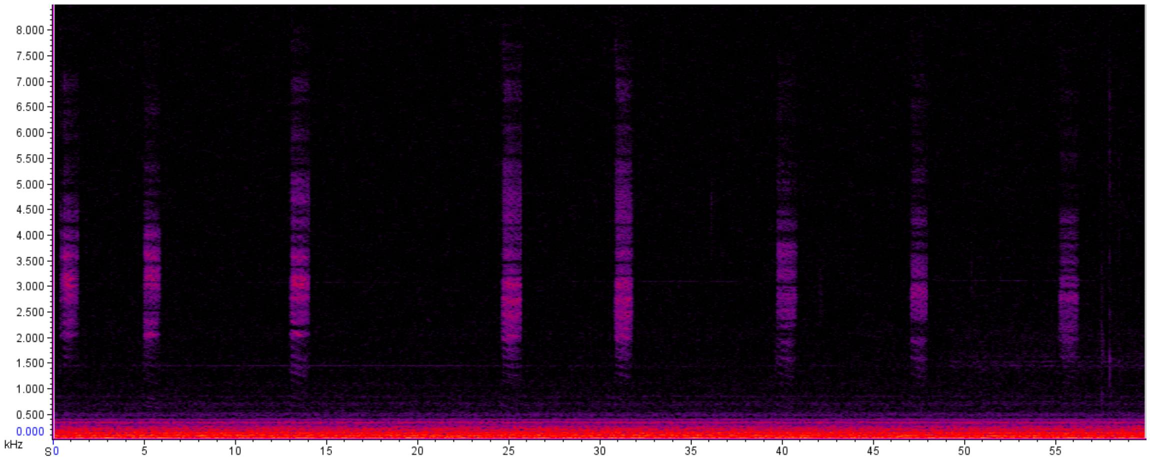

Figure 15: Audiospectrogram of a series of *Dendrobates tinctorius* calls.

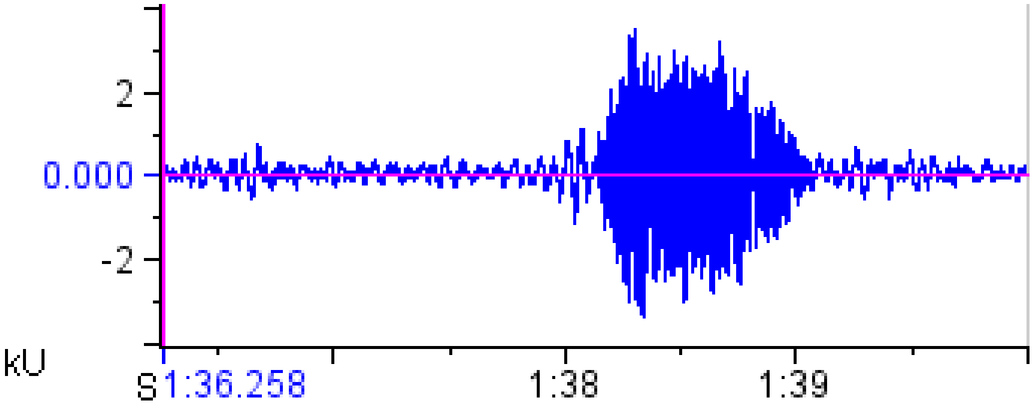

Figure 16: Oscillogram of a *Dendrobates tinctorius* call.

Figure 17: Oscillogram of individual clicks within a call

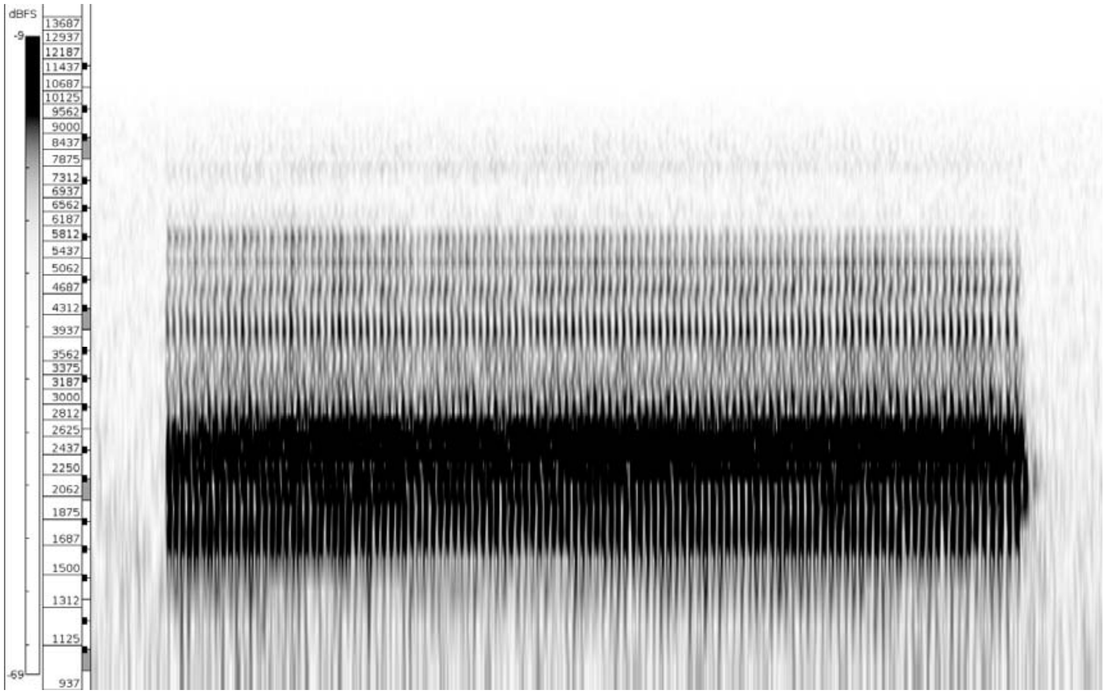

Figure 18: Audiospectrogram of an uninterrupted click series.

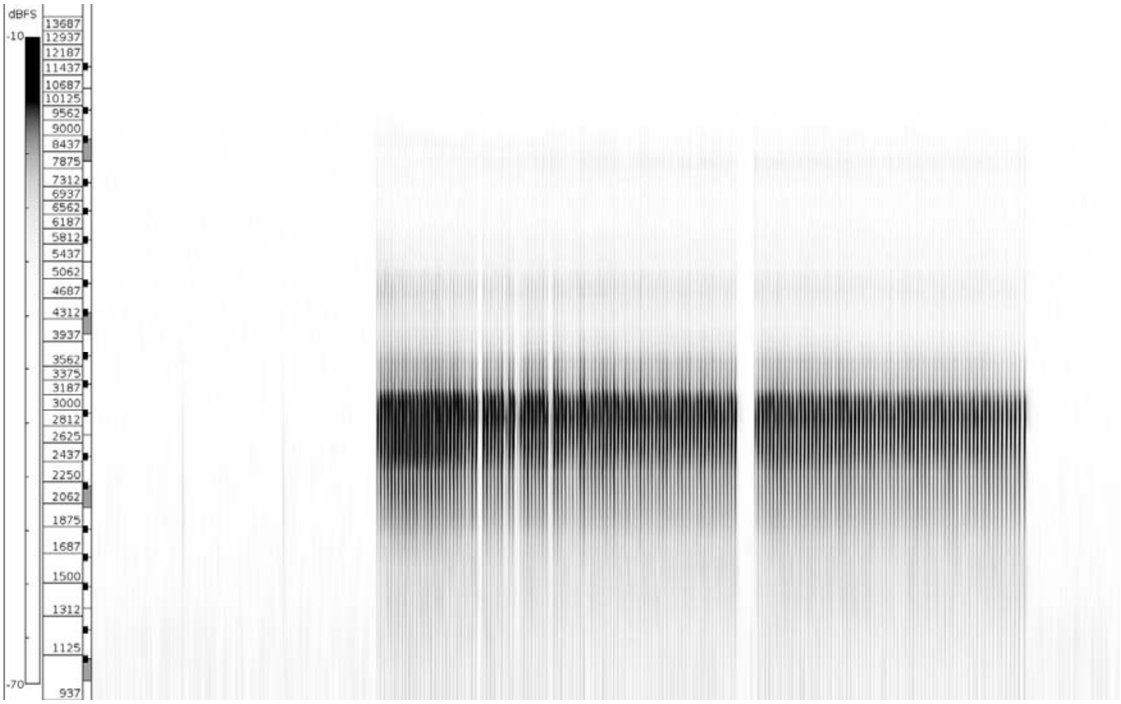

Figure 19: Audiospectrogram of a “choppy” click series.

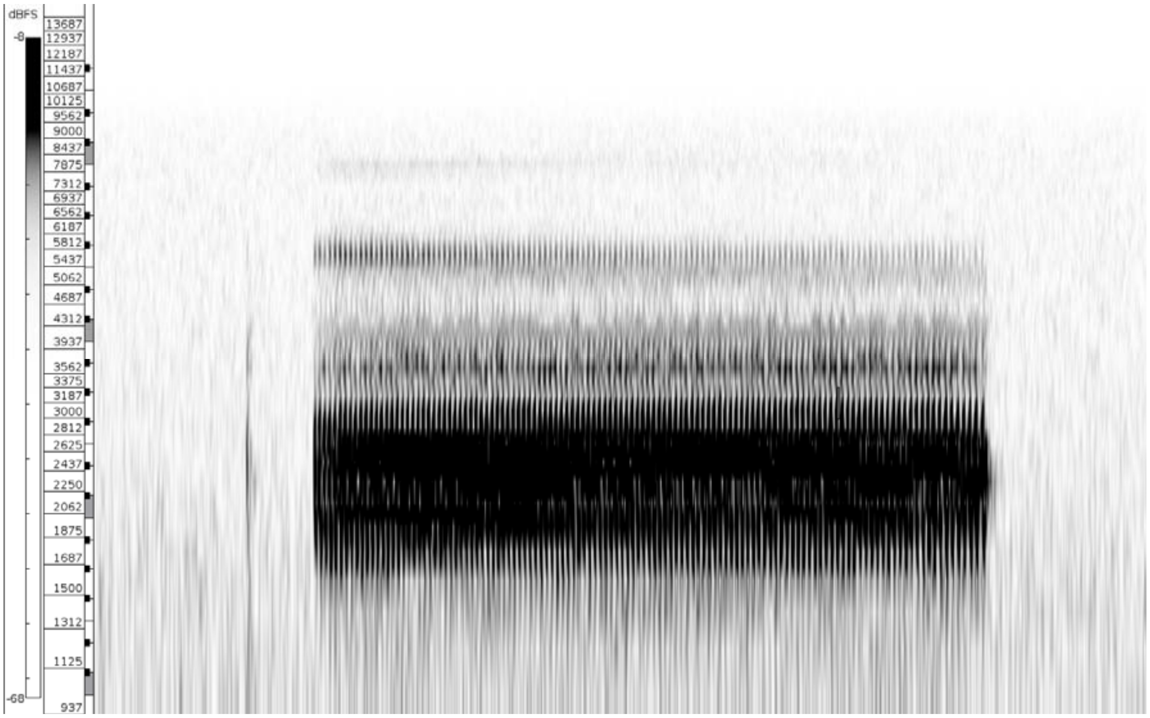

Figure 20: Audiospectrogram demonstrating a “pre-click”.

CHAPTER 4

DISCUSSION

In this experiment the sexual conditioning procedure was shown to be successful at improving breeding behavior in an amphibian species. The pairs in the experimental group produced more eggs and showed shorter latencies to positive breeding behaviors such as calling and contact than the control groups. Other breeding behaviors increased in frequency, allowing for close examination of call characteristics and possible instances of visual signaling.

Breeding Related Behaviors and Reproductive Success

The number of eggs produced in the three groups was significantly different with the experimental group producing more eggs than the two control groups. Although most eggs produced during the experiment were infertile, the only fertile eggs produced were from experimental pairs. However, sexual conditioning in this species did not result in decreased latency to reproduction, as has been demonstrated in some other species (Hollis, Pharr, Dumas, Britton, and Field, 1997).

On several latency measures the active control group showed significantly reduced time to assumed breeding behaviors than the control group. However, both groups produced similar numbers of eggs. The important behaviors of interest in this procedure should then be the measures that do not differ significantly between the control groups but do differ in the experimental group. Two measures most clearly fit the criteria. Latency to kneading behavior and latency to pursuit by the female were significantly different only between the experimental group and the two control groups. Other measures had data suggestive of this pattern, although the relationships were not always significant. For example, the time spent within one frog length was significantly different between the experimental and active control group and with only a marginal difference between the experimental group and the control group, but there was no difference between the two control groups. Latency to call showed a similar pattern, with a marginal difference only between the experimental group and the two control groups.

Another measure that may be of interest is symmetry of pursuit latencies. Only the experimental group had differences in pursuit that were grouped significantly in a non-random manner. All differences were 60 seconds or less whereas in the other two groups differences in pursuit were as large as ten minutes. Overall, female latencies to pursuit are longer than males; this procedure may be bringing the female breeding behaviors closer in time to male breeding behaviors. It is possible that synchrony of onset of breeding behavior in the two sexes increases reproductive success.

However, when looking at correlations in the reproductive success data, symmetry in pursuit values was not significantly correlated with laying eggs. It is possible, that while synchrony is a result of this procedure, it is not the behavior responsible for the increased reproductive success seen in the experimental group. Instead the procedure may be effective through, for example, reducing female breeding behavior latencies, measures that were significantly correlated with egg-laying. More research will need to be done to tease out the details of this procedure.

Behaviors Predictive of Egg-Laying

Behaviors during testing were examined to see if any behaviors were predictive of egg-laying. Several behaviors were uncorrelated with egg-laying including: latency to first call, number of calls, latency to male mount, symmetry (male and female latencies to pursuit differing by less than one minute), and time spent by male in breeding hut. One behavior, female in breeding hut, was actually negatively correlated with egg-laying, perhaps being in the hut during the first 30 minutes is more indicative of avoiding the other frog than interest in breeding. Several other behaviors were significantly correlated with egg-laying including latency to first contact, latency to male pursuit, latency to female pursuit, latency to kneading, and time within one length.

One interesting way of comparing these outcomes with experimental outcomes is to correlate the behaviors with group membership and see where the differences lie. For example, while the relationship between latency to male pursuit and egg-laying is not strong, male pursuit is highly correlated with group membership, with the shortest latencies occurring in the experimental group. Time spent within one length shows an opposite pattern, highly correlated with egg-laying, but not with group membership.

Conditional Approach

Proximity to the CS is considered one of the best indicators of successful sexual conditioning in other species (Pfaus, Kippin, and Centeno, 2001). However, in this experiment, amount of time spent proximate to the CS did differ between groups, but not as much as would be expected given previous experiments (e.g. quail, Hilliard and Domjan, 1995).

One possible reason for the unimpressive conditional approach figures may be the nature of the UCS in this experiment. Conditional approach behavior is more exaggerated when copulation rather than visual exposure is used as the UCS (Holloway and Domjan, 1993a). In fact, only one trial of conditioning is necessary, when using copulation as the

UCS, to create a large difference in conditional approach behavior (e.g. 0% vs. 60% after one trial in quail, Hilliard, Nguyen, and Domjan, 1997).

It is also possible that in this species other behavioral measures would prove a better predictor of sexual conditioning. One possible alternate measure to conditional approach is the number of calls produced during CS presentation. Prior to conditioning calling occurred only in the presence of other frogs. After conditioning, males were observed to orient towards the light and begin calling during the CS period. These calls were observed during the CS presentation much more often in the experimental group than in the active control group. Although this behavior is obviously under stimulus control, it is not being emitted in form that would be captured by measuring conditional approach. In the future alternative measures of conditional approach (including alternate measures for other forms of the conditional response) should be employed.

Sex Differences

Unlike in most prior experiments using sexual conditioning, both sexes were explicitly included in the conditioning procedure (i.e. not just used as a UCS for the opposite sex) (but see, Hollis, Cadieux, and Colbert, 1989). This allows for some interesting observations to be made regarding sex differences in sexual conditioning. Given the different roles sexes in most species have in the breeding process it is possible that the sexes would respond differently to the conditioning procedure. For example, one experiment that used female quail found that females responded differently to sexual conditioning, showing increased receptive behavior rather than approach behavior (Gutiérrez and Domjan, 1997). Evidence of sex differences was also found in this experiment. For example, independent of experimental group, females entered the CS area more often than males.

Conditioning in the Active Control Group

One unexpected result of this experiment was that some latencies to breeding behavior were shorter in the active control group than in the other control group. This is likely a result of increased familiarity with the procedure; the active control group frogs had experience with a frog being on the other side of the barrier. It is also possible that the raising of the barrier served as a CS for the active control group. The noise and visual stimuli of the raising of the barrier were predictive of access to another frog, as predictive as the flashing light in the experimental group. The only difference is the length of the CS, 90 seconds in the case of the experimental group, and less than a second for the active control group. The interesting question is how the extra predictive time translates into reproductive success. Perhaps, for example, the extra time allows for the development of sign- or goal-tracking behavior, which in turn decreases latency to contacting the other frog, which allows for behavior to be more effectively brought under discriminative control by the light.

The variables that differ between the active control group and the straight control group may clarify the issue. Two variables, latency to male pursuit and latency to contact, were significantly different between all three groups and one measure, latency to male mount, had the unique distinction of showing a significant difference only between the control group and other two groups. These variables (especially the male mount behavior) could represent aggressive behaviors. It might be possible that the raising of the barrier is predictive of the presence of a frog, but the shorter CS time prevents the manifestation of sex-specific interactions.

Conditioning in the active control group could also be interfering with the conditional approach measures. Some sexual conditioning experiments did not use true randomization because it appeared to cause excitatory conditioning (general increase in activity levels) in the control group (e.g. Hollis, Cadieux, and Colbert, 1989). In this experiment the number of times the CS area was entered did not differ between the females of the experimental and control groups. This might be expected if excitatory conditioning was occurring with the active control group.

Breeding Harnesses

Sexual conditioning is used outside of academia as well, even if it is not labeled as such. One interesting example of this is the phenomenon of using breeding harnesses with stud horses (Sue McDonnell, personal communication). While this technique has not been reported in the peer-reviewed literature it is widely used in horse breeding.

With horses, different types of halters are used for various activities, for shows, for just around the barn and pasture, and, of interest here, sturdier harnesses for breeding. Over time the horse's behavior comes under stimulus control of the different types of harnesses.

To accelerate conditioning to the breeding harness, some people attach a "breeding bell" to the halter. When it is time to breed, the bell is attached to the halter, increasing the salience of the harness as a discriminative stimulus. Stallions are difficult to handle when sexually aroused and so it is advantageous to bring sexual behavior under stimulus control. Additionally, it is practical to train stallion to remain unresponsive sexually to females at show or just when being led around the farm. Then the breeding bell can be used as a salient signal for the stallion for when breeding behavior is and is not imminent.

Of course, special cues for breeding are often not required. Stallions have been reported to become aroused in advance of the actual breeding situation based on a variety of environmental cues. The direction the horse is led from their stall, other equipment used for breeding (e. g. type of lead rope, leg wraps), or specific people (the stud groom vs. the show groom) can all come to elicit sexual behavior. People who trailer their stallions to breeding facilities report that the stallion starts to show arousal even when the trailer makes a certain turn off the highway.

Using Sexual Conditioning to Study Sexual Behavior

Another benefit of the sexual conditioning procedure is that behaviors related to breeding may be more easily studied. This procedure condenses the period of time during which breeding behavior can be expressed, and possibly exaggerates the behaviors as well, allowing for their more efficient study. This study allows for the opportunity to describe breeding behaviors in this species as well as to compare them to behaviors exhibited in closely related species. Also, as breeding behavior in captivity has been shown to be similar to breeding behavior in the field for several species of dendrobatids (Summers, 1992) these results can give insight into wild populations as well.

One example of this experimentally increased behavior is calling. The experimental group produced significantly more calls during the CS period than the active control group and the number of calls produced during the UCS increased over time in the experimental group, but not in the active control group. However, there is also evidence that breeding behavior is simply being condensed in the experimental group. For example, there was no significant difference in number of calls produced overall during the testing period. Instead the procedure may just be shifting the behavior closer to the start of the UCS period, condensing it. However, the small number of males that called during testing in each of these groups must be taken into consideration (3, 2, 2) when making such speculations.

Reproductive Behavior in the Dyeing Dart Frog

The behaviors exhibited during this procedure were similar to those reported for related species. Examples of strokes, toe-trembling, limb-shaking, upright posture and body-lowering were observed (Wells, 1978; 1977; Dunn, 1941). Additionally, like in *D. auratus* the behavior of moving both front feet up and down was seen, but unlike in *D. auratus* was emitted only by the females. This kneading behavior may be particularly important for breeding in this species; a female that did not exhibit this behavior never went on to lay eggs. However, some of the more elaborate tactile interactions, such as the female climbing on top of the male and touching him with her front feet or drumming on his back with her hind feet, were not observed in this species.

During this experiment, in *D. tinctorius*, both sexes pursued each other and both sexes initiated tactile contact, although most touches were initiated by the female. This is very similar to courtship in *D. auratus* and *D. leucomelas* which can include elaborate tactile interactions between partners, with the female taking the more active role (Wells, 1978; 1977; Dunn, 1941; Summers, 1992). Also, as in related species, the males called intermittently during courtship. Of interest, males were observed to jump on the female's back, but this did not seem to be a breeding related behavior. Evidence from the reproductive success results as well as the pattern of behaviors conditioned in the active control group suggests this behavior may have a different function.

Visual Signaling

A variety of types of visual signaling during courtship has been reported in some frog species (Hödl and Amézquita, 2001). These can include elaborate visual displays or simple interactions, such as changes in posture. While visual signaling has been reported in *D. tinctorius* and in other related species, tactile interactions have historically been emphasized (Hödl and Amézquita, 2001; Zimmerman and Zimmerman, 1988). Additionally, which sex performs these behaviors as well as under which contexts has not been entirely clear.

Visual signaling appears to be much more prevalent in this species than has previously been reported. Males and females were observed to exhibit a variety of potentially visual interactions from limb-shaking to posture changes. Several behaviors exhibited during this experiment merit mention. First of all, one visual signaling behavior observed in this experiment that was not previously reported was body-lowering. The kneading behavior, due to its rhythmic performance, also appears to be distinct from other previously described visual signaling behaviors, and may in fact be a combination of visual and tactile interactions. Sex differences in performance of these behaviors are of interest as well. While many behaviors are exhibited by both sexes, there was often one sex which performed them more often. For example, while limb-shaking was seen equally between sexes, upright posture was exhibited mostly by males and the kneading behavior and body-lowering were seen only in females.

Dyeing Dart Frog Call

Given the call characteristics obtained in this study, the *D. tinctorius* call should be most likely classified as a "buzz" call along with the calls given by *D. truncatus* and *D. auratus*. (Lötters, Reichle, and Jungfer, 2003). While differences were seen between individuals within this species, there does not appear to be any consistent differences between the populations, as would be expected. While both the Cobalts had the shortest call refresh time they were also the two smallest males in the study, which may have influenced the rate. In support of this, they also had two of the shortest average call lengths.

The variation in click distribution within the call lends itself to future study. It is possible that a "choppy" call might be less preferred than a more regular one. Additionally, the function of the "pre-clicks" is unknown. One way to test these possibilities is through manufacturing different variations on a call and testing female response to them. With the audio software currently available even the single 10ms clicks could be removed individually from a call and the results examined. The opportunity for future research in this area is practically limitless.

During this study, calling behavior in males was enhanced and sustained in the experimental group. Calls could be predictably elicited from this group which made recording the calls relatively easy. Often recordings of frog calls are obtained serendipitously, long hours may go into trying to obtain even one call (Elliott, personal communication). Having a technique by which calls are produced in a manner of minutes greatly simplifies the process.

Sexual conditioning could be used in other species to elicit other sexual behaviors that may not occur frequently enough for easy study. Additionally, sexual conditioning can be used to elicit sexual behavior that has never been seen in an individual before. For example, in Cutmore and Zamble (1988) rats that had never successfully copulated before showed improved copulatory performance following sexual conditioning. Additionally, in this study, several males who were not observed to call during the control procedures were later induced to call through exposure to the experimental procedure.

Male-Male Interactions

It could also be interesting to study conditioning of male-male interactions, in addition to male-female, as certain types of calls may only be present during same-sex interactions (Tobias, Barnard, O'Hagan, Horng, Rand, and Kelley, 2004). In fact, physical contact between males could produce even more interesting call variations. Context of environment (physical, social and physiological) can have dramatic effects not only on the expression of different types of calls but also the characteristics of those calls.

Possible Modifications to the Methodology

During this procedure pairs of frogs were exposed to five training sessions a day for five days. Five sessions within a day may have been too many; it is possible that later trials were providing diminishing returns. However, there was no evidence of diminishing returns, neither latency to contact, nor number of trials where contact occurred, differed between the first and last trials for the experimental group.

While the number of trials per session does not appear to be problematic, it is recommended that more sessions overall be conducted. Examination of Figure 4 shows an increase of calls during the UCS that does not appear to have reached an asymptote. Additional sessions may have yielded further increases in breeding related behaviors.

Requiring additional sessions in this procedure seems reasonable. Conditioning under this procedure may take longer to occur than in other procedures due to differences in the UCS. In other studies where conditioning proceeded rapidly (including examples one-trial conditioning) the UCS was defined as successful copulation (e.g. Hilliard, Nguyen, and Domjan, 1997). In fact direct comparisons between using copulation and unconsummated exposure as a UCS show that copulation produces more rapid conditioning, although both procedures are effective (Crawford, Holloway, and Domjan, 1993; Holloway and Domjan, 1993a).

If future experiments in this same vein were conducted, a pre-test for response to sexual stimuli would be recommended. Equating groups on variables such as latency to contact (or some other variable reflective of sexual motivation) could help clarify results by reducing variability. Some past experiments have conducted pre-tests for copulatory behavior, and not used animals who failed to copulate (Hilliard, Nguyen, and Domjan, 1997). However, the goals of this procedure were to both examine the process of sexual conditioning as well as increase breeding behavior in amphibians. If application is the goal then pre-screening might not be of interest, as generality of the procedure is very important.

If this procedure were to be conducted again, both the behaviors tracked, and how conditional approach was measured would be modified. The initial behaviors of interest were obtained from pilot data and existing incomplete breeding descriptions. Now that other behaviors are known to be of interest, they too should be tracked. For example, visual signals such as posture changes were not tracked during training sessions; there was no way of knowing a priori that they would be important. Additionally, as conditional response behavior appears to differ in this species from some of the more commonly used subjects (e.g. quail), it should be calculated differently as well. A more comprehensive measurement of where and when the animal is located in the tank could provide valuable goal- vs. sign-tracking information.

One aspect of this experiment that was not ideal was the conditions under which the call recordings were conducted. Most calls recorded in this study were taken opportunistically during testing sessions, while subjects were housed in a colony room. Additionally, the lab area, where most of the recordings were taken, was near other active areas in the Atlanta Botanical Garden. Although care was taken to record calls at low-activity times (usually by recording calls between 7:00-7:30AM) external noise was inevitable. To minimize disruptions in the future, recordings could be taken in a sound-proofed room, or external noise could be canceled out using active mechanical means.

Another modification that would be recommended is greater isolation of the experiment from other, extraneous influences. For example, the use of one way glass or video cameras for observation could minimize the presence of the experimenter as a variable. It is possible that the experimenter's presence contributed to the excitatory conditioning seen in the active control group. Auditory isolation of the frogs from environmental noise is also recommended. Noise levels at Atlanta Botanical Gardens were occasionally excessive, and as artificial noise has been shown to disrupt calling behavior in other frogs (e.g. Lengagne, 2008) care should be taken to minimize background noise in an experimental situation. Additionally, as the research on behavioral facilitation and suppression in frogs demonstrates, the frogs should also be isolated from each other. If this is not practical (the current subjects were housed in a colony room, where the experiments were also conducted), subjects could be exposed to white noise or a control play-back of frogs calls during the experiment.

One other possible modification, especially if subject numbers remained low, would be to conduct the experiment as a within-subjects design. With the limited number of subjects available power could have been increased by conducting the design as within-subjects. For example, all groups could be exposed to the experimental procedure and during testing half of the groups could be tested with the CS and half without. After a period of time the groups could be re-exposed to the experimental training and then re-tested with the groups being switched in relation to CS exposure. If behavior is indeed under stimulus control, differences should arise during the test period dependent on whether the CS was presented.

Future Directions

While the conditioning procedure was successful in increasing breeding behaviors and improving egg laying many questions remain. Unexpected outcomes within the procedure as well as correlations and anecdotal reports led to many new questions. This area of research is rife with opportunity for future study.

Sex Recognition

One important consideration in the sexual conditioning procedure is the cues used in any particular species for sex identification. While it is apparent that visual cues serve an important role in communication in a number of anuran species (Hödl and Amézquita, 2001), based on the pilot study data it appeared that males and females could not recognize the sex of another frog based on visual cues alone. This finding is surprising, and should be followed up with further experimental examination. *Dendrobates* *tinctorius* are actually classified as having a rather large visual signal repertoire for a frog. The evolution of reliance of visual signals would complement their reliance on other visual cues for communication (e.g. assumed aposematic coloring) and their diurnal nature (Hödl and Amézquita, 2001). However, a study using simulated dendrobatid males found that electromechanical models were only effective in eliciting attack from another male if both visual and auditory signals were present (Narins, Hödl, and Grabul, 2003). Multiple cues during the UCS period may be necessary for proper sex identification.

However, sex recognition by visual cues could have been complicated by the lack of other breeding behaviors exhibited during the pilot study. For example, based on results from other studies, it is possible that had males called during the pilot studies that the females would have been able to identify them based on the addition of a an expressed visual cue (e.g. inflation of the vocal sac) (Kelley, 2004). Further testing of the exact stimuli required for sex identification would be interesting.

The cues used in sex recognition can vary greatly, even when just looking within reptiles and amphibians. Auditory cues are usually considered the most relevant cue for sex recognition in anurans, however other cues such as pheromones have been shown to be important for various species as well (reviewed in Woolley, Sakata and Crews, 2004). A variety of amphibians and reptiles have been shown to rely on visual, thermal, or even somatosensory feedback while attempting to differentiate between sexes. Combinations of cues can be important too, red-sided garter snakes use pheromones as well as visual and thermal cues to locate and identify females.

However, it is obvious from a review of the literature that sex recognition, even within frog species, is extremely varied. For example, in *Bufo andrewsi* males identify potential mates through a combination of visual and auditory cues (Liao and Lu, 2009). Comparatively in *Chaunus marinus* identification of males requires actual synchrony between auditory cues and vibrations, whereas females may be identified by the texture of their skin (Bowcock, Brown, and Shine, 2008). Male *Dendrobates pumilio* may identify females by the female's lack of calling while approaching (Bunnell, 1973). Which of course begs the question, if the female were paired with a call, would she be treated differently? Other species are sensitive to chemicals extruded through the surface of the skin or through specialized glands (Wabnitz, Bowie, Tyler, Wallace, and Smith, 2000; Byrne and Keogh, 2007). Any given species might then rely on auditory, visual, tactile, chemical cues or even any combination of the four. When designing any study where sex recognition is key, as is the case in sexual conditioning, such factors need to be taken into consideration.

Differences in Calls Produced During CS and UCS Periods

Studies in Pavlovian conditioning have shown that the characteristics of responses elicited by the CS and UCS can differ greatly. In one extreme example if the UCS in an experiment in insulin (with an unconditional response of decrease in blood sugar level) the conditional response will be exactly the opposite, an increase in blood sugar level (Siegal, 1975). While there were not enough calls recorded during the CS period in the present study, it would be interesting to look for differences in CR and UCR calls in the future.

It would not be surprising to find such differences, frog calls have been found to be sensitive to a number of variables. For example, using hormone supplementation can have an effect on the characteristics of male frog calls (Marler, Chu, and Wilczynski, 1995). It is possible that this procedure as well could change the types of calls produced. It would be interesting to look at not only the differences in calls produced during the CS and the UCS, but also the differences between calls produced by experimental and control males, or even see if calls change accordingly when males are moved between control and experimental conditions.

Classical Conditioning in Amphibians

Results of this research also add to our knowledge of classical conditioning in frogs. Studies of classical conditioning in frogs are fairly sparse and no one had previously attempted to show sexual conditioning in a frog. In fact the conditioning research done thus far has mostly focused on conditioning of responses to aversive stimuli such as electrical shocks, prodding of the nictitating membrane, immersion in hypertonic solutions, and exposure to distressed tadpoles (Zavala, 1968; Goldstein, Spies and Sepinwall, 1964; Daneri, Papini and Muzio, 2007; Ferrari and Chivers, 2008). In the current experiment non-aversive stimuli and more “active” responses were used and still positive results were obtained.

During this experiment the frequency of the flashing of the CS was tied to the frequency of frog behaviors. As the procedure produced behavioral differences it can be assumed that the CS was salient to the subjects. In future learning research, this method could be used as a guide when selecting salient stimuli for amphibians. However, it would also be interesting to vary characteristics of the stimuli to see if particular stimuli control behavior more effectively, as in: is there a “super stimuli”? However, using such a stimulus might once more blur the lines between neutral and non-neutral stimuli use in Pavlovian conditioning.

Use of Species-Specific Stimuli in Sexual Conditioning

Sexual conditioning already has a long and varied history (Pfaus, Kippin and Centeno, 2001). However, studying this technique in additional species allows us to examine the generality of the methods as well as identify the peculiarities of working with this particular species. One potential benefit of future research in this area could be in the use of neutral vs. species-specific stimuli in sexual conditioning.

Domjan (2005, 1997) has argued that the success of sexual conditioning is very much dependent on using ecologically valid (or species-specific) stimuli. In fact he has used sexual conditioning as a counter-example to equipotentiality. According to Domjan (2005) conditioning is not done in a vacuum, stimuli that are ecologically relevant will produce faster, better conditioning. The ecology of a particular species should help guide stimulus selection. In his experiments he has shown that Japanese quail react best to sexual conditioning when the head and neck of a female quail is used as the CS.

Different stimuli can evoke behaviors of very different forms and whether or not an ecologically valid stimulus is used in sexual conditioning does affect the results. For example, species-specific stimuli (e.g. stuffed quail head and neck) actually elicit approach to the stimulus over repeated exposures, independent of pairing with exposure to a member of the opposite sex (Akins, 2000). However, the greatest approach and copulatory responses are obtained by actually pairing the species-specific stimulus with exposure to the UCS (Cusato and Domjan, 1998). Additionally species-specific cues have been shown to elicit consummatory behaviors (sexual contact or copulatory responses) whereas non-species-specific cues elicit approach behaviors (search, identification and approach).

In another study, Domjan (1994) found that copulatory responses were only directed at the CS by male quail if the CS included some species-specific characteristics. However, through sexual conditioning, initially neutral cues can come to elicit improved sexual behavior during the UCS. So, while copulatory behavior (consummatory) will most likely be directed only at species-specific cues during the CS period, sexual behavior can come under the control of initially neutral stimuli. Interestingly, when species-specific cues are used, approach is controlled by visual cues of the stimulus; conditional approach follows the CS even when it is moved. Comparatively, when a neutral cue is used, conditional approach is instead controlled by spatial cues (Domjan, O'Vary and Greene, 1988).

The important question at this point might be not which is more effective but how big the difference is. Akins (2000) did show that the type of stimuli used influenced the form of the conditional behavior, species-specific cues elicit consummatory behavior and arbitrary cues elicited approach behavior. However, sexual conditioning, even to the point of clear differences in reproductive success, has been demonstrated using initially neutral colored lights as stimuli (Hollis, Pharr, Dumas, Britton, and Field, 1997; Hollis, 1990). Which is more effective becomes a very important issue when dealing with exotic species whose ecology may not be well known. The problem becomes a trade-off in time: identify the proper cues needed for a specific species or be able to implement the process quickly. Obviously being able to use arbitrary stimuli would be potentially more useful in a situation where time is of the essence and details of breeding behavior may be unknown, such as in the amphibian decline.

However, sexual conditioning might only be properly categorized as Pavlovian if the CS is initially neutral. The use of non-neutral stimuli (and the increased efficacy of the procedure) might be better categorized as some other process, such as sensitization. Several experiments provide support of sensitization to an existing stimulus-response relationship. For example, presentation of the non-neutral CS unpaired with the UCS will come to elicit copulatory behavior in quail, although further increases can be seen when pairing is implemented (Cusato and Domjan, 1998). Similarly, mere repeated exposure to a female (through mesh) is enough to reduce ejaculation latency during actual testing in rats (De Jonge, Oldenburger, Louwerse and Van De Poll, 1992).

The distinction between neutral and non-neutral stimuli may be better thought in terms of gradations, rather than categories. The ability to condition particular things together might lie along a spectrum from easily connected to impossible (Domjan, Cusato, and Krause, 2004). In fact, one could argue that some of the Pavlovian procedures in use are already non-neutral in some respects. For example, certain modalities of stimuli are necessary for successful taste-aversion conditioning. By restricting the CS to the modality of tastes or smells we are most likely already making that stimulus "non-neutral".

Instrumental Effects in Classical Conditioning

While sexual conditioning is considered a Pavlovian procedure it is important to remember that other processes may be at work as well. For example, it is well documented that it is often difficult to clearly define certain procedures as either operant or Pavlovian (Schwartz and Gamzu, 1977). Operant procedures often include elements of Pavlovian procedures and vice versa. One classic example is that of auto-shaping in pigeons where the mere presentation of a lighted key followed by food can regularly induce pecking.

Sexual conditioning is likely also influenced by more than just classical conditioning. For example, one of the major outcome measures of sexual conditioning is approach of the CS. It would seem that conditioned approach is likely the combination of classical and operant conditioning. Pairing of the UCS and the CS causes the CS to take on properties of the UCS (in this case properties of a receptive mate) making the CS something to be approached. Additionally, due to the temporal pairing of CS-UCS exposures any approach to the CS would be reinforced by the presentation of the UCS.

The control over sexually conditioned behavior by either Pavlovian or operant contingencies has been tested. In one experiment the conditioning procedure was conducted with an omission procedure, approach to the CS omitted exposure to the UCS (Crawford and Domjan, 1993). Omission of access to mates contingent on approach of the CS did not significantly retard the acquisition of conditional approach behavior, suggesting strong Pavlovian control.

However, there was anecdotal evidence for operant effects from this procedure. In one example, after a number of paired trials a male began to call during CS presentation. The female heard the male and approached the barrier. Thus when the barrier was raised latency to contact was reduced, which may have improved the result of that particular trial. For application purposes this entanglement of contingencies is likely not troublesome. However, to further explore the issue, an omission procedure, such as the one described, could be conducted to test for the instrumental effects of sexual conditioning with amphibians.

Goal-Tracking vs. Sign-Tracking

One interesting outcome of this study was that conditional approach did not seem to be a good indicator of successful sexual conditioning for this particular procedure. Amount of time spent proximate to the CS was not greatly different between the two groups. One explanation of this result is that individuals may not have been showing only sign-tracking behavior. Some individuals appeared to be exhibiting a mixture of sign- and goal- tracking behavior. Sign-tracking is exhibited as approach to the CS whereas goal-tracking is inferred by proximity to the location where the UCS will appear. For example, using auto-shaping, differences in goal- and sign-tracking can be examined by placing the key light and the food magazine in different parts of the experimental chamber (Brown, Hemmes, Cabeza de Vaca, and Pagano, 1993). During CS presentation individuals might stand by the light (sign-tracking) or by the food magazine (goal-tracking). With food based Pavlovian conditioning, introducing a trace delay can shift behavior from sign- to goal-tracking.

It has generally been found that in sexual conditioning in quail the propensity is to elicit sign-tracking (approach CS) instead of goal-tracking (approach UCS) behavior (Burns and Domjan, 1996). Indeed, quail continue to exhibit sign tracking even after increasing the distance between the CS and the UCS or using a trace interval. However, in many sexual conditioning experiments the location of the CS and the UCS is confounded. For example, the experimental context may serve as the CS (there is a separate room/cage for pairing trials) (e.g. Domjan, Blesbois, and Williams, 1998) or the CS and UCS may be accessed through the same door (Holloway and Domjan, 1993b).

The lack of differentiation between goal and sign in these experiments make comparisons with the current study difficult.

However, in this experiment, sign-tracking may not have been the dominant response to the procedure. The experimental and active control groups did not differ greatly in how much time they spent near the CS during the training trials. Instead, in this experiment, individuals appeared to be exhibiting a mixture of sign- and goal-tracking, individuals would not only approach the light during CS presentation, but also move back and forth along the barrier. In addition, the details of the current procedure may have caused goal-tracking behavior to manifest as pacing the barrier during CS presentation. Since the barrier separating the halves of the tanks runs the entire length of the tank, the location in which the other frog appears may be extremely variable, there can be no "magazine training". This variability could potentially be reduced in the future simply by having a smaller area where the UCS could be presented.

It is also possible that sign- or goal-tracking is confounded by collapsing data across pairs. The sexes may be reacting differently to the conditioning procedure. The types of behaviors shown by males and females have already been shown to differ in sexual conditioning. For example, in quail, while males will show greater approach of the CS and of the UCS, female quail show mainly increases in squatting behavior (Gutiérrez and Domjan, 1997). In this experiment as well it is possible that male and female frogs were responding to the sexual conditioning procedure differently. While females tended to approach the light but then pace back and forth along the barrier (and indeed, females entered the CS more times than males) males would often approach the CS only after a period of calling. These behaviors would be reasonable in the context of the natural behaviors of these species. Male frogs tend to call to attract females, and approach the females when they come into view while female frogs tend to seek out calling males (Wells, 2007). However, if females paced the goal line and males called at the light, neither would be captured by the existing way of measuring conditional approach.

An alternative way of thinking about goal- vs. sign- tracking is as a manifestation of the separation of appetitive and consummatory behavior. Sexual behavior can be separated into appetitive behavior (search, identification and approach) and consummatory behavior (sexual contact or copulatory responses) (Domjan, O'Vary and Greene, 1988). These two parts of sexual behavior can respond to conditioning in different ways. One example is the separation of sexual motivation and copulatory behavior in rats using counter-conditioning (Ågmo, 2002). Even when males reduced their copulatory behavior with scented females paired with lithium chloride (LiCl) shots they still emitted an equal amount of female seeking behavior. Satiation also affects appetitive and consummatory behaviors differently. Even if copulatory behavior is suppressed, search behavior will still be exhibited (Hilliard, Domjan, Nguyen, and Cusato, 1998).

The propensity to goal- vs. sign-track has also been shown to vary greatly based on a number of factors including species, conditioning paradigm, characteristics of the UCS and CS and pre-exposure to the CS (Brown, Hemmes, Cabeza de Vaca, and Pagano, 1993; Boughner and Papini, 2003; Flagel, Akil, and Robinson, 2009; Boughner and Papini, 2003). Individual differences may also come into play (Flagel, Watson, Robinson, and Akil, 2007). Some researchers have even separated individuals into three groups: those who approach and manipulate the CS as if it were the UCS, those who withdraw from the CS and wait where the UCS has been presented and an intermediate group who vacillate between the previous two behavior patterns (Flagel, Akil, and Robinson, 2009). If such groups existed in the current experiment, it would also explain the lack of clear-cut sign-tracking demonstrated in this experiment.

Possible physiological correlates of the conditioning procedure

Hormones

One potentially interesting area for future research is in exploring the physiological correlates of sexual conditioning. How the physiology of an organism varies under these procedures could potentially inform future application attempts. One possible physiological correlate to the conditioning procedure is a change in hormone levels. It has already been demonstrated that sexual conditioning can cause a previously neutral stimulus to be as effective as the presence of a female in promoting secretion of luteinizing hormones (Graham and Desjardins, 1980). Additionally, hormone suppression has been shown to diminish but not eliminate sexual conditioning outcomes (Hilliard and Domjan, 1995; Hilliard, Domjan, Nguyen, and Cusato, 1998). It could be interesting to examine the interaction of hormone levels with the conditioning procedure in amphibians as well. For example, hormone levels could be tracked, or manipulated, during the course of conditioning. On a purely application level it would be interesting to see if the results of the procedure could somehow be maximized by, for example, by not only prepping an animal hormonally but also using non-neutral stimuli.

Hormone levels in frogs have been shown to vary predictably with expressions of breeding behavior (Moore, 1983). This has been shown even in aseasonal breeders who tend to maintain lower maximum androgen levels than seasonal breeders (Emerson and

Hess, 1996). Additionally, hormone supplementation has been successful in promoting breeding behavior in a number of anuran species. For example, in *Physalaemus pustulosus* breeding behavior in females can be elicited by injections of estradiol (Chakraborty and Burmeister, 2009). Additionally, human chorionic gonadotropin (HCG) has been shown to increase female *Physalaemus pustulosus* responses to both conspecific calls as well as to calls that were a synthetic blend of males from their species and males from a closely related species (Lynch, Crews, Ryan, and Wilczynski, 2006). Lynch, et al. further demonstrated that this increase in “permissiveness” (responding more often to calls then without supplementation) was not due to a decrease in the female’s ability to discriminate between calls. Tests of discrimination did not differ under different levels of hormone injections. Similar results were demonstrated in an experiment using female *Hyla versicolor* (Gordon and Gerhardt, 2009). The influence of hormones on breeding behavior is complex, however. Hormonal state likely affects not only the expression of breeding behavior and signals, but also the reception of those communications as well (Arch and Narins, 2009).

The expression of sexual characteristics, outside of the breeding season, can be brought about by the addition of hormones. For example, in *Hyla chrysoscelis* muscle mass in males (for use in calling) is gained during the breeding season and lost once the season is over (Girgenrath and Marsh, 2003). Supplementation with testosterone can cause the male to return to his breeding season muscle mass and even cause females (who do not call) to have similar muscle growth.

Supplementation can also change how breeding behaviors are expressed, but possibly in a positive manner. For example in *Acris crepitans* injections of the peptide hormone arginine vasotocin (AVT) not only increased the probability of calling in males but also changes in call structure (Marler, Chu, andWilczynski, 1995). AVT injected males produced calls characteristic of less aggressive males. Experimental males also began calling sooner than control males, although whether this is a facilitation of calling behavior or a blocking of the effects of handling is unclear. However, if AVT was inhibiting the detrimental effects of handling on breeding behavior this would have important implications for captive breeding.

Anecdotal observations from these experiments suggest that hormone supplementation might be an effective mechanism for improving outcomes of sexual conditioning in this species. One female in this study was carrying lots of eggs. This individual showed the most extreme responses to the conditioning procedure. Also, due to the interactive nature of the experiment, the male involved also showed extensive conditional behavior. Both animals readily approached the light and the male called extensively during CS presentation. During testing this pair had the shortest latency to contact and calling and the shortest latency to pursuit and kneading by the female. Additionally they had the highest percentage of time spent within one length (97% of the first 30 minutes). Anecdotally, it appears that the extreme outcome for this pair was due at least in part to the starting condition of the female.

Cautions when using Hormone Supplementation

If the addition of hormones is to be used in breeding experiments, caution should be taken. In some cases hormonal injections have led only to the production of infertile eggs (Bishop, Haigh, Marshall, and Tocher, 2009). In other cases the implications are more subtle. For example, in *Physalaemus pustulosus* females, phototaxis behavior (an appropriate breeding behavior) can be elicited by injections of estradiol (Chakraborty and Burmeister, 2009). However, preferences for various calls of these supplemented females were also tested, and while preference was similar to that of naturally breeding females there were also important differences. For example, estradiol injected females did not continue to respond to the calls as long as the naturally breeding females. Additionally, supplemented females have been shown to respond to calls they previously would not have responded to (Lynch, Crews, Ryan, and Wilczynski, 2006). Similar results have been demonstrated with female *Hyla versicolor*, where hormone injected females will respond more than those that are not supplemented, but even supplemented females do not respond as much as a female that is naturally in a breeding state (Gordon and Gerhardt, 2009). Hormonal induced behavior may also manifest differently than behavior that in naturally expressed. Additionally, calls produced by males that have been supplemented with AVT are shifted in dominant frequency from naturally occurring calls (Marler, Chu, and Wilczynski, 1995). So while hormonal supplementation may elicit sexual behavior, care must be taken to insure that the behavior being elicited is sufficiently similar to naturally occurring behavior for the purposes of that particular experiment.

Hormone levels may also not have a simple relationship with the expression of breeding behaviors. For example, playbacks of calling frogs increased androgen levels overall in wild frog populations of *Hyla cinerea* (Burmeister and Wilczynski, 2000). However, while overall calling also increased in this population in response to the playbacks, even frogs who did not call at all had similar increases of hormone levels as to those who increased their calling behavior. So while recordings of choruses increased both circulating levels of hormones and calling behavior, the relationship between the two is by no means a straightforward causal relationship. Additionally, different hormones may act synergistically to promote breeding behavior in amphibians (Moore, Boyd, and Kelley, 2005). For example, androgens alone may fail to elicit breeding behavior, interactions of the hormone with prolactin and corticosterone are also important.

Another aspect to be considered is the strength of the role of hormones in any particular species. Tropical frogs, with less well defined breeding seasons, actually maintain lower levels of androgens than their more seasonal relatives (Emerson and Hess, 1996). Emerson and Hess (1996) suggest that maintaining high levels of androgens during an expended potential breeding season is energetically expensive. This could have several implications for using hormone supplementation with dendrobatids. For example, hormones may be less effective in promoting breeding behavior than in more seasonal breeders, hormones may have more of a "permissive" rather than an activating role in the aseasonal breeders, or possibly smaller increases in androgen levels for this species might actually have a larger effect than in a species where higher levels occur more frequently.

CNS Suppression

Another interesting possibility is that sexual conditioning is accompanied by suppression of the sympathetic nervous system (SNS). Activation of the SNS keeps an animal in a general state of fight or flight. A stimulus that signals that upcoming interactions are not antagonistic, and thus activation of the SNS is not necessary, could suppress activation and allow for the expression of other behaviors, such as breeding.

Some stimuli have been shown to suppress general behavioral levels. For example, in some amphibian tadpoles, stimuli from conspecifics are thought to produce general behavioral quiescence through suppression of the hypothalamo-pituitary-adrenal (HPA) axis (Fraker, Hu, Cuddapah, McCollum, Relyea, Hempel and Denver, In Press). Suppression of the HPA axis allows the animal to stay in a still state, and possibly reduce the chance of being detected by a predator.

Additionally, stress (as measured by corticosterone) is known to have negative effects on reproduction (Moore, Boyd, and Kelley, 2005). As such, suppression of these reactions would be assumed to have positive effects on breeding behavior. When amphibians are exposed to stressful situations (including captivity) corticosterone levels rise and reproductive behaviors are suppressed (Moore, 1983). Decreases in reproductive behavior can be ameliorated through administering metyrapone, which inhibits synthesis of corticostreone.

Studies using amphibians and reptiles have found that different species and even individuals can have differential corticosterone responses to the same stimuli (Moore and Jessop, 2003). Additionally differences in environment and history can also affect these responses. It is possible that some of these differences might arise from either desensitization to stimuli or learned suppression of stress responses.

Aggressive responses are also a known issue for successful captive breeding. Inappropriate aggression can even result in injury or death (e. g. Augustus, Casavant, Troxel, Rieches and Bercovitch, 2006). Aggressive behaviors are partially under control of the central nervous system (Haller, Makara, and Kruk, 1998). Conditioning could possibly result in the suppression of expression of aggressive behaviors.

There is some evidence that conditioning can result in suppression of aggression. Pavlovian conditioning procedures conducted with blue gouramis show that CSs can be used to signal whether a rival is likely or unlikely to appear, and consequently the male's behavior shifts accordingly (Hollis, Martin, Cadieux, and Colbert, 1984; Hollis, Cadieux, and Colbert, 1989).

Environmental Stimuli

Behavioral suppression and facilitation.

During pilot testing one group of frogs produced eggs while not exhibiting behaviors generally correlated with egg-laying in other groups (e.g. calling, high level of proximity). Another group had started testing an hour previously and that male was still engaged in calling behavior. The tanks are not sound-proof and calls from one male can be heard in adjoining tanks. It is possible that the behavior of the second group was either suppressed or facilitated by the calls from the first group. For example, the second group may have shown typical breeding behaviors, had those behaviors not been suppressed by the other male calling, or the second group's egg-laying might have been facilitated by the calling of the first male, even in the absence of calls by the second male.

Suppression of calling behavior by the calls of other males has been demonstrated in several species of frogs (e.g. Tobias, Barnard, O'Hagan, Horng, Rand, and Kelley, 2004). Calling may also not be necessary for breeding to occur. Indeed, in other frog species, there are non-calling males that still father offspring through sexual parasitism (e.g. Forester and Lykens, 1986). Additionally, in this experiment, several pairs that produced eggs had non-calling males.

When the amphibian literature is examined, frog calling behavior appears to be influenced mostly by facilitation rather than suppression (but see, Tobias, Barnard, O’Hagan, Horng, Rand, and Kelley, 2004). Socially facilitated behavior can be defined as a frequency or intensity increase of a response that is already in an animal’s repertoire, when that animal is in the presence of another animal engaged in the same behavior at the same time (Clayton, 1978). Especially in species where the breeding season is constricted by temperature limitations or the availability of transient water pools, social facilitation of breeding behavior would be extremely beneficial. Synchrony of breeding behavior would be expected in species with limited opportunity for breeding. While environmental variables might give a large scale view of when breeding might occur, stimuli from conspecifics could allow for timing on a more fine temporal scale. This would allow for large enough groups to gather to promote a successful breeding outcome. In fact, the importance of these social stimuli can be seen in several species, especially those who breed in large flocks, such as flamingos. Even if environmental conditions are appropriate for breeding in these species, breeding behavior may not occur until a sufficient number of birds are present in the area.

Even in species that are not the most gregarious (i.e. for birds, the yellow-eyed penguin), social cues can still have positive effects on facilitating breeding behavior (Setiawan, Davis, Darby, Lokman, Young, Blackberry, Cannell, and Martin, 2007). Playbacks of combinations of social calls were broadcast in parts of a colony of yellow-eyed penguins, which are considered to be a fairly solitary nesting species. Pairs in areas where the calls were broadcast tended to lay eggs sooner, have more synchrony in egg laying, and have higher levels of circulating androgens that pairs in control areas.

Examples of calls facilitating calls in other males abound in the frog literature. For example, in *Hyla microcephala* calls can be elicited from males by playing calls from conspecifics (Schwartz and Wells, 1985). Both recordings of aggressive and advertisement calls elicit aggressive calls from the males as well as an increase in calling rates. Frog calling in field situations has also been facilitated by playbacks of conspecific choruses in *Cophixalus ornatus* (Brooke, Alford, and Schwarzkopf, 2000) and *Hyla cinerea* (Burmeister and Wilczynski, 2000). Additionally, exposure to choruses can increase androgen levels in some frogs (Chu and Wilczynski, 2001) independently of whether or not the males actually call during the experiment (Burmeister and Wilczynski, 2000). So, even if playbacks had no external effect, hormones levels can still be affected.

Breeding behavior can also be facilitated by chemical cues from conspecifics. For example, in *Pseudophryne bibronii* odors from females elicited advertisement calling from male frogs (Byrne and Keogh, 2007). Comparatively when the males were exposed to male odors there was an increase in territorial calling.

In some anuran species females call in addition to the males. In fact female calling in *Xenopus laevis* can have a dramatic effect on male behavior (Tobias, Viswanathan, and Kelley, 1998). One a female has begun calling it is rarely more than a second or two before she is accompanied by a male. In addition, it would be expected that male calling behavior should also have effects on the females of the frog species. For example, exposure to recorded frog choruses increased circulating levels of androgens in male *Physalaemus pustulosus* and increased levels of estradiol in females (Burmeister and Wilczynski, 2000; Lynch and Wilczynski, 2006). Exposure to random tones had no such effect.

It is apparent from the existing literature that males of different species react differently to calls from conspecifics. For example, *Bufo punctatus* males would call in response to playbacks of recordings taken from conspecifics while *Bufo woodhousei* males reduced their call rate (Sullivan, 1985). As such it would be hard to predict how *Dendrobates tinctorius* males respond. However, studies using related dendrobatids *Colostethus talamancae* and *Dendrobates pumilio* suggest that playback experiments should be able to elicit calling from this species (Bunnell, 1973). However, calls were only produced once the recorded call ceased, suggesting either suppression while the call is being produced or perhaps an attempt at asynchrony. Future research could be conducted using playback experiments to clarify behavior interactions present in this species.

In fact, the effects of social facilitation in frog behavior are fairly conducive to experimental examination. Many social facilitation studies suffer from complications due to the compound effects inherent in the mere presence of a conspecific (Clayton, 1978). For example, comparing behavior of a primate while alone or with a conspecific can be confounded by the detrimental effects of isolation on the study animal. Studying social facilitation of pecking behavior in chicks can be complicated as well. While the presence of another chick may facilitate pecking behavior it is hard to isolate the effects of the chick's presence from the effects of the chick's behavior. Further complications arise with the demonstration that a chick may act differently when its social companion is sleeping compared to when it has been anesthetized.

In comparison, studying the effects of frog calls should be very simple. Calls can be recorded and synthesized to make "average" calls or calls with specific properties (e.g.

Gerhardt, 2008). Additionally the effects of calls can be measured without complications from signals from other modalities. Playbacks of calls have been shown to be effective at changing behavior in a variety of species both in the lab and in the field (e.g. Gerhardt, 2008; Schwartz and Wells, 1985; Brooke, Alford, and Schwarzkopf, 2000). Studying calls as a way of facilitating breeding behaviors in sexual conditioning should also be achievable. Indeed, if calls of other males are facilitators of breeding behavior, as has been suggested, then frog calls might serve as an appropriate non-neutral stimulus to improve sexual conditioning for use in applied settings.

Temperature Effects

Another suggested several potential areas for future research in this area is looking at the effects of temperature. While some amphibians do not appear to behaviorally thermoregulate (e.g. Bogert, 1952), most frogs appear to exploit the temperature gradients available in their environments (Carey, 1978). Individuals that have just eaten seek out higher temperatures than those that have not, and a variety of behaviors are restricted to rather narrow bands of temperatures. Frogs may have a variety of temperature modulation behaviors available ranging from moving between microhabitats of different temperature to changing the amount of moisture released through their skin. However, as has been shown in other species, preferred temperature is also a function of acclimation.

Temperature-dependent learning has been demonstrated in both reptiles (Krekorian, Vance, and Richardson, 1968) and fish (reviewed in Prosser and Nelson, 1981). Two interesting findings emerge from these studies. First of all, learning proceeds faster as temperature increases, up to a point (usually when temperature starts exceeding the maximum operating temperature of the organism). Secondly, this quality interacts with what might be described as a context-dependent learning effect, the greater the difference in temperature between the training and testing situations the worse the recall. These factors could be used to advantage if, for example, training and testing trials were both held at a higher temperature than during other times. This would combine the increased learning effect with further differentiation of the contextual difference between conditioning times and other periods of time.

The effects of temperature on behavior have already been demonstrated in a number of amphibians. For example, in *Rana temporaria* performance of both swimming and jumping behaviors were found to be temperature dependent (Navas, James, Wakeling, Kemp, and Johnston, 1999). Calling behavior has also been shown to be coupled with temperature in a variety of species. In *Xenopus laevis* trill rates of male calls increase linearly with increase in temperature (Yamaguchi, Gooler, Herrold, Patel, and Pong, 2008). In *Hyla versicolor* not only do characteristics of males' call change with temperature but females also prefer calls with the properties appropriate to their current temperature (Gerhardt, 1978).

Many amphibians have also been shown to have temperature-dependent breeding behavior. For example, breeding aggregations of both male and female *Hyperolius marmoratus* can be predicted based on a number of environmental factors, including temperature (Henzi, Dyson, Piper, Passmore, and Bishop, 1995).

Other Environmental Cues

Another environmental incident of interest occurred after the experiments had concluded. Three groups of frogs had been left with access to their partners over a weekend. Rain fell heavily all weekend and on Monday all three groups were found to have laid eggs. Other groups that had been left together on other occasions had not produced similar results. The frogs in this study usually reproduce during the rainy season (Lötters, et al., 2007) which is why they were misted daily during experiments. It is possible that other stimuli associated with the rainy season may also be useful in encouraging breeding behavior. In fact, placing a dripping water feature in a captive environment has been known to increase breeding behavior in some other frog species (R. Hill, personal communication). The changes in pressure associated with impending precipitation might also have been a salient stimulus (e.g. Obert, 1976) although pressures changes would prove difficult to reproduce in the lab.

Rainfall and humidity have been suggested as possible seasonal cues for some amphibians, cues that may be especially relevant in equatorial environments where temperatures may not drastically differ throughout the year (Bogert, 1952). In fact, amphibians that breed over a longer period of time may be more sensitive to a variety of environmental stimuli than frogs with shorter breeding seasons (Oseen and Wassersug, 2002). Some frog species' breeding patterns can be partially accounted for by a variety of environmental factors including barometric pressure, wind, light intensity, day length, rainfall and humidity (e.g. Henzi, et al. 1995; Brooke, Alford, and Schwarzkopf, 2000; Obert, H.-J., 1976). However, even just between the sexes, the relative strength of these predictive factors can vary. Additionally, using absolute measures of these factors can be misleading. While amount of rainfall is important for expression of breeding behavior in *Physalaemus pustulosus* whether breeding behavior is facilitated or suppressed by rainfall is a function of the overall rainfall for that year (Marsh, 2000).

Wind, as a potential environmental factor, is quite interesting. It might be assumed that all frogs would avoid windy days due to the increase chance of desiccation and a reduction in call fidelity, as wind noise should interfere with call reception (Oseen and Wassersug, 2002). However, it appears that only frogs with relatively low frequency calls have calling behavior suppressed by low levels of wind (the frequency that wind noise is most prevalent at) suggesting that desiccation at low levels of wind is probably not a large contributing factor in the timing of breeding behavior.

Environmental and social cues may be of particular importance to this species. As anurans with less well defined breeding seasons are suggested to be less dependent on a small number of stimuli for activation of breeding behavior (Emerson and Hess, 1996), a range of environmental cues may be of importance. It is likely that a large number of events are involved in optimally promoting breeding behavior. In fact examination of sensory pathways in anurans in general suggests that there are separate pathways for information pertaining to social and environmental cues that influence the secretion of gonadotropin releasing hormone (GnRH) (Wilczynski, Allison, and Marler, 1993). It appears and that these two sets of cues provide parallel, but independent and potentially different information that influences breeding behavior.

During pilot testing another potentially interesting event occurred. In the beginning the frogs seemed to be laying very few eggs. Serendipitously there were not enough plastic breeding huts for the experiment and so coconut huts were used for one of the groups and that group proceeded to produce eggs. While the correlation may have been coincidental, coconut huts were used for oviposition sites during the course of these experiments.

It is possible that without a proper egg-laying site frogs will be less likely to lay eggs. As such, it is imperative that the relevant environmental cues be identified. Literature on captive breeding in this species suggests that many cues might be important, including such things as daily misting, proper temperature, appropriate oviposition sites (which for some species may be as simple as a black film canister) the right social grouping, and the calling of other males (Lötters, et al., 2007).

General Conclusions

Captive Breeding of Exotics.

The positive results obtained in this study have important implications for the captive breeding of exotics. Often captive animal facilities have issues breeding some of their animals. Captive animal facilities are not the optimal context for the breeding of exotic animals and breeding success can be very low (McDougall, Réale, Sol, and Reader, 2006). As such, a procedure with the potential to increase reproductive success would have wide-spread application.

As mentioned earlier, captive breeding is often a difficult process with a myriad of potential issues that range from a lack of potential reproductive partners, to social hormonal reproductive suppression, to offspring being conceived that are not viable (Augustus, Casavant, Troxel, Rieches and Bercovitch, 2006; Daleum, Creel and Hall, 2006; Swaisgood, Dickman, White, 2006). One of the most common hurdles in breeding exotic animals in captivity is a lack of proper breeding behavior on the animal's part (Munkwitz, Turner, Kershner, Farabaugh, and Heath, 2005; Zhang, Swaisgood, and Zhang, 2004). Inappropriate breeding behavior has actually been implicated as a major problem in successful captive breeding for a variety of species from birds to pandas.

Obviously, overcoming these behavioral issues should be a major objective for captive animal facilities.

Results from this study support earlier sexual conditioning studies that have shown that classical conditioning could be effective in battling many of the aforementioned issues. Not only has sexual conditioning been shown to promote positive breeding behaviors and decrease aggressive behaviors (e.g. Cutmore and Zamble, 1988; Hollis, Cadieux and Colbert, 1989) but it has also been shown to increase the number of viable offspring produced in a successful encounter (Hollis, 1990). Earlier success, combined with the current data, suggests that indeed sexual conditioning might prove a successful tool for improving breeding behavior in other captive exotics.

Additional anecdotal evidence suggests that this procedure may be especially beneficial for breeding captive exotics, as the results may not be limited to the experimental period. Pairs that were exposed to the experimental condition were reported by animal staff (who were blind to which animals had been exposed to which contingencies) to be spending more time in proximity to one another and showing increased incidences of breeding behavior as compared to prior to the experiment. Additionally several pairs who had been housed together prior to this experiment and had never produced eggs have now begun to lay eggs on a regular basis. It appears as if the procedure may have additional benefits of facilitating introductions, or possibly fostering long-term breeding relationships between individuals.

Such changes in partner preference would not be surprising given past studies on conditional preference in sexual conditioning. Sexual conditioning has been shown to have the effect of influencing preference for particular stimuli. For example, sexual conditioning has been shown to influence preference for sexual partners (Kippin et al., 1998) and increase the reinforcing properties of stimuli used in operant procedures (Everitt, Fray, Kostarczyk, Taylor and Stacey, 1987).

Amphibian Decline

Improving breeding success is especially important for amphibian populations. As previously mentioned, amphibian numbers are in decline and action must be taken quickly to prevent an extreme loss of diversity (Norris, 2007). One possible solution in action is the creation of assurance colonies for these species that can later be used to repopulate affected areas. Creation of assurance populations requires breeding these amphibians in captivity on a large scale, and in a relatively short period of time. The advantages seen in other species from sexual conditioning would be particularly important for the creation of amphibian assurance colonies, where time is of the utmost importance. The results from this study could add sexual conditioning to the repertoire of techniques currently in use for promoting reproduction in captive amphibians (e.g. Browne, Clulow, Mahony and Clark, 1998).

The current focus on improving reproduction in amphibians is on using assistive reproductive techniques such as harvesting and preservation of gametes, hormone manipulations, artificial insemination, and even cloning (Edwards, Mahony, and Clulow, 2004; Browne, Clulow, Mahony, and Clark, 1998; Kouba, Vance and Willis, 2009; Holt, Pickard, and Prather, 2004). To the best of my knowledge there are no other attempts to use purely behavior techniques to improve breeding behaviors. The current technique could be useful in species where hormone manipulations have only produced infertile eggs (e.g. Bishop, Haigh, Marshall and Tocher, 2009) or this technique could be combined with others, potentially improving outcomes even more.

Other results from this study could also help improve captive breeding techniques. The correlation of behaviors shown during testing with reproductive success has resulted in a list of behaviors that can help determine whether a breeding attempt will be successful, just based on the first 30 minutes of interaction. For example, if the female did not perform the kneading behavior within the first 30 minutes the chance of her laying eggs in this experiment was zero. This predictive power is especially useful in a species such as *D. tinctorius* because often it would take three or four days before eggs were laid. Those three or four days could have been spent pairing that frog with another partner with whom them might have better success. If time is important, being able to determine whether breeding will be successful, based on the first 30 minute interaction, has great application value.

Overall, this procedure has proved successful at increasing reproductive success in *D. tinctorius*. Sexual conditioning, which is known to be very flexible in its application, appears to be applicable to amphibian species as well. The extension to amphibians provides evidence that this procedure could potentially generalize to a variety of other species. In addition to reproductive success, sexual conditioning also increased the expression of breeding behaviors. This increased expression, along with the condensed time in which it occurs, could allow for easier observation of breeding behavior in any species of interest. Sexual conditioning appears to be an area rife with opportunity for continued study on a variety of effects within Pavlovian conditioning, as well as for application to captive breeding or the study of sexual behavior.

APPENDIX A

BEHAVIOR DESCRIPTIONS

Contact: Physical contact occurs between the two frogs.

Pursuit: Frog moves within one body length of other frog (male and female) or frog commences calling upon seeing other frog (male only).

Call: Only seen in males. Body of frog inflates and deflates, accompanied by a rasping sound. Throat sac may or may not inflate.

Male mount: Male places his ventral surface on female's dorsal surface with both heads oriented in the same direction.

Kneading: female raises and lowers her front feet, either individually or in succession, rhythmically and repeatedly. The feet may be resting on the male or on substrate directly adjacent to him. Movement is slower than in limb-shaking.

Toe-trembling: Fourth toe of back foot vibrates up and down rapidly. (Hödl and Amézquita, 2001)

Limb-shake: front or back foot is raised and lowered quickly (Hödl and Amézquita, 2001).

Body-lowering (Crouching): Either whole body or anterior part of the body is pressed against the substrate (Hödl and Amézquita, 2001).

Upright posture: Arms are extended and the anterior part of the body raised (Hödl and Amézquita, 2001). In this species the arms may even be over-extended.

Stroke: Frog touches other frog with front foot.

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