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This thesis describes studies that investigated 1) the effects of courtship on the neuroendocrine system of female rough-skinned newts, Taricha granulosa, and 2) whether the observed courtship-induced neuroendocrine changes affected female sexual receptivity.

Sexual behaviors of female T. granulosa changed dramatically during courtship. Initially, females exhibited unreceptive behaviors to a courting male. After several hours of courtship, females displayed receptive behaviors, and males responded by attempting to transfer a sperm cap. Once courtship, insemination and post-insemination behaviors were complete, the female no longer became receptive to future courtship.

Both telencephalic irLHRH and plasma estradiol concentrations changed as females became receptive. Early in courtship, when females were unreceptive, irLHRH

concentrations in the telencephalon were elevated, but by the time females became receptive irLHRH concentrations were low. The change in telencephalic irLHRH concentration occurred in the nervus terminalis, a little understood cranial nerve that may influence reproduction. Plasma estradiol levels were low at courtship initiation, but were elevated by the time females became receptive. The observations, that irLHRH and estradiol concentrations were associated with changes in female sexual behaviors and that these hormones influence female sexual receptivity in other vertebrates, suggested that courtship by a male activates female sexual behavior by stimulating endogenous changes in LHRH and estradiol.

Increasing or decreasing plasma estradiol concentrations or blocking the action of this steroid in the brain were found to have no effect on female receptivity. Also, injecting LHRH or LHRH analogs into brain was found to have no effect on female receptivity. Progesterone implantation inhibited receptivity suggesting that this steroid may be responsible for the post-insemination decrease in female sexual behavior. These results suggest that although courtship induces physiological changes in female T. granulosa, the changes in LHRH and estradiol may not be influencing receptivity.

Courtship-induced Changes in Female Sexual Receptivity: A Neuroendocrine Study in an Amphibian

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CHAPTER 1: GENERAL INTRODUCTION

The timing of reproduction is under intense selection pressure: any organism that reproduces at an inopportune time loses progeny and consequently has a lowered reproductive success. Alternatively, an organism that breeds under favorable conditions maximizes reproductive success. Environmental cues play crucial roles in influencing the timing of reproduction. The ability to receive, process, and translate these cues into physiological messages is essential to successful reproduction.

Organisms use several categories of environmental information to fine-tune the timing of reproduction (Wingfield, 1983). "Initial predictive information" such as seasonal changes in photoperiod and temperature allow organisms to predict the arrival of favorable periods for reproduction. "Supplementary information," provides cues that initiate the last stages of the reproductive effort. For instance, the establishment of a territory or behavioral interactions with a mate provide cues that induce final gonadal maturation. However, timing reproduction based strictly on predictive and supplementary information may be limiting since rapid

changes in local environmental conditions may diminish the value of using only this information to initiate reproduction. Therefore, an organism should respond to "modifying information" from the environment such as unpredictable changes in weather and food availability. Once all of the cues indicate favorable reproduction conditions, "synchronizing and integrating information," such as sexual interactions exchanged during courtship, can lead to insemination. The behavioral exchange of courtship becomes an integral part of the environmental information an organism receives and integrates through neurological and physiological mechanisms.

Not only does courtship synchronize and integrate sexual behaviors, but mating displays often facilitate overall reproductive function. Some of the first documented examples of behavioral facilitation of reproductive function comes from work with birds (Brockway, 1964; 1965; Lehrman, 1965; Kroodsma, 1976;). In many bird species, male courtship (particularly vocalizations) stimulates female ovarian development and the onset of reproductive behavior. Likewise, in reptiles, the behaviors and displays of courtship by males can activate reproductive functions in females. For example, the dewlap display of courting males facilitates photoperiod-induced ovarian recrudescence in female Anolis carolinensis (Crews, 1975). Thus, the behaviors and

displays of courtship can act as supplementary and synchronizing information to regulate the exact timing of reproduction in a number of species (recently reviewed by Moore and Deviche, 1987 and Wingfield and Marler, 1988).

The facilitating and synchronizing effects of courtship are not limited to the effects of male behavior on female physiology and behavior; male reproductive function is also mediated by social behavior of females. The presence of female brown-headed cowbirds facilitates a rise in testosterone concentrations and gonadal growth in male cowbirds under laboratory and field conditions (Dufty and Wingfield, 1986). Male white-crowned sparrows exposed to both a stimulatory photoperiod and sexually active females also show higher testosterone and luteinizing hormone concentrations and larger gonads than do males exposed to the photoperiod alone (Moore, 1983). Furthermore, free-living white-crowned sparrow males exhibit sexual behavior and elevated androgen concentrations if their mates continue to solicit copulations once the normal sexually active season is over (Moore, 1982). The results of these studies indicate that behavioral interactions induce physiological and behavioral effects in males and females of many species.

Although the above studies have demonstrated that long-term or repeated exposures to courtship behaviors can stimulate reproduction in conspecific mates, to my

knowledge no study has investigated the potential neuroendocrine changes that mediate courtship-stimulated behaviors. The rough-skinned newt Taricha granulosa offers the opportunity to investigate whether male courtship behaviors trigger sequential changes in female behaviors and neuroendocrine state and whether any of the observed neuroendocrine changes are responsible for the sequential changes in behavior.

The following review describes what is known about courtship behaviors of T. granulosa. Since changes in female sexual behavior during courtship may be mediated by changes in the female neuroendocrine system, several hormones known to regulate female sexual behavior are also discussed with respect to their behavioral actions.

Courtship in Taricha granulosa

Courtship in salamanders has been subdivided into a series of behavioral interactions (Salthe, 1967). First, a male contacts and identifies a conspecific female (Stage A). Next, the male captures the female in amplexus or performs a visual display while exposing the female to olfactory, visual, and tactile stimuli (Stage B). The male then releases and moves away from the female who may or may not follow (Stage C). If the female follows the courting male and provides appropriate behavioral cues,

the male deposits a spermatophore on the substrate (Stage D). Last, insemination occurs when the male leads the female forward so her cloaca comes in contact with the spermatophore (Stage E). It is interesting that Salthe fails to mention post-insemination clasping as phase F. Although not described by Salthe (1967), many species of salamander continue courtship by recapturing or displaying to the female after the initial attempt at insemination (Arnold, 1976; 1977; Halliday, 1977; Verrell, 1982). Little is understood about the function of post-insemination courtship in an already inseminated female, but these behaviors may help to prevent insemination by a new male.

Within this general framework, urodele amphibians show a great diversity of courtship patterns and behaviors (Salthe, 1967; Salthe and Mecham, 1974; Arnold, 1977; Halliday, 1977). In particular, the type and duration of the display by the male to the female (stage B) can vary greatly (Arnold, 1976). Some species remain in physical contact for a long time during the entire courtship process, while others have little or only brief physical contact prior to sperm transfer. Arnold (1976; 1977) proposed that the probability of successful insemination may increase with the amount of time and energy invested in courtship by the male and has found that among salamander species there is a correlation between the

amount of time a male spends in courtship and the probability of the female finding and being inseminated by the male's sperm package. Whether courtship in T. granulosa fits into this correlation across species will be discussed in Chapter 2.

For T. granulosa in western Oregon, the breeding season begins in late winter after males and females migrate to breeding ponds. Males arrive first, and females migrate to the ponds later, usually beginning in February during a period of warmer rains (Pimentel, 1952; Propper, pers. obs.). Shortly after the females enter the ponds, courtship begins.

There are no published accounts describing in detail the behaviors of both males and females during courtship for any member of the genus Taricha, although there are anecdotal accounts for Taricha torosa (Smith, 1941) and Taricha rivularis (Davis and Twitty, 1964). Several male behaviors are common to courtship in Taricha. A male always initiates courtship by capturing a female in a dorsal amplectic clasp (Stage A). Soon after courtship initiation Stage B begins and, a male performs a clutching reflex by contracting his hind legs against the abdomen and cloaca of the female. Also, the male rubs the submandibular gland of his lower jaw over the nares of the female. Both the nares rubs and the hind limb contractions have been reported to become more intense as

courtship progresses (Smith, 1941; Davis and Twitty, 1964). Not as much is known about the behavior of the female Taricha during courtship. T. rivularis females stop swimming and raise their heads to male nares rubs (Davis and Twitty, 1964). As well, in T. rivularis, courtship prior to insemination is reported to last about one hour (Davis and Twitty, 1964).

Preliminary observations on T. granulosa (Propper, pers. obs.) suggest that the female's behaviors change during courtship. Initially, the female appears to attempt to escape from the male. Furthermore, she moves her head down and away from his nares rubs. As courtship progresses, however, the female stops moving her nose away from the male, and, as in T. rivularis, she raises her head in response to the nares rubs. Soon after the female lifts her head the male releases her (Stage C) and places a spermatophore in front of her nares (Stage D). The male moves away from the spermatophore, and the female follows him closely. If the sperm package touches the edges of the female's cloaca, it is drawn into the cloaca, and the female is inseminated. Therefore, during courtship a female's behavior changes from unreceptive to receptive.

Of interest is the fact that courtship does not end with insemination. After completion of a sperm transfer attempt, the male turns and recaptures the female, and the pair remains in amplexus for several hours. This post-

insemination clasping had not been documented before for T. granulosa, and its full significance was not known until the studies in Chapter 2 were conducted.

Chapter 2 not only quantifies the changes in behavior that occur during courtship in the rough-skinned newt, but it also determines the amount of time a pair invests in courtship and the probability of successful sperm transfer (insemination) for each courtship event. Furthermore, this chapter investigates the significance of post-insemination amplexus.

Patterns of Reproduction

The changes in female T. granulosa sexual behavior may be associated with endocrine or neuroendocrine events. A specific internal endocrine environment may be necessary for courtship to activate sexual receptivity. Furthermore, the behavioral exchanges of courtship may facilitate physiological changes that mediate the onset of receptivity.

Crews (1984) defined three reproductive patterns commonly seen in vertebrates: associated, dissociated, and constant. These patterns are useful in attempting to categorize how the endocrine system may regulate reproductive behaviors. Species with an associated reproductive pattern exhibit seasonal gonadal development,

gamete production, and increased steroid secretion at the time of year when mating occurs. Species with a dissociated reproductive pattern exhibit reproductive behaviors during seasons when gamete maturation and steroid secretion are low. Last, species with a constant reproductive pattern are ready to reproduce at any time the environment becomes favorable. These patterns are points of reference that help determine whether specific endocrine events are important to the activation of sexual behavior.

What type of reproductive pattern does the female rough-skinned newt exhibit? When female T. granulosa enter ponds to mate, their ovaries contain mature follicles (McCormack, 1979). However, females do not ovulate until 12-32 days after mating, and will not ovulate unless they have been inseminated (McCormack, 1979; Moore et al., 1979; Spielvogel and Moore, unpublished data). These results mean that while mating behavior occurs when the gonads are mature, receptivity is temporally dissociated from ovulation. Whether hormones normally associated with ovarian development and ovulation play a role in reproductive behavior in such a system has not been determined.

The Endocrine System and Female Sexual Receptivity: Ovarian Steroids and Sexual Receptivity

The associated reproductive pattern is found in a majority of species investigated. Endogenous, seasonal or cyclic changes in plasma ovarian steroid concentrations are correlated with changes in sexual receptivity. Examples include the rat, Rattus norvegicus (Feder, 1984), rhesus monkey Macaca rhesus (Gordon, 1981), ruffed lemur, Lemur variegatus (Shideler et al., 1983), several bird species (Hinde and Steel, 1978), and the lizard, A. carolinensis (Crews, 1980; Jones et al., 1983). This strong correlation between sexual behavior and endocrine state suggests that changes in plasma steroid concentrations, associated with gamete development and ovulation, may serve as activational regulators of appropriate mating behavior.

The effects of ovarian steroid hormones on sexual receptivity have been studied extensively in many species, and especially in rats (see reviews by Feder, 1984; Arnold and Breedlove, 1985; Pfaff and Schwartz-Giblin, 1988). Boling and Blandau (1939) demonstrated that ovariectomy abolishes female sexual receptivity in rats, and estrogen alone or in combination with progesterone treatment restores receptivity. Since this early study, further investigations have demonstrated that treatment with

estradiol followed by progesterone is more effective in facilitating sexual receptivity than is estradiol or progesterone alone (Feder and Marrone, 1977; Glaser et al, 1983; Green et al., 1970) suggesting that there is a synergistic effect on behavior by the two hormones.

The actions of gonadal steroids on female sexual behavior are similar in many different mammalian species (reviewed in Feder, 1984; Feder and Marrone, 1977). Estradiol treatment at a high dose induces sexual receptivity in primates (Kendrick and Dixson, 1985a), ferrets (Baum and Schretlen, 1978), and several rodent species (McDermott et al., 1980; Huck et al., 1982; Steel, 1983). In many species, a dose of estradiol that is too low to stimulate sexual behavior primes the female to progesterone or a second low dose of estrogen which then activates receptivity (Feder and Marrone, 1977; McDermott et al., 1980; Sodersten et al., 1981; Burley et al., 1983; Clark and Roy, 1983; Moreines and Feder, 1983; Steel, 1983; Feder, 1984; Carter et al., 1987). These results suggest that estradiol may act to prime females to later exposure of estradiol and progesterone.

In birds, steroids also play an important role in sexual behavior. In female ring doves, ovariectomy eliminates sexual responsiveness to male courtship (Cheng, 1973a), and treatment with estradiol restores behavior in a dose-dependent manner (Cheng, 1973b). Also, in ring

doves, estradiol injections into intact females stimulate sexual solicitation or proceptivity (Lehrman, 1965). Indeed, when free living female white-crown sparrows (Moore, 1982) and song sparrows (Runfeldt and Wingfield, 1985) are implanted with estradiol, the females continue to solicit copulations from males long after the period for normal seasonal courtship has ended. These results indicate that in many bird species, ovarian steroids influence the expression of sexual behavior.

As in mammals and birds, sexual receptivity is under steroidal control in many ectothermic vertebrates. Receptivity in the lizard A. carolinensis can be activated by a high dose of estradiol, or a low dose of estradiol followed by a single injection of progesterone (McNicol and Crews, 1979; Wu et al., 1985). Furthermore, the antiestrogen, CI-628, effectively blocks estradiol-mediated receptivity in this species (Tokarz and Crews, 1980). Receptivity in the lizard, Eumeces laticeps, also is induced by treatment of estradiol (Cooper, et al., 1986). Ovariectomy eliminates receptivity in the frog, Xenopus laevis, and both estradiol and progesterone are necessary to reinstate sexual receptivity to male courtship (Kelly, 1982). Studies on fishes also demonstrate a dependence on ovarian hormones to maintain receptivity (Liley and Donaldson, 1969; Liley, 1972). The results of these studies indicate that ovarian hormones

affect female sexual behavior in many vertebrates.

Many of the studies investigating endocrine control of sexual behavior have focused on species that exhibit an associated reproductive pattern. However, in some species studied, mating behavior is dissociated or not correlated to the ovarian cycle (Crews, 1984). In these species, the physiological control of behavior remains a puzzle. For example, some primates exhibit the dissociated pattern. Human females offer an example of a case where sexual activity takes place during all phases of the ovarian cycle (Urdy and Morris, 1968). In gorillas (Gorilla gorilla), female sexual receptivity also appears to be dissociated with gonadal steroid cycle (Nadler et al., 1983).

Many other species besides primates do not follow the common associative pattern. In female musk shrews, Suncus murinus, although ovariectomy eliminates receptivity (Rissman and Bronson, 1987), females show no single period of behavioral estrus (Rissman et al., 1988). In female garter snakes, circulating concentrations of estradiol are low at the time of mating (Crews and Garstka, 1982; Whittier et al., 1987), and several other reptiles show a pattern of reproduction in which the ovarian state and steroid hormones are temporally dissociated from sexual receptivity (Crews, 1984; 1987).

In addition to a lack of correlation between sexual

behavior and plasma steroid cycles, in many species, ovarian steroids do not play a strong activational role in sexual receptivity. For example, human females do not show a decline in sexual activity after ovariectomy (Kinsey et al., 1953). In the Djungarian hamster, Phodopus campbelli, receptivity is dependent on estradiol, but progesterone does not affect receptivity or peak at the time of ovulation (Wynne-Edwards et al., 1987). Furthermore, this dissociation of hormones and behavior is not limited to mammals since one species of frog, Rana pipiens, will show receptive behavior after ovariectomy (Diakow et al., 1978). These results suggest gonadal steroids are not always associated to the activation of sexual behavior.

Luteinizing Hormone-Releasing Hormone and Sexual Receptivity

Luteinizing hormone-releasing hormone (LHRH) has been found to affect gonadotropin release from the pituitary (reviewed in McCann, 1978), and sexual receptivity (Moss and McCann, 1973; Pfaff, 1973). Especially exciting is the fact that the effects of LHRH on female sexual receptivity appear to be independent of the hypothalamus-pituitary-gonad axis (Moss and McCann, 1973; Pfaff, 1973).

Sexual receptivity in females of several species is

facilitated by LHRH. In the LHRH deficient hypogonadal mouse, LHRH injections in estrogen-primed females stimulates sexual behavior (Ward and Charlton, 1981). LHRH injections also enhance sexual responsiveness in the common marmoset, Callithrix jacchus (Kendrick and Dixson, 1985b), ring dove, Streptopelia risoria (Cheng, 1977), green anole, A. carolinensis (Aldrete et al., 1980), and South African clawed frog, Xenopus laevis (Kelley, 1982), indicating that the behavioral effects of LHRH are widespread among vertebrates.

Male sexual behavior is LHRH-facilitated. This peptide stimulates male sexual behavior in rats and man (reviewed by Moss et al., 1979; Mauk et al., 1980), the vole, Microtus canicaudius (Boyd and Moore, 1985), and the rough-skinned newt (Moore et al., 1982). These results indicate that the effect of LHRH on behavior is not sex specific.

Studies of LHRH localization have helped to localize where in the brain this neuropeptide has its behavioral effects. In mammals, LHRH has a wide distribution in the brain (see Schwanzel-Fukuda et al., 1987 for review). Cell bodies with LHRH immunoreactivity are found in the nervus terminalis, medial preoptic area, basal hypothalamus, and in the organum vasculosum of the lamina terminalis (OVLT). Luteinizing hormone-releasing hormone-containing fibers are found in the accessory olfactory

bulb, olfactory bulb, medial septal nucleus, hypothalamus, preoptic area, and median eminence. As well, LHRH-containing fibers project to the amygdala, infundibulum (Jennes, 1987), OVLT, and the mesencephalic grey (Shivers et al., 1983).

The locations of LHRH in the amphibian brain has also been the subject of several studies (Alpert et al., 1976; Doerr-Schott and Dubois, 1976; Nozaki and Kobayashi, 1979; Jokura and Urano, 1985; Jokura and Urano, 1986; Wirsig and Getchell, 1986; Crim, 1987; Muske and Moore, 1987). Cell bodies containing luteinizing hormone-releasing hormone are found in the nervus terminalis, olfactory nerve, preoptic area, medial septal nucleus, diagonal band of Broca, and medulla. Besides projecting to the median eminence, LHRH-containing fibers are also found to terminate in the preoptic area, medial septal region, stria medullaris, habenula, and lamina terminalis. In Hyla regilla and T. granulosa a diffuse network of LHRH-containing fibers is also found in the ventral and dorsal pallium, tectum, thalamus, midbrain tegmentum, and torus (Muske and Moore, 1987).

Injection of LHRH into brain regions containing endogenous LHRH cells and fibers enhances female sexual behavior. For example, injection of LHRH into the medial preoptic area (MPOA) or the mesencephalic grey, but not into the arcuate nucleus or the superior colliculus,

enhances female lordosis in the rat (Moss et al., 1975; Riskind and Moss, 1979; Sakuma and Pfaff, 1980). Moreover, an injection of LHRH antiserum into the mesencephalic grey disrupts sexual behavior (Sakuma and Pfaff, 1980, 1983). These results suggest that LHRH may mediate sexual behavior by acting in specific areas of the brain.

The fact that the nervus terminalis contains LHRH immunoreactivity (Schwanzel-Fukuda and Silverman, 1980), suggests that this nerve plays a role in reproduction and possibly sexual behavior. In support of this hypothesis is the finding that electrical stimulation in the area of the nervus terminalis stimulates sexual behavior in two species of teleost fish (Doving and Solset, 1980; Satou et al., 1984). Likewise, and severing the nervus terminalis in male hamsters inhibits sexual behavior (Wirsig and Leonard, 1987).

Demski and Northcutt (1983) suggested that since the nervus terminalis is closely associated with the olfactory system it may function as a chemosensory system responding to pheromonal communication during reproduction. Evidence in support of this hypothesis comes from the finding that LHRH concentrations in the olfactory bulbs (presumably the nervus terminalis) of female voles increase in response to male urine (Dluzen et al., 1981). In male mice exposed to females a similar result is found (Dluzen and Rameriz,

1983). Possibly, these changes in LHRH concentration mediate subsequent changes in sexual behavior.

The finding that LHRH is located in the nervus terminalis of female T. granulosa (Muske and Moore, 1987), and the fact that newt courtship has a pheromonal component, suggests that the rough-skinned newt may make a good model to examine the effects of mating on LHRH in the nervus terminalis. Furthermore, since evidence suggest that this little understood cranial nerve may affect sexual behavior in vertebrates, and LHRH influences receptivity in many vertebrates, it is possible that LHRH in the nervus terminalis mediates female sexual receptivity in the newt.

Summary

The above review 1) describes what is known about courtship behaviors in the rough-skinned newt T. granulosa, and 2) discusses the relationship between several hormones and female sexual behavior. Courtship in the rough-skinned newt not only acts to synchronize male and female behavior, but it also appears to activate sexual receptivity in the female. The physiological mechanisms activated by courtship to produce this change in behavior are largely unknown, but studies conducted in many different species suggest that several hormones may be involved. The ovarian hormones, estradiol and

progesterone, have long been known to be associated with female sexual receptivity. More recently, several neuropeptides have been found to affect female sexual behavior. One of these hormones, luteinizing hormone-releasing hormone is known to have behavioral effects in many species.

Objectives

The main goal of this thesis was to determine if estradiol, progesterone, and LHRH act as chemical mediators between male courtship and female sexual receptivity in T. granulosa. To achieve this goal, several objectives were formulated:

1. To determine the changes in female and male behavior occurring during courtship, and to determine whether pheromonal communication is necessary for courtship-induced changes in female sexual behavior (Chapter 2).
2. To determine if there are changes in plasma steroid and brain peptide concentrations associated with changes in female sexual behavior (Chapter 3).
3. To determine if estradiol, progesterone or LHRH affect courtship-induced sexual receptivity (Chapter 4).
4. To determine if plasma steroid and brain peptide concentrations are associated with migration, mating, and oviposition, three distinct stages of reproduction during the breeding season for female T. granulosa (Chapter 5).

CHAPTER 2: COURTSHIP IN THE ROUGH-SKINNED NEWT *TARICHA GRANULOSA*

INTRODUCTION

Male salamanders face two distinct problems in gaining successful reproduction. First, insemination in most salamander species is indirect: a male deposits a spermatophore on the substrate, and the female must follow him to find the sperm cap with her cloaca (Salthe and Mecham, 1974). Second, male-male mate competition is intense in many salamander species (Arnold, 1976; 1977; Halliday, 1977; 1978), possibly as a result of skewed sex ratios in the breeding ponds (Halliday and Verrell, 1984). This paper focuses on how the rough-skinned newt, *Taricha granulosa*, "deals" with these two problems.

If a courting male is unable to induce sexual receptivity in a female, then his time and energy investment in the insemination effort are lost. Furthermore, if the behaviors of the individuals in the pair are not synchronized, the insemination attempt will fail even if the female is receptive. These two functions of courtship, persuasion and synchrony (Tinbergen, 1953), are quite evident in the mating habits of many salamander species (Arnold, 1976, 1977; Halliday, 1977; Verrell, 1982). By investing time in courtship, a male may be better able to synchronize reproductive behaviors with his mate and to evaluate her receptivity.

Arnold (1977) hypothesized that the time and energy a male spends per spermatophore is directly related to the probablility that the sperm cap will inseminate the female. Studies of a few species of salamander (Arnold, 1976; Halliday, 1977; Verrell, 1982) suggest that there is a correlation between courtship time investment and insemination success (Arnold, 1977). Although this generalization appears to hold true between species (Arnold, 1977), and may also be the case within species (Verrell, 1982), in order to determine if this correlation represents a general pattern it is important to determine if this relationship holds true when more information from a number of different species is provided.

The success of courtship also depends on the behavior of competing males. Sex ratios in many salamander breeding ponds are skewed towards males (Halliday and Verrell, 1984), and this fact may be responsible for male-male mate competition in salamanders (Arnold, 1976; 1977; Verrell, 1983; 1984; Massey, 1988). In salamanders, male-male mate competition can take the form of sexual interference (Arnold, 1976). For example, in Ambystoma tigrinum, a male uses female mimicry to induce spermatophore depositions from a rival, and then covers the spermatophore with one of his own (Arnold, 1976). Such behavior not only prevents insemination of the female by the original suitor, but also causes him to lose energy

and time in wasted spermatophores and courtship effort. Another form of competition is direct physical interference, such as wrestling a female away from a courting male. This method is commonly seen in members of the genus Taricha (Smith, 1941; Pimentel, 1952; Davis and Twitty, 1964; Propper, pers., obs.).

Another form of male-male competition is sperm competition (Parker, 1970) which occurs when more than one male inseminates a female. Sperm competition has been demonstrated in the plethodontid salamander Desmognathus ochrophaeus (Houck and Schwenk, 1984; Houck et al., 1985). The potential for sperm competition in nature exists for female Triturus vulgaris and Notophthalamus viridenscens since females will become receptive to, and pick up the sperm cap from, more than one male in the laboratory (Halliday and Verrell, 1984). Males that have mechanisms to limit or prevent any of these forms of mate competition may gain a greater overall reproductive success.

Courtship in Taricha

There are no published accounts, for any member of the genus Taricha, describing in detail the behaviors of males and females during courtship, although there are brief anecdotal descriptions of courtship behaviors of Taricha torosa (Smith, 1941) and Taricha rivularis (Davis and Twitty, 1964). Several male behaviors are common to

courtship in Taricha. Males always initiate courtship by capturing a female in a dorsal amplectic clasp. Then while in amplexus, a male performs bouts of contracting his hind legs against the abdomen and cloaca of the female and bouts of rubbing the glands on his lower jaw over the nares of the female. Both of these behaviors become more intense and frequent as courtship progresses. Not as much known about the behavior of the female Taricha during courtship, although it is reported that a T. rivularis female stops swimming and raises her head to the chin rubs of the male (Davis and Twitty, 1964). In T. rivularis, courtship prior to insemination is reported to last about one hour (Davis and Twitty, 1964).

Sperm transfer in Taricha occurs when a male releases the female and places the spermatophore in front of her nares (Smith, 1941; Davis and Twitty, 1964; Pimentel, 1952). The male then pivots about one forearm so that his body moves through an arc away from the female. She follows the male closely, keeping her nares near his cloaca. This rotation places the cloaca of the female in the vicinity of the spermatophore. If the sperm cap touches the edges of the female's cloaca, it is partially drawn into the cloaca, and sperm transfer is successful. Shortly after rotating away from the sperm cap, the male will reclasp the female in amplexus.

Much is unknown about courtship in Taricha. First,

there are no published accounts that follow female behavior throughout the entire course of courtship. Second, the amount of time spent in courtship prior to insemination has not been reported for either T. granulosa or T. torosa. Third, the probability of courtship leading to insemination is unknown for any member of the genus Taricha. Fourth, although pheromones are known to play an important role in salamander courtship (Salthe and Mecham, 1974; Arnold and Houck, 1982), it is not know whether sex pheromones are used by Taricha. Two lines of evidence support the hypothesis that pheromones are involved in reproductive behavior and possibly female receptivity in Taricha. The chin rubbing behavior of courting Taricha males certainly suggests that pheromones play a role in the induction of female sexual responsiveness. Furthermore, when compared to the gland of unmated males, the gland found under the chin of male T. torosa that have recently completed courtship is reduced in size and apparently exhausted (Smith, 1941).

Another fact about Taricha courtship is that pairs exhibit post-insemination amplexus, a behavior common to all three species of Taricha. The function of this behavior is unknown.

The purpose of this investigation was to quantify the behavioral interactions that lead to successful insemination in the rough-skinned newt Taricha granulosa

and to determine some of the factors that influence changes in female sexual behavior. Changes in behavior during courtship were quantified for both male and female newts, and the probability of courtship culminating in successful sperm transfer was determined. Furthermore, the role of pheromonal stimuli in inducing the changes seen in female sexual receptivity was investigated. Last, post-insemination courtship was investigated and a novel function for this behavior is suggested.

METHODS AND MATERIALS

General Methods:

Animals: From February-April in 1984, 1985, and 1988, female T. granulosa were collected in pit traps as they migrated toward water at Soap Creek Ponds, which are located 12 miles north of Corvallis (Benton County), Oregon. The collection procedure insured that females had not mated during the current season. Females were kept in a large tank (160 x 60 x 20 cm) in the laboratory until used for the study. Light:Dark cycle was adjusted regularly to approximate the natural photoperiod. Temperature was maintained at 5-10°C. The tank contained dechlorinated tap water, dried oak leaves, and a platform for the females to leave the water. Females were regularly fed mealworms and earthworms.

The day before each study, males were collected from the Soap Creek Ponds and kept 1 to 2 per standard 40 liter observation aquarium that contained 20 liters of water in a controlled-environment room (simulated natural photoperiod and ambient temperature of 12-15°C). Females were placed in buckets with 1 liter of water and left in the room containing the observation tanks.

Initial Study: Behaviors, Timing, and Success of Courtship

The purpose of this study was to determine the behavioral changes that occur during courtship, the length of courtship, and the probability of courtship resulting in insemination.

Between 8:30 a.m. and 10:30 a.m. on study days, one female was placed in each tank that contained a single male. Each pair was observed for a one minute focal period once every 20 minutes for a 12 hour period. During each focal period, several parameters of courtship were recorded. First, the pair was checked to determine if a sperm transfer attempt had been made during the unobserved period. A sperm transfer attempt is readily discernible because the sperm cap is visible for several hours on the substrate or in the cloaca of the female. Any sperm transfer attempt was noted as "successful" if the female picked up the sperm cap in her cloaca and as "unsuccessful" if the female had not picked it up in her cloaca. Second, for each pair, the number of male hind limb contractions performed during the focal minute was recorded. Third, the position of the female's head was noted and assigned a rank value as follows: if the female moved her head down at a sharp angle after the male attempted to rub her nares, the head position was scored as zero. If her head was one-half of the angle between being level with her body and position score 0, the

position was scored as 1. If she held her head level with her body the position was scored as 2. Last, if her head was raised in response to a chin rub, the position was scored as 3.

After the initial 12 hour observation period, pairs were not observed for 12 hours. After this time period, the females and the substrate were again checked to determine if a sperm transfer attempt had taken place during this unobserved period. The females and substrate were examined for attempted sperm transfers each 12 hours for a total of five days of observation. A total of 29 pairs were observed.

By using the focal observation method, it was possible to determine 1) the number of pairs that attempted a sperm transfer within a given unit of time, 2) the approximate length of time a pair spent courting before the male attempted to transfer a sperm cap, 3) the number of transfer attempts/pair during the experimental period, 4) the length of time spent in courtship between sperm transfer attempts, 5) whether a sperm transfer attempt was successful, and 6) if male and female behaviors changed during courtship. In order to quantify behavioral changes, at three times during each courtship, I used the male hind limb contraction rate and female head angle score for pairs that successfully transferred a sperm cap during the first 12 hours of observation. The

focal observation minutes chosen to make this determination were 1) within the first 20 minutes of courtship initiation, 2) one-half of the time between the initiation of courtship and a successful sperm transfer, and 3) just prior to sperm transfer.

Statistics: Differences in female head position scores and the rate of male hind limb contractions at the different courtship periods were determined using a Friedman's 2-Way Analysis of Variance followed by a Wilcoxon matched-pairs, signed-ranks test (Siegel, 1956). The correlation between head position score and the number of palpitations/minute was determined by Spearman's Rank Correlation Test (Siegel, 1956).

Experiment 1: The Importance of Olfactory Stimuli to Female Sexual Receptivity

This study was designed to determine whether the olfactory system plays a role in courtship-induced female sexual receptivity. In March, 1988, 60 females were assigned to one of three treatments (N = 20/treatment). The experiment was conducted over two days with one-half of the females in each treatment being tested on each day. Females in the "Untreated Control" group received, using a micropipettor, 0.5 ul dechlorinated water over each nares. Females in the "Unilateral Cover" group received 0.5 ul dechlorinated water over one naris and 0.5 ul

cyanoacrylate (Krazy Glue, Inc. Itasca, Ill.) over the other naris. Females in the "Bilateral Cover" group received 0.5 ul glue over each naris. To confirm that the glue covered the nares completely, the nares of females in the two cover treatments were checked under a dissecting microscope before and after the experiment. Any female without a complete cover over the nares was eliminated from the analysis. The final sample sizes (N) for each treatment were: Untreated, N=20; Unilateral Cover, N=7; Bilateral Cover, N=13.

After the treatment, females were placed in buckets containing 0.5 liters of water and left overnight in the experimental room (15^{o}C). The next morning, individual females were placed in the aquaria containing 2 males collected the day before. When one male captured a female in amplexus the remaining male was removed from the tank. Pairs were checked every 15 minutes for 12 hours, recording the following: 1) incidence of amplexus, 2) the position of the females' head, 3) attempts at sperm transfer, and 4) the outcome for each sperm transfer attempt (successful or unsuccessful). To monitor male behaviors, the number of times a male attempted to rub a female's nares and the number of hind limb contractions were quantified for 1 minute every 3 hours for one-half of the pairs in each treatment. Pairs were left unobserved for another 12 hours (overnight) and then checked again

for any sperm transfer attempts. The presence of a sperm cap in the female's cloaca or on the substrate indicated that a pair had attempted sperm transfer during the night (hours 12-24 of courtship). The experiment was ended 24 hours after the initiation of courtship.

Statistics: The number of pairs successfully transferring sperm caps in each treatment group, at 12 or 24 hours after the initiation of courtship, was compared between treatments using a Fisher's Exact Test. Differences in male behavior between the three treatments were determined using a Kruskall-Wallis One Way Analysis of Variance (Siegel, 1956).

Experiment 2: Effects of Courtship, Insemination, and Post-insemination Clasping on Subsequent Sperm Transfers and Female Sexual Receptivity.

The purpose of this study was to determine if females remain receptive following mating and to find which component of courtship and insemination may be responsible for any decrease in receptivity.

On the first day of the study, females were assigned to one of four treatments: Group 1 females (No Courtship Controls) were placed alone in a 10-gallon aquarium containing 20 liters of water. These females were not exposed to a male and, therefore, had not been courted or

inseminated on the first day of the experiment. Group 2 females (Courted only) were placed individually in a 10-gallon aquarium with a single male. Pairs were allowed to court until there was an attempt to transfer a sperm cap. The spermatophore was manually removed from the substrate before a female could pick it up in her cloaca, taking care not to disturb the animals. The male was removed from the tank at this time, and the female was left alone until the following morning. Group 3 females (Inseminated) were treated as Group 2 females except they were allowed to pick up the sperm cap in her cloaca. At sperm transfer the male was removed, and the female was left alone until the next morning to prevent the female from receiving post-insemination courtship. Group 4 females (Full Courtship Control) were treated as females in group 3 except the males were allowed to resume amplexus (post-insemination amplexus) after sperm transfer. Males in this treatment were left in the tank with the females overnight. On day 2 of the experiment (24 hours after the start of the first courtship for females in groups 2, 3, and 4), all males from day 1 in group 4 were removed.

To test whether the experimental treatments on day 1 had an effect on female sexual receptivity, a new male was placed in each tank containing a female. The pairs were checked visually each 15 minutes, and sperm transfer

attempts were recorded. All pairs were allowed to court undisturbed for 24 hours. All incidences of sperm transfer attempts were recorded.

Statistics: Differences between treatments in sperm transfer attempts on day 2 were determined by Fisher's Exact Test (Siegel, 1956).

RESULTS

Initial Study:

Courtship in the rough-skinned newt involves a series of behavioral changes in both males and females. At the initiation of courtship (Table 1.1), the female newt arches her back and keeps her head bent down so that the male has difficulty rubbing his chin over her nares (score 0). At a time one-half of the period between courtship initiation and sperm transfer, the female does not move her head to such a sharp angle, but she still moves her head away from the male's rubs (score 1). By 20 minutes prior to sperm transfer, the female holds her head at an angle that is parallel to the substrate and does not move it in response to a chin rub (score 2). Because of the one-minute sampling method females were not always observed to raise their heads prior to sperm transfer in this experiment, however, in every case in this experiment and others (Experiments 2 and 3, this chapter; Chapter 3), where pairs were under continuous observation, females always lifted their heads to the angle score 3 before a male would make a sperm transfer attempt. Also, shortly before sperm transfer, in conjunction with a raised head posture, females stop swimming, stretch their forelimbs in front of their heads, and release a single air bubble from their mouth.

The behavior of the female is associated with whether a pair will transfer a sperm cap. Fifty-two percent of the pairs that transferred a sperm cap had females raise their heads by 20 minutes before the transfer. Since observations were made for only 1 minute every 20 minutes, this percentage is probably an underestimate. Only 10% of the pairs that did not transfer a sperm cap in the first 12 hours of courtship had a female demonstrate a raised head posture. This result indicates that when females signal with a head raised position, a male will usually respond with a sperm transfer attempt.

Male newts also exhibit changes in behavior as courtship proceeds to sperm transfer (Table 1.1). The frequency of hind limb contractions is initially slow, but as courtship proceeds, the hind limb contraction rate increases until soon before sperm transfer, when the rate can be more than an order of magnitude higher than it was at courtship initiation. Indeed, the contraction rate from one male was 82 contractions/minute shortly before a sperm transfer attempt. Immediately after a sperm transfer attempt, a male holds the female in amplexus but does not perform hind limb contractions.

The rate of the chin rub bouts also changes during courtship, with the number of rubs/minute being highest in the middle of the courtship period. Also, soon before a sperm transfer attempt, apparently in response to the

raised head position of the female, the male will rub his chin vigorously side-to-side over the nares of the female. Within the first 20 minutes after sperm transfer, the male does not perform any chin rubs (Table 1.1).

The behavior of the female is correlated with that of the male during courtship. The hind limb contraction rate is correlated with the female head position: the contraction rate is fastest when the female's head is held highest (Spearman's Rank Correlation: $P < 0.001$).

During a sperm transfer attempt, the male completely releases the female and moves forward over her head. He then places himself perpendicular to her, and she places her nares near to his cloaca. The male's sides contract while he presses his cloaca to the substrate. After about 15 seconds the male deposits a sperm cap on the substrate and begins to pivot his cloaca away from the female and sperm cap. The female remains oriented to the male's cloaca and follows it as the male moves away from her. The male stops pivoting when the female's cloaca is close to the spermatophore. At this point, the female starts a back and forth side-stepping motion that brings her cloaca directly in contact with the sperm cap. Once the sperm cap makes physical contact with the cloaca, it appears to stick and be partially drawn into the female. The pair remains in the perpendicular orientation with respect to each other for a number of seconds more, and then in 100%

of the cases, regardless of whether the female picked up the sperm cap, the male turns and in one rapid movement, recaptures the female in amplexus. The pair remains in amplexus for at least two hours and will sometimes attempt another sperm transfer (see below).

Not only is courtship comparatively long in the rough-skinned newt, but the probability of courtship leading to fertilization is very high if females have not been previously mated during the breeding season (see experiment 2, this Chapter). The median amount of time spent in amplexus before sperm cap transfer was 7 hours, and pre-insemination courtship in this species takes from less than two hours to several days. The cumulative incidence of successful sperm transfer across the time of the experiment is shown in Fig. 1. Sixty-five percent of the pairs successfully transferred sperm caps within 12 hours after the initiation of courtship, and 76% of all pairs successfully transferred sperm caps within 24 hours after courtship initiation. A total of 93% of all pairs transferred sperm caps within the five day observation period, while 7% of the pairs never transferred a sperm cap during the study.

The probability of success for the first sperm transfer attempt was also high. Of the 19 pairs that attempted a sperm transfer within the first 12 hours of courtship, 89.5% of the first attempts were successful.

Only two pairs exhibited at least one unsuccessful attempt: one male made 3 unsuccessful attempts at sperm transfer because during each attempt the female swam away. In the other case, the female swam away during the first attempt, but followed the male and picked up the sperm cap in her cloaca during the second attempt.

Even if the first sperm transfer attempt inseminates the female, the male resumes amplexus, and pairs sometimes attempt sperm transfer again. Forty-seven percent of the pairs in which insemination was successful within the first 12 hours attempted one to three more sperm transfers. Of the 27 animals that successfully transferred sperm caps during the course of the study, 37% attempted to transfer another sperm cap as was indicated by presence of a new sperm cap on the substrate or in the cloaca of the females. A maximum of 4 spermatophores/ total courtship sequence were deposited by any one male, but the median was 1 spermatophore deposited per courtship sequence.

Only two of the second attempts succeeded in inseminating the female (7% of all second attempts). The low success rate of these second attempts is not due to lack of female receptivity. In all of the observed cases of a second sperm transfer attempt, the female had signalled the male with a raised head posture, and followed the male to the spermatophore. However, the

first sperm cap was still in the female's cloaca and blocked the acquisition of a second cap. In the two cases where females received a second sperm cap, the first did so 7 hours after she was inseminated by the first sperm cap, and the second received the second package more than 24 hours after the first successful transfer. In these cases, the first female had already partially absorbed the first sperm cap, and the second female had entirely absorbed the first sperm cap.

Experiment 1:

The results of this experiment suggest that the female requires an intact olfactory system to achieve successful sperm transfer. Table 2.2 shows that obstructing the nares of females reduces the incidence of successful sperm transfer. Significantly fewer females in the Bilateral Cover group were successfully inseminated during courtship when compared to the Untreated group and the Unilateral Cover group, (After 12 hours of courtship: Bilateral Cover compared to Unilateral Cover, $P = 0.004$ and compared to Untreated, $P = 0.031$; after 24 hours of courtship Bilateral Cover compared to Unilateral Cover or Untreated, $P < 0.001$). There was no significant difference in the incidence of successful sperm transfer between the Untreated and the Unilateral Cover groups 12 hours ($P = 0.338$) or 24 hours ($P = 0.450$) after the

initiation of courtship, indicating that the glue alone had no effect on female sexual receptivity.

Covering the nares of the females did not prevent all males from attempting to transfer a sperm cap (Table 2.3). Thirty-eight percent of the pairs in the Bilateral Cover treatment did attempt a sperm transfer. However, by 24 hours after the initiation of courtship, significantly fewer pairs in this treatment attempted sperm transfers compared to the Untreated treatment ($P = 0.025$). One possible explanation for some of the males attempting a sperm transfer in the Bilateral Cover treatment is that a female sometimes gave one or more momentary raised head signals before moving her head down, but no female in this treatment ever exhibited an extended raised head posture. The momentary raised head posture appeared to excite the male, and he would release the female and deposit a spermatophore. At this time, the female either remained in one spot or swam away. In no case did a female follow a male. The results of this experiment further support the hypothesis that a female must have an intact olfactory system for courtship to elicit a full receptive response.

Covering the nares of the female had no measurable effect on male courtship behaviors. A male performed the same number of chin rubs and hind limb contractions toward a female regardless of the treatment group (Table 2.4). Therefore, the difference in the incidence of sperm

transfer among the treatment groups is apparently due to the effect of the treatment on the female.

Experiment 2:

Courtship, including insemination and post-insemination amplexus, inhibits sexual receptivity in female rough-skinned newts. Table 2.5 summarizes the results of this experiment. Previous insemination causes a significant decrease in female receptivity in response to courtship by a new male. Forty percent of the females in Group 3 (Inseminated, no post-insemination amplexus day 1) were unreceptive to a new male ($P = 0.05$). This result demonstrates that insemination causes a decrease in sexual receptivity in some, but not all females.

No pairs formed on day 2 from females in group 4 (Full Courtship Controls) transferred sperm caps. Although all of the females were courted by the new males, females never raised their heads to the males' chin rubs, and males did not attempt to transfer a sperm cap. Group 4 pairs had significantly fewer attempts at sperm transfer when compared to Group 1 (No Courtship Controls; $P = 0 < 0.001$), Group 2 (Courted Only; $P < 0.001$), and Group 3 (Inseminated; $P = 0.005$). Therefore, although insemination alone blocks receptivity in some females, insemination and post-insemination amplexus together are effective in inhibiting female sexual receptivity in all

females.

Courtship that does not culminate in insemination is not sufficient to inhibit female sexual receptivity. One-hundred percent of the females in Group 2 (Courted Only on day 1) were inseminated by new males on day 2. This result is the same as Group 1 (No Courtship Control) that also had 100% receptivity among females on day 2 ($P > 0.05$), therefore indicating that courtship alone does not inhibit sexual receptivity.

Courted Only (Group 2) females had similar latencies to sperm transfer on day 1 as on day 2 (Wilcoxon matched-pairs test, $T = 9$, $P > 0.05$). This result indicates that courtship, without insemination and post-insemination amplexus, does not prime a female to future courtship, at least when subsequent courtship episodes are separated by a 12 hour interval.

DISCUSSION

The results of these studies demonstrate that not only is courtship long-lasting in T. granulosa, but a courting male also has a high probability of inseminating the female. Furthermore, once a male has successfully inseminated a female, his paternity is secured because post-insemination amplexus, a behavior that is performed after 100% of the inseminations, induces a decline in female sexual receptivity. Last, pheromonal communication apparently facilitates female sexual receptivity.

Courtship in T. granulosa

The amount of time a pair spends in courtship is ultimately a result of the costs and benefits incurred during that time. The obvious benefit of courtship is that, if it culminates in insemination, it leads to increased reproductive success. A lengthy courtship may enhance the probability of successful sperm transfer for a male salamander by giving the male more time to evaluate and enhance the receptivity of the female.

Arnold (1977) hypothesized that, in salamanders, the amount of time invested in courtship may increase the probability of successful insemination. Using data from several species, Arnold compared the amount of time spent in courtship to sperm transfer success; he found that there was a significant correlation between invested time

and the probability of successful insemination. The results presented in initial study for T. granulosa fit into this pattern. The median amount of time spent in courtship before sperm transfer for pairs of rough-skinned newts is 7 hours. To my knowledge, no other salamander species studied invests so much time in the courtship of a single female (Arnold, 1977; Halliday, 1977; Verrell, 1982; Massey, 1988), nor do any of these species have the success rate for courtship demonstrated in initial study for T. granulosa. In T. granulosa, 90% of the first spermatophores deposited by the males inseminate the females, while 93% of all courtships culminate in insemination.

The increased time investment in courtship could be adaptive if it increases the probablility of successful insemination (Arnold, 1977). First, a lengthy courtship may give a male more time to evaluate the receptive state of the female before he attempts a sperm transfer. Second, courtship may enhance the receptivity of the female, thereby increasing the probability that she will attempt to receive the sperm cap. Third, a long courtship period may help the individuals of the pair synchronize behaviors during sperm transfer. In T. granulosa all of these factors probably help to enhance the probability of insemination.

The behavior of a courted female newt appears to

signal receptivity to the male. A female is initially unreceptive to courtship and signals this state with a head down position. Soon after the initiation of amplexus, a male attempts to rub the nares of the female and performs hind limb contractions against her sides. After several hours to many days of courtship, a female will raise her head and stop moving in response to the male's chin rubs. Often at this time the female also releases an air bubble which may act as a visual or tactile signal for the male. In 90% of the cases where a female was observed to exhibit these behaviors, the males soon released the female and deposited a spermatophore. In 90% of these attempts, insemination was successful. This result suggests that the female's combined behaviors act as a signal of receptivity to the male. However, on occasion, a male releases a female from amplexus and swims away without attempting a sperm transfer, and in these cases, almost invariably, the female has remained in the head down position for the entire period of amplexus (Propper, pers. obs.).

Courtship in T. granulosa also acts to synchronize mating behaviors and to enhance female receptivity. First, there are consistent changes in female and male behaviors probably reflecting reciprocal behavioral interactions that synchronize internal and external events leading to mating. Second, the changes in female behavior

are prevented by blocking a component of male courtship: pheromonal stimulation (experiment 1). Pheromones are known to be an integral part of courtship in many species of salamander (Rogoff, 1927; Malacarne, 1984; reviewed by Arnold and Houck, 1982). Perhaps a lengthy courtship allows salamanders more time for pheromonal stimulation to enhance receptivity.

If a lengthy courtship insures insemination, why is courtship relatively short, and often unsuccessful, in many other salamander species? There are costs associated with a long courtship. One cost of courtship is an increased risk of predation. Certainly, the more time a pair spends in courtship, the more time they are exposed to predators. T. granulosa is not subject to this constraint since this species of newt is highly toxic (Brodie, 1968) and has only one known predator, the snake Thamnophis sirtalis. Therefore, while predation risk may limit the benefits of a lengthy courtship in some species, it may not be restrict courtship length for T. granulosa.

For males, another cost of a lengthy courtship is the time it takes away from searching for and courting other females. In other words, a male may increase his overall mating success by courting many females for a short peroid, even though the success per individual female may be low, instead of courting one female intensely. However, if the operational sex ratio of a breeding area

is skewed toward males, a male will increase his mating success more by courting one female until she is fully receptive, than he will by spending time finding a new female.

The operational sex ratio in T. granulosa breeding ponds is skewed towards males. At the peak of the breeding season, the sex ratio may be as high as 50 males for every female (Moore, pers. obs.). First, males enter the ponds earlier than do females, so that once females begin arriving at the ponds, most of the males are already in the water searching for a mate (Pimentel, 1960). Second, only newly arriving females are receptive when courted since, once a female mates, she is no longer receptive (experiment 2). Indeed, by the time females ovulate, they are not even attractive to males, and they can swim freely in ponds without males initiating courtship (Propper and Moore, pers. obs.). Therefore, at any time during the breeding season, there are many more sexually active males than females. Such a skewed sex ratio for T. granulosa breeding populations would favor males investing more into insuring paternity with a current partner rather than searching for another mate.

There may be advantages for a female T. granulosa to become receptive only after a long courtship period. Halliday (1977) proposed that since fitness may be reflected in a male's ability to maintain a display for

longer time periods, it would be to the female's advantage to test this ability. Besides testing the male's courtship ability, a lengthy courtship may test a male's competitive ability. In this species, once a male captures a female in amplexus, the female is unable to displace him. Apparently active female choice is not a potential mechanism for sexual selection. However, if females prolong courtship, the probability of male-male mate competition may increase. There is competition for mates in T. granulosa and many other salamander species (Pimentel, 1952; Propper, pers. obs.; Arnold, 1976; Verrell, 1983; 1984; Massey, 1988). In T. granulosa, competition takes the form of mating balls where up to 20 males may surround and wrestle for one female. Nothing is known about the chance of successful displacement of an amplectic male under these circumstances in this species, but in N. viridenscens there is a positive correlation in the number of displacement attempts made by rival males and the amount of time a pair spends in amplexus (Verrell, 1983). Therefore, female T. granulosa that remain unreceptive for longer periods may facilitate more male-male mate competition. If competition results in the female mating with the fittest male, then it is possible that the slow rise to behavioral receptivity is a selected trait.

Mechanisms to Assure Paternity in T. granulosa

Insemination of a female does not secure paternity for a male. If another male also succeeds in mating with a female, then an opportunity for sperm competition exists (Parker, 1970), and may result in a potential reduction of reproductive success for the first male. A male can decrease the probability of sperm competition by decreasing the probability of the female being inseminated by another male. A male can decrease this probability by directly guarding his mate, by blocking her genital aperture with some type of a plug, or by inhibiting her receptivity (Parker, 1970). Male T. granulosa decrease the risk of multiple insemination of their mates by using all of these mechanisms. First, post-insemination amplexus itself acts as a barrier against courtship by other males. Second, the sperm cap in the female's cloaca blocks further insemination for about four hours after insemination (initial study). Sperm plugs have been documented in snakes (Devine, 1975), rodents and insects (see Dewsbury, 1984), and have been suggested as a possible short term mechanism to prevent sperm competition in the salamander Plethodon jordani (Arnold, 1976). However, to my knowledge, this is the first documentation of a sperm plug successfully preventing insemination in a salamander.

However, the sperm plug does not act as a long-term

barrier against future insemination. Once a sperm cap has been absorbed, a female can pick up a second spermatophore. Indeed, 60% of the females that were inseminated did become receptive and were inseminated by a new male if they had not received post-insemination amplexus from their first mate.

Rough-skinned newts have a previously undocumented mechanism for preventing future inseminations by a new male: post-insemination amplexus causes a decrease in female sexual receptivity (experiment 2). Post-insemination amplexus is not merely an extension of preinsemination courtship, or simply a physical barrier to female access by a new male, it also acts to insure paternity by decreasing female sexual receptivity. To my knowledge, there are no other reports suggesting that post-insemination association with a female inhibits female receptivity.

Comparison of Mating Systems of Taricha

The hind limb contractions and chin rubs exhibited during courtship by male T. granulosa appear to be similar to the courtship behaviors described for T. torosa (Smith, 1941) and T. rivularis (Davis and Twitty, 1964). Female behaviors are less well described. As found in this study for T. granulosa, a T. rivularis, female will lift her head shortly before a sperm transfer attempt (Davis and

Twitty, 1964). This result suggests that for Taricha the raised head posture is a common signal of female receptivity.

There is variability in the amount of courtship time invested by a pair before a sperm transfer attempt. Davis and Twitty (1964) found that courtship in T. rivularis lasts only about one hour before a pair attempts to transfer a sperm cap. Courtship in T. granulosa lasts a median of seven hours before a sperm transfer attempt (initial study). It would be quite interesting to determine whether this difference in preinsemination courtship time investment between the two Taricha species is correlated with the success of insemination as is suggested by Arnold (1977). Multiple insemination appears to be much more common in T. rivularis (Davis and Twitty, 1964) than it is in T. granulosa. T. rivularis females will pick up a sperm cap from a new male as early as one hour after receiving a previous one. In T. granulosa, it is not physically possible for a female to receive a second sperm cap so soon after insemination since the first cap effectively blocks the female's cloaca. This result suggests that either there is a difference in the sperm cap between the two Taricha species, or female T. rivularis can absorb the sperm cap faster than T. granulosa. Although T. rivularis males also recapture females in amplexus after insemination,

this behavior does not appear to influence female receptivity since a female remains receptive even after a male has released her. Indeed, Davis and Twitty (1964) reported a decline in female sexual receptivity as the breeding season progresses, but they also mention finding fresh sperm caps in the cloaca of females that have already ovulated. In T. granulosa, I have never found a sperm cap in the cloaca of a female with oviducal eggs, nor have I found oviducal eggs in any female that was being courted (Chapter 5). These results suggest that while the courtship behavior of Taricha appears to be similar among the three species, their overall reproductive biology is different in several respects.

In summary, courtship in T. granulosa lasts longer than courtship in any other species of salamander studied. The probability of courtship leading to insemination is higher in this species than is reported for any other salamander. Furthermore, pheromones are important to the induction of female receptivity in T. granulosa. Last, not only does a sperm plug block insemination by a new male, but post-insemination clasping in T. granulosa also prevents other males from courting a female and insures paternity by inducing a decline in female sexual receptivity.

Table 2.1. Behaviors of male and female rough-skinned newts during courtship. Please see text for a full description of behaviors.

Behavior	Period of Courtship*			
	Initiation of courtship	1/2 Time to sperm transfer	20 min. before sperm transfer	20 min. after sperm transfer
Median female head orientation score	0 [a]	1 [b]	2 [c]	--
Median # hindlimb contractions/ min. by male	2 [a]	14 [b]	29 [c]	0 [a]
Median # rubs/min.	2.5 [a]	3.5 [b]	3 [a]	0 [c]

*For each behavior, values followed by different superscripts are significantly different across courtship period.

Table 2.2. Percent pairs succeeding at transferring a sperm cap in each treatment group at 12 hours and 24 hours after the initiation of courtship.

		Percent Pairs with Successful Sperm transfer*	
Treatment	N	12 Hours	24 hours
Untreated Control	20	45% [a]	85% [a]
Unilateral Cover	7	42% [a]	86% [a]
Bilateral Cover	13	0% [b]	0% [b]

*Superscripts of different letters indicate significant differences between treatments within a time period.

Table 2.3. Percent pairs attempting a sperm transfer in intact nares cover, unilateral naris cover, and bilateral nares cover treatment groups by 12 hours and 24 hours after the initiation of courtship.

		Percent Pairs Attempting Sperm transfer*	
Treatment	N	12 Hours	24 hours
Untreated Control	20	55%	95% [a]
Unilateral Cover	7	42%	86% [ab]
Bilateral Cover	13	38%	38% [b]

*Superscripts of different letters indicate that there are significant differences between treatments within a time period.

Table 2.4. Male-typical behaviors directed toward females during courtship. There was no significant difference (Kruskall-Wallis) due to covering the nares of females in the number of nares rubs or the number of palpitations a female received.

	Male Behavior	
Treatment	Median # Chin Rubs/ Minute	Median # Palpitations/ Minute
Untreated Control	3.5	13
Unilateral Cover	4.0	10
Bilateral Cover	3.5	9

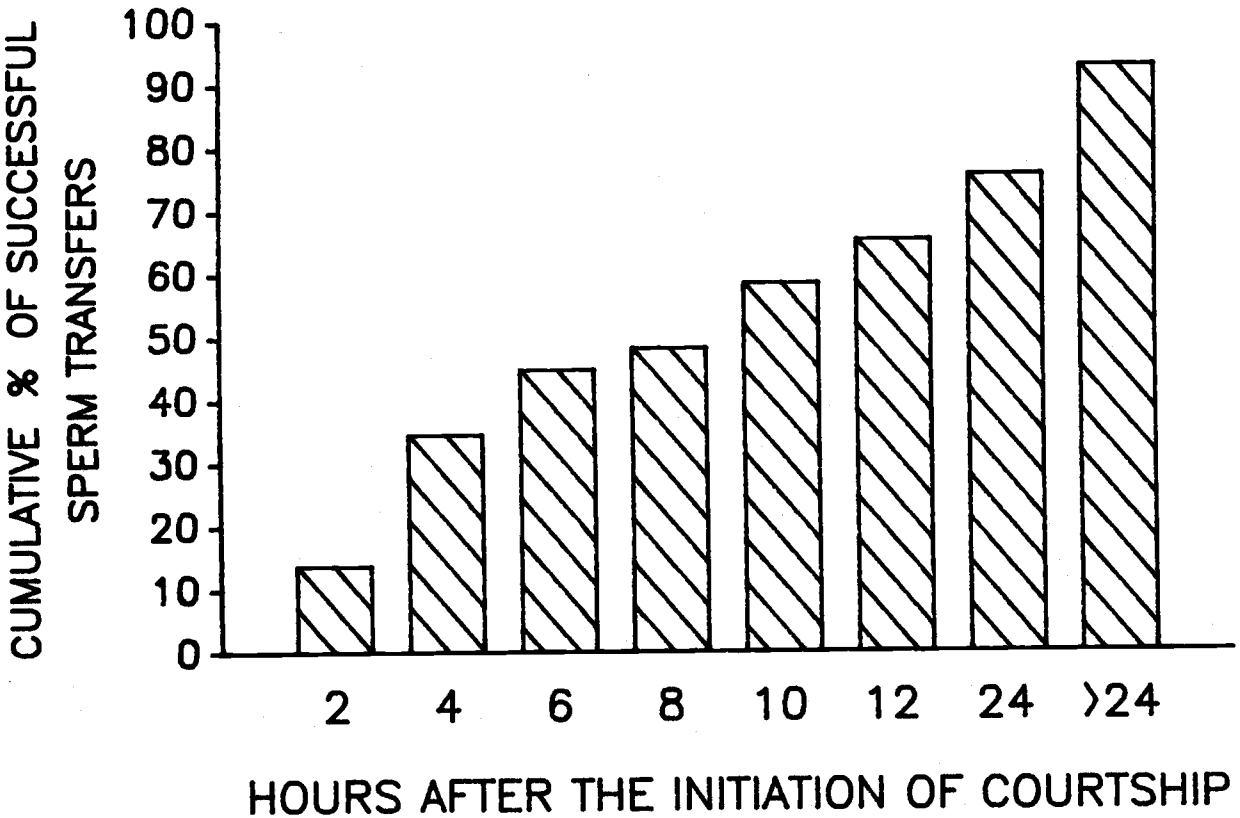
Table 2.5. Effect of courtship, insemination and post-insemination clasping on female sexual receptivity.

Treatment Day One	N	% Females Receptive to New male on Day 2*
No courtship No insemination No post-insemination clasping	9	100% [a]
Courtship No insemination No post-insemination clasping	9	100% [a]
Courtship Insemination No post-insemination clasping	10	60% [b]
Courtship Insemination Post-insemination clasping	10	0% [c]

*Superscripts of different letters indicate significant differences between treatments.

Figure 2.1. Cumulative percent of successful sperm transfers for pairs of T. granulosa. The median time from the initiation of courtship until sperm transfer is 7 hours.

Figure 2.1


CUMULATIVE % OF SUCCESSFUL SPERM TRANSFERS
100
90
80
70
60
50
40
30
20
10
0
2
4
6
8
10
12
24
>24
HOURS AFTER THE INITIATION OF COURTSHIP

CHAPTER 3: EFFECTS OF COURTSHIP ON BRAIN LUTEINIZING HORMONE-RELEASING HORMONE AND PLASMA STEROID CONCENTRATIONS IN FEMALE *TARICHA GRANULOSA*

INTRODUCTION

Courtship involves a series of reciprocal behavioral interactions. In many species, a male directs behaviors toward a female, and the female either does or does not respond. If the female responds appropriately, the male may, in turn, perform the same or another behavior involved in the courtship ritual. These behavioral exchanges continue until insemination or the potential mate is rejected. For many species, without these reciprocal cues of courtship, the necessary synchronization for mating would not be possible.

The behavioral cues of courtship, in the form of visual, tactile, olfactory, auditory, and sometimes electrical stimuli, are processed by the central nervous system (Kelley, 1981). These signals are transduced into specific neurochemical signals which trigger appropriate behavioral responses by the partner. Little is understood about how the ritualized behavioral exchanges of courtship influence physiological changes in the brain and the rest of the body.

Many salamander species have courtship rituals that demonstrate clearly a series of behavioral exchanges that lead to insemination (Arnold, 1972; 1976; 1977; Halliday,

1977; Verrell, 1982; Chapter 2), The rough-skinned newt, Taricha granulosa, makes an excellent model to investigate the functional relationship between courtship-induced changes in reproductive physiology and female sexual behavior. Courtship in T. granulosa follows a consistent pattern of behavioral transitions (Chapter 2). When first captured in a dorsal amplectic clasp, a female exhibits unreceptive behaviors by arching her back, moving her head down, and making several vigorous attempts to free herself. The male attempts to rub his submandibular gland over the female's nares and contracts his hind limbs against the female's abdomen and cloaca. If the female continues the unreceptive display for a long time, the male releases her and swims away without depositing a spermatophore (Propper, pers. obs.). If the female stops moving her head away from the male, the frequency of the male's hind limb contractions increases (Chapter 2). After several hours, the female signals that she is receptive by raising her head, remaining motionless in response to the male's chin rubs, and releasing an air bubble (Chapter 2). The male releases her and places a spermatophore on the substrate. The female picks up the sperm cap in her cloaca. The male turns and recaptures the female in amplexus, and after several hours, the female again signals that she is unreceptive. Eventually, the male releases his mate and swims away

(Propper, pers. obs.). In summary, within 24 hours the female's behavioral responses to male courtship change from unreceptive to receptive and then, back to unreceptive. The question remains: what are the neuroendocrine mediators of these behavioral changes?

The hormones of the hypothalamus-pituitary-gonad axis are well known for their involvement in reproductive behaviors. In particular, the ovarian steroid hormones, estradiol and progesterone, influence female sexual receptivity in many mammals (Boling and Blandau, 1939; reviewed in Feder, 1984; Arnold and Breedlove, 1985; Pfaff and Schwartz-Giblin, 1988), the ring dove, Streptopelia risoria (Cheng, 1973a, 1973b), the lizards, Anolis carolinensis (McNicols and Crews, 1979; Tokarz and Crews, 1980; Wu et al., 1985) and Eumeces laticeps (Cooper et al., 1986), the frog Xenopus laevis (Kelley, 1982), and the guppy, Poecilia reticulata (Liley, 1968). The neuropeptide, luteinizing hormone-releasing hormone (LHRH) also induces female sexual receptivity in rats (Moss and McCann, 1973; Pfaff, 1973), S. risoria (Cheng, 1977), A. carolinensis (Alderete et al., 1981), and X. laevis (Kelley, 1982). The behavioral actions of these hormones make them candidates to act as chemical mediators of courtship-induced receptivity in female rough-skinned newts.

Although studies on the endocrine control of female

sexual receptivity in amphibians are limited, there is some correlative and experimental evidence suggesting a relationship between specific hormones and sexual receptivity. Seasonal studies found that plasma estradiol and androgen concentrations are high during the breeding season in female Rana catesbeiana (Licht et al., 1983), Pachymedusa dacnicolor (Iela, et al., 1986), Pleurodeles waltl (Garnier, 1983) and Euproctus asper (Clergue et al., 1985). Estradiol and progesterone have been found necessary for the expression of female sexual receptivity in X. laevis (Kelley, 1982). However, in R. pipiens, ovariectomy does not block female sexual receptivity, and replacement treatments with estradiol and/or progesterone fail to facilitate receptivity (Diakow et al., 1978). These studies indicate that there are species differences in the effects of plasma steroids on receptive behaviors in anuran amphibians. To the best of my knowledge, no study has examined the effects of any hormone on sexual receptivity in female urodele amphibians.

Luteinizing hormone-releasing hormone also is involved in amphibian reproductive behaviors. In the presence of estradiol and progesterone, LHRH induces female sexual receptivity in X. laevis (Kelley, 1982), and in the male rough-skinned newt an injection of LHRH induces sexual behavior (Moore et al., 1982).

Previous research (Chapter 2) indicates that male

courtship behaviors induce sexual receptivity in T. granulosa females. Such behavioral changes are probably mediated by, as yet undiscovered, changes in the neuroendocrine system. Since estradiol, progesterone and LHRH affect sexual behavior in many vertebrates, including amphibians, these hormones are likely chemical mediators of the observed behavioral changes in T. granulosa females. If male courtship behaviors induce female sexual receptivity through these hormones, then changes in female behaviors may be correlated with endogenous changes in one or more of these hormones.

In T. granulosa, not only does courtship induce receptivity, but post-insemination behaviors cause a decline in female sexual receptivity (Chapter 2). Mating-induced suppression of female sexual receptivity has been reported. Guinea pigs (Zucker and Goy, 1967; Goldfoot and Goy, 1970), hamsters (Carter and Schein, 1971), and other rodents (reviewed by Feder and Marrone, 1977), as well as several nonmammalian vertebrates and insects (see Valenstein and Crews, 1977) exhibit a post-mating decline in sexual receptivity. In guinea pigs the post-mating decline in receptivity may be due to a "biphasic" response to progesterone (Zucker, 1968). Progesterone, in conjunction with estradiol, initially induces receptivity, but after mating progesterone apparently mediates the post-mating decline in receptivity. Valenstein and Crews

(1977) have also suggested that progesterone may play a role in the post-mating decline in receptivity seen in A. carolinensis. These results suggest that an increase in progesterone secretion may be responsible for the post-mating decline in female sexual receptivity.

The steroid hormone, corticosterone, has been shown to decrease male sexual behavior in T. granulosa: mild stress or a single injection of corticosterone inhibits sexual behavior in male T. granulosa (Moore and Miller, 1984). If corticosterone has similar actions in female newts, it is possible that the post-mating decline in female sexual receptivity is mediated by an increase in plasma corticosterone concentrations.

The purpose of this study was to learn whether courtship induces changes in brain LHRH concentrations and plasma estradiol, progesterone, or corticosterone concentrations. These hormones were measured by radioimmunoassay in female T. granulosa at different behavioral and reproductive stages during and after courtship.

METHODS AND MATERIALS

General Methods

Animals: Females were collected in pit traps along a drift fence paralleling a small pond at Soap Creek Ponds, 12 miles North of Corvallis, Benton County, Oregon. This collection procedure insured that females had not previously mated during the current season.

Until the day of the experiment females in experiment 1 were kept in a small outdoor artificial pond in Corvallis. Females for experiment 2 were housed in an environmentally-controlled room with a simulated natural photoperiod and a temperature of 5 - 10°C. Females were regularly fed earthworms and mealworms.

Males were collected from Soap Creek Ponds and were transported to the laboratory the day before the experiment. Males were placed one or two per aquarium. Each aquarium contained 20 liters of water and was housed in an environmentally-controlled room with a simulated natural photoperiod and ambient temperature. The night before the experiments, females were placed 10 per holding tank that contained 10 liters of water.

Initial Tissue Preparation: Females were sacrificed by decapitation. Their heads were heated in a water bath at 60°C for 10 minutes, and then placed on ice. Within 1

hour, the brains were removed, frozen separately in O.C.T. Compound (Tissue Tek), and stored at -80°C until sectioning. Trunk blood was collected in tubes containing 0.05 cc 3% heparin in amphibian Ringers, and stored on ice until centrifugation at 1200 g for 15 minutes. After centrifugation, the plasma was removed and stored at -80°C until the steroids were extracted, chromatographed, and assayed by radioimmunoassay.

Preparation of Brain Tissue for Peptide Assays: The microdissection-punch technique used was that of Palkovitz and Brownstein (1982) as modified by Zoeller and Moore (1985). Briefly, brains were sliced at 100 um in a cryostat at -14°C and the sections were thaw-mounted to microscope slides. Specific brain regions were removed using a punch needle modified from a stainless-steel hypodermic needle with an i.d. of 350 um. Punches of individual brain regions were placed in 300 ul ice cold 1N acetic acid in polypropylene test tubes and sonicated for 20 seconds. Each sample was vortexed for 10 seconds and centrifuged at 21,000 g for 20 minutes. Two hundred fifty microliters of the supernatant was removed, lyophilized, and stored at -80°C until immunoreactive LHRH (irLHRH) was measured by radioimmunoassay (RIA). The remaining 50 ul of supernatant and the pellet from centrifugation were dried under air at 40°C and stored frozen (-20°C) until

assayed for protein content using a modified Bradford assay (Bradford, 1976).

Bradford Assay: Two hundred microliters 0.1N NaOH were added to the dried protein samples tubes, which were then vortexed and allowed to stand at room temperature for at least 15 min. Five hundred microliters concentrated Bradford Reagent was added to the tubes and samples were read on a spectrophotometer at OD 595 within 1 hour of adding the Bradford Reagent. The Bradford Reagent consisted of 200 mg Coomassie brilliant Blue G-250 dissolved in 50 ml 95% ethanol. To this solution, 10 ml of Phosphoric Acid, 85% w/v, was added followed by 840 ml distilled, deionized water. This solution was vortexed for 1 hour and filtered through Whatman #1 filter paper. The reagent gave consistent results for 2 weeks.

Luteinizing Hormone-Releasing Hormone (LHRH) RIA: The assay procedures were the same as in Zoeller and Moore (1985). The antiserum used for experiment 1 was R722 (supplied by Dr. A. Arimura) at a final dilution of 1:300,000 in phosphate-buffered saline with gelatin. For experiment 2 the antibody was EL-14 (supplied by Dr. W. Ellenwood) at a final dilution of 1:1,500,000 in the same buffer. The EL-14 antiserum is known for its specificity to the mammalian form of LHRH (Ellenwood *et al.*, 1985) which is the form found primarily in *T. granulosa*

(Sherwood et al., 1986). To validate the E1-14 antibody, two whole salamander brains were homogenized in 1 N Acetic Acid, spun at 21,000 g for 20 minutes, and the supernatant was lyophilized and reconstituted in assay buffer. A five point serial dilution of this extract was found to be parallel to an LHRH standard curve.

For all LHRH assays, standards were prepared in triplicate using synthetic LHRH (Sigma) at concentrations from 2 to 200 pg. The label was ^{125}I-LHRH (New England Nuclear). Standards and samples were incubated for 24 hours at 4°C in antiserum before 4500 cpm of ^{125}I-LHRH (New England Nuclear) was added. All tubes were incubated for another 72 hours at 4°C. Bound from free label was separated by the addition of 1.5 ml of ice cold 95% ethanol to all standards and samples, which were then centrifuged at 1500 g for 10 minutes. The supernatant was decanted, the tubes dried, and radioactivity counted in a Beckman 5500 Gamma Counter. The specific binding for the assay was 31.8%, and the sensitivity was 1.6 pg. The intra- and interassay coefficients of variation were 10% and 20% respectively.

Steroid Extraction and Chromatography: Progesterone, corticosterone, and estradiol were separated from extracted plasma using LH-20 column chromatography after the method of Archambault, Begue, Faure, and Gandin

(1984). Briefly, 75 ul of plasma was spiked with 1000 cpm of each steroid in 25 ul of ether. Plasma steroids were extracted in 2 x 1 ml ethyl-ether, and the ether extracts were dried. LH-20 columns (Isolab, QS-44) were prepared by pouring 10 ml of solvent (Chloroform:Heptane:Methanol, 5:5:1) through the columns. Samples were dissolved into 100 ul of the solvent and loaded onto the columns. Two hundred microliters additional solvent was added to the sample tubes, vortexed, and loaded onto the columns. Separation of the steroids was accomplished using the same solvent. Solvent volumes 1.5 to 3 ml contained the progesterone fraction; volumes 3.5 to 6.0 ml contained corticosterone; volumes 7 to 11 ml contained estradiol. Steroid samples were dried under air and stored at -20^{o}C until assayed (within 48 hours).

Steroid Radioimmunoassay:

Progesterone: Progesterone was measured as described in Moore, et al. (1979). The antiserum used was #337 (from Dr. G. Niswender) at a final dilution of 1:4000. Standards were made in duplicate (4 to 1000 pg). Standards and samples were incubated in 200 ul or 300 ul of assay buffer, respectively, and placed on an orbital shaker at 250 rpm for 10 minutes. From each sample, 100 ul of buffer was removed to determine recovery. To the remaining sample, 7000 cpm/100 ul buffer of ^{3}H-[1,2,6,7]-

progesterone (Amersham) was added. All samples, for each experiment, were assayed together. Tubes were incubated for 90 minutes, and bound was separated from free using dextran-coated charcoal in phosphate-buffered saline. All samples were run in the same assay. The specific binding was 36.1%, sensitivity was 13.4 pg/tube (0.18 ng/ml), and the intraassay coefficient of variation was 11.6%.

Corticosterone: Corticosterone was measured as described in Zoeller and Moore (1985). The antiserum used was #337 (supplied by Dr. G. Niswender) at a final dilution of 1:4000. The label was ^{3}H-[1,2,6,7]- corticosterone at 10,000 cpm/tube in a final dilution of 300 ul. The assay was otherwise the same as for the progesterone assay. Standards ranged between 4 and 1000 pg/ tube. All samples for each experiment were run in the same assay. Specific binding was 21.8%, sensitivity was 4.25 pg/tube (0.06 ng/ml), and the intraassay coefficient of variation was 4.4%.

Estradiol: The antiserum used in this assay was anti-estradiol #277 (supplied by Dr. G. Niswender) at a final dilution of 1:10,000 (experiment 1) or 1:100,000 (experiment 2). Standards ranged from between 4 and 1000 pg/tube, and all tubes were incubated in 10,000 cpm ^{3}H-[2,4,6,7,16,17]-estradiol (Amersham). Serial dilutions of plasma showed parallelism with the standard curve. The

assay was conducted as the other steroid assays, and all samples for each experiment were measured in one assay. In experiment 1 the intra-specific coefficient of variation was 6%, sensitivity was 57 pg/tube (.76 ng/ml), and specific binding was 75%. In experiment 2, the specific binding was 35.1%, the sensitivity was 1.7 pg (0.02 ng/ml), and the intraassay coefficient of variation was 6%.

Statistics: All data were analyzed using a Two-way Analysis of Variance. Data were log or square root transformed when necessary to remove heteroscedasticity. When an ANOVA showed a significant interaction effect, data were further analyzed using a Fisher's Least Significant Difference Test. Significance for the two-tailed test was set at $P = 0.05$.

Experiment 1: The Effects of Courtship on Brain Peptide and Plasma Steroid Concentrations

Female newts at the initiation of courtship exhibit unreceptive behaviors (see Chapter 2). After several hours of courtship, however, females' behaviors change: they exhibit sexual receptivity. Several hours after insemination and post-insemination amplexus females become sexually unreceptive. The purpose of this study was to determine if these changes in female receptive behaviors

were associated with changes in brain LHRH or plasma steroid concentrations.

Experimental Design: Females were collected between February 26, 1985 and April 7, 1985. On the day of the experiment, all females were brought into the laboratory and placed into aquaria, one per aquarium. One half of the aquaria contained males as described in the general methods. Each female in a tank with a male was assigned to one of the following treatments and sacrificed by decapitation at the end of the treatment time: group 1, courted by a male for 3 minutes (N = 5); group 2, courted by a male until sperm transfer (N = 10); group 3, courted by a male until sperm transfer and allowed to remain with the male until 24 hours after courtship initiation (N = 4); and group 4, courted until sperm transfer, allowed to remain with the male until 24 hours after courtship initiation at which time the females were placed individually into a five gallon bucket with 10 liters of water and a platform for leaving the water. Females in group 4 were left for 2 weeks (N = 10). Time-matched controls were unpaired females that were sacrificed at the same time as each courted female. There was a total of 8 groups: four groups with females paired to males, and four time-matched control groups of unpaired females. Brains were collected for regional LHRH concentration

determination, and plasma samples were collected for progesterone, estradiol, and corticosterone concentration determination.

Brain Regions and Peptides Assayed: Immunoreactive LHRH (irLHRH) was measured in the ventral hypothalamic nucleus, ventral preoptic area, anterior telencephalon, infundibulum, and the olfactory nerves. Anatomical descriptions of brain regions are from Herrick (1948). All samples from a given brain region were analyzed in a single assay.

Experiment 2: Changes in Brain Peptide and Plasma Steroid Concentrations During the Early Stages of Courtship

Since the results of experiment 1 demonstrated that irLHRH concentrations in the anterior telencephalon of female newts change during courtship, this study was conducted to replicate experiment 1 and to determine whether these changes were occurring in the nervus terminalis, a major LHRH system in the telencephalon of vertebrates (Demski and Schwanzel-Fukuda, 1987).

Experimental Design: Females were collected between February 7, 1987 and February 24, 1987, and maintained in a large tank in the laboratory. The light:dark cycle was adjusted regularly to approximate natural photoperiod, and the temperature was maintained between 5 - 10^{o}C. All

animals were fed earthworms and mealworms. Females were placed one per aquarium into aquaria the night before the experiment. At the initiation of the experiment, males were placed, one per tank, into one-half of the aquaria containing females. Once courtship began, females were assigned to one of the following treatments where courtship allowed for: group 1, 5 minutes of courtship; group 2, 20 minutes of courtship; or group 3, 60 minutes of courtship (N = 10 for all treatments). In order to determine if the number of chin rubs was correlated with irLHRH concentration in the nervus terminalis, the number of chin rubs a female received during the treatment period was quantified by direct observation. At the end of the assigned courtship time females were separated from males and sacrificed. Brain tissue was prepared as above, and plasma was collected for estradiol, progesterone, and corticosterone concentration determination. Time-matched control females, females not exposed to courtship, were sacrificed at the same time as each courted female. There was a total of six treatment groups: three groups with females paired to males, and three time-matched control groups.

Brain Regions Assayed: The nervus terminalis and the infundibulum were assayed for irLHRH.

RESULTS

Experiment 1:

Effects of Courtship on Brain irLHRH Concentrations:

Exposure of females to male courtship behaviors influenced brain irLHRH concentrations. In the anterior telencephalon-olfactory bulb region, concentrations of irLHRH were initially high in courted females, but decreased by the time of sperm transfer (Fig. 3.1A).

There was no effect of courtship on irLHRH concentrations in any other brain region (Figs. 3.2A, 3.2B) including the infundibulum (Fig. 3.1B). This result indicates that the changes in the anterior telencephalon are regionally specific. There were no changes in irLHRH concentrations along the main LHRH pathway to the pituitary.

Effects of Courtship on Plasma Steroid Concentrations:

Courtship stimulated a change in plasma estradiol concentrations. Between the initiation of courtship and sperm transfer estradiol concentrations rose significantly (Fig. 3.3A). By 24 hours after the initiation of courtship, estradiol concentrations were significantly lower than they had been at sperm transfer, but they still remained elevated above unmated controls. By two weeks after courtship, there was no difference in plasma

estradiol concentrations between mated and unmated females. Male courtship behaviors had no effect on plasma progesterone or corticosterone concentrations in females (Fig. 3.3B and 3.3C).

Experiment 2:

Effects of Courtship on Brain irLHRH Concentrations:

Exposure to male courtship behaviors affected irLHRH concentrations in the nervus terminalis. Immunoreactive LHRH levels were higher in courted females than in uncourted controls (Fig. 3.4A), as was found in the entire anterior telencephalon in experiment 1. Furthermore, as in experiment 1, courtship had no effect on irLHRH concentrations in the infundibulum (Fig. 3.4B). This result again demonstrates that the effect of male courtship behaviors on irLHRH concentration is regionally specific.

There was no correlation between the number of chin rubs a female received and irLHRH concentrations in the nervus terminalis in any of the courted treatment groups (Regression analysis: $P > 0.1$ for all comparisons) suggesting that these courtship-induced changes in irLHRH concentrations are not mediated by male pheromones.

Effects of Courtship on Plasma Steroid Concentrations:

Also as found in experiment 1, male courtship behaviors influenced circulating concentrations of estradiol (Fig 3.5A). Estradiol was elevated significantly, compared to controls, at 20 minutes after the initiation of courtship. There was no significant effects of courtship on plasma concentrations of progesterone or corticosterone (Fig. 5B and Fig. 5C).

DISCUSSION

This study provides the first evidence in any vertebrate for courtship-induced changes in female sexual behavior being correlated with changes in neuropeptide concentrations in the brain. Telencephalic irLHRH concentrations in females tend to be elevated during the early stages of courtship, which is when females are unreceptive. The irLHRH concentrations in the anterior telencephalon decrease dramatically by the time a female exhibits receptive behavior. The specific location of these changes in irLHRH concentration is the nervus terminalis, a cranial nerve that contains LHRH in many vertebrates (Demski and Schwanzel-Fukuda, 1987), including amphibians (Muske and Moore, 1987; Wirsig and Getchell, 1986). The function of the nervus terminalis is poorly understood (Demski and Schwanzel-Fukuda, 1987).

Concentrations of irLHRH in the nervus terminalis are higher in courted females than in controls during the first stages of courtship. Although previous studies have provided some evidence that the nervus terminalis has a reproductive function (Doving and Solset, 1980; Schwanzel-Fukuda and Silverman, 1980; Demski and Northcutt, 1983; Stacey and Kyle, 1983; Demski and Dulka, 1984; Satou et al., 1984; Wirsig and Leonard, 1987), the observed changes in irLHRH concentrations in the nervus terminalis of

female newts represents the first documentation of physiological changes in the nervus terminalis in response to naturally occurring reproductive stimuli.

Changes in irLHRH concentrations in the nervus terminalis may have behavioral significance. In a number of vertebrates, LHRH injections activate female sexual receptivity (Moss and McCann, 1973; Pfaff, 1973; Cheng, 1977; Alderete et al., 1980; Ward and Charlton, 1981; Kelley, 1982; Kendrick and Dixson, 1985b). Furthermore, LHRH injections increase male sexual responsiveness in T. granulosa (Moore et al., 1982). The correlation between the changes in irLHRH concentration in female T. granulosa and the courtship-induced change in sexual receptivity suggest that LHRH may be having a behavioral role in female sexual behavior. However, a single intracerebroventricular injection of LHRH or LHRH analog had no effect on female sexual receptivity (Chapter 4). Nevertheless, it is possible that, since the site of injection was the third ventricle, the exogenous LHRH failed to reach target cells of the nervus terminalis. There is evidence that, in the nervus terminalis of sharks, an efferent current runs from the ganglion, through the olfactory nerve, and toward the olfactory epithelium (Bullock and Northcutt, 1984). Furthermore, in mice (Jennes, 1987), the nervus terminalis has projections directly into the nasal epithelium, and, in most other

vertebrates including T. granulosa (Muske and Moore, 1987), it is likely that this nerve has direct olfactory connections (Silverman, 1988).

Because there is an obvious anatomical connection between the nervus terminalis and the olfactory system, Demski and Northcutt (1983) proposed that responses to pheromonal stimuli are activated by the nervus terminalis. However, a few of lines of evidence suggest that pheromones do not influence the nervus terminalis. First, in T. granulosa, in spite of evidence in support of pheromonal activation of female sexual behavior (Chapter 2), there was no correlation between the degree of male pheromonal application and irLHRH concentrations in the nervus terminalis of a female. Furthermore, recent research in goldfish demonstrates that there is no electrophysiological response in the nervus terminalis to sexual pheromones, while there is a response to tacrks, several ch electrical activity in the nervus terminalis, whereas water movement over the lateral line does affect an electrical response (Bullock and Northcutt, 1984). The results of these studies do not eliminate the possibility of a pheromonal connection to LHRH in the nervus terminalis. Instead of pheromonal application causing changes in the nervus terminalis, changes in the nervus

terminalis may affect nasal epithelium sensitivity to pheromones. For example, LHRH from the nervus terminalis released in the nasal epithelium may modulate sensitivity to odors and pheromones in T. granulosa.

Fibers of the nervus terminalis project to the medial septal nucleus, and from the medial septal nucleus, through the hypothalamus and infundibulum to the median eminence (Muske and Moore, 1987). These fibers may affect behavior by acting on target neurons along this pathway or they may lead to the release of LHRH in the median eminence to stimulate gonadotropin secretion. The gonadotropins, in turn, would influence the synthesis and release of ovarian steroids. However, although there are changes in plasma estradiol concentrations in courted female newts, there are no courtship-related changes in irLHRH concentrations in the pathways leading to the median eminence. This finding suggests that the elevation of plasma estradiol concentrations seen in females during courtship is not the result of hypothalamic LHRH stimulating luteinizing hormone release into the circulation. The anatomical location of the nervus terminalis suggests a couple of other mechanisms through which LHRH may effect the pituitary and subsequent estradiol release. Many of cell bodies of the nervus terminalis are found on the ventral surface of the telencephalon, in the subarachnoid space (Muske and Moore,

1987). Also, there is evidence from mice (Jennes, 1987) and other mammals (Silverman, 1988) that irLHRH-containing neurons may be terminating on capillaries. The nervus terminalis may stimulate release of luteinizing hormone from the pituitary by releasing LHRH into the cerebral spinal fluid or the circulatory system. Luteinizing hormone would then affect ovarian release of estradiol.

A correlation between courtship-induced changes in sexual receptivity and estradiol concentrations also has never been documented before in vertebrates. Estradiol concentrations in courted females were the same as controls at courtship initiation when females are unreceptive. By the time a female is receptive, however, the concentrations had risen above control levels, and estradiol remains elevated for at least 12 hours after insemination. By two weeks after the initiation of the experiment, mated females had lower estradiol concentrations than did unmated females. Courtship, therefore, appears to trigger changes in plasma estradiol concentrations, changes that persist long after courtship ends.

As with LHRH in the nervus terminalis, the role of the courtship-induced changes in estradiol concentrations also remains to be determined. Estradiol is known to induce female sexual receptivity in many vertebrate species (for reviews see Feder, 1984; Arnold and

Breedlove, 1985; Pfaff and Schwartz-Giblin, 1988). However, in anuran amphibians, the effects of steroids on sexual behavior appear to be species-dependent. In X. laevis, estradiol and progesterone are necessary for the expression of female receptivity (Kelley, 1982), but in R. pipiens, neither steroid is essential for the induction of receptivity (Diakow et al., 1978; Diakow and Ramondi, 1981). There are no published reports of the effects of estradiol on reproductive behaviors in female urodeles. In T. granulosa, ovariectomy does not eliminate, and estradiol does not enhance, courtship-induced receptive behaviors (Chapter 4). These negative results with estradiol treatment in female newts suggest that the observed changes in endogenous estradiol during courtship may not mediate the courtship-induced increase in female receptivity in T. granulosa.

The post-mating decline in receptivity in T. granulosa also is correlated with plasma steroid levels. The fact that estradiol concentrations remain elevated above controls for at least 12 hours after insemination, suggests that elevated estradiol concentrations may act to decrease sexual receptivity in female T. granulosa (Chapter 2). However, in many other species, estradiol has been shown to enhance, not inhibit female receptivity (reviewed by Feder, 1984; Arnold and Breedlove, 1985; Pfaff and Schwartz-Giblin, 1988). Furthermore, female

rough-skinned newts implanted with estradiol remain receptive, demonstrating that estradiol alone does not inhibit sexual behaviors of female newts (Chapter 4).

Estradiol may act in concert with other hormones to mediate sexual behavior. Estradiol in conjunction with progesterone affects a decrease in receptivity under some treatment regimes in some mammals (Zucker and Goy, 1967; reviewed by Feder and Marrone, 1977; Pfaff and Schwartz-Giblin, 1988), and in T. granulosa, progesterone inhibits sexual behavior both in the presence and in the absence of estradiol (Chapter 4). However, there were no observed changes in plasma progesterone concentrations in courted females. This result suggests that progesterone may not influence female sexual behaviors.

There are several lines of evidence that do suggest that in combination with estradiol or alone, endogenous progesterone may affect the mating-facilitated decrease in receptivity. The fact that, in female newts, progesterone concentrations were not observed to change during or after courtship does not prove that the endogenous steroid has no effect on receptivity. First, because of the sampling schedule, a transient rise in progesterone may have been missed. Indeed, Moore et al. (1979) found differences in plasma progesterone concentrations between mated and unmated female T. granulosa, but only when females were sampled at very specific times after courtship. Second,

changes in sensitivity to progesterone, and not changes in progesterone concentrations, may act to inhibit sexual behavior. If the rise in estradiol, seen in courted female newts, sensitizes the brain to progesterone by increasing progestin receptors, as reported for rats (MacLusky and McEwen, 1980), then progesterone can inhibit behavior through increased receptor availability without any increase in progesterone in the plasma. It remains to be determined whether the elevated estradiol concentrations found during courtship interact with progesterone to facilitate the mating-induced decline of sexual receptivity in T. granulosa.

Although corticosterone may provide another means for blocking courtship-induced receptivity in previously mated females, there is no evidence in support of such a role for this steroid. However, this hormone inhibits male T. granulosa sexual behavior (Moore and Miller, 1984), and may be partly responsible for the courtship-induced decline in female sexual behavior. There was no change in plasma corticosterone concentrations in females at any time during courtship, but as with progesterone, it is possible that mating caused undetected, transient changes in corticosterone concentrations or sensitivity that then influenced behavior. Further investigations into the endocrine mediation of the mating-induced decline of receptivity may yet demonstrate a role for estradiol,

progesterone, and corticosterone.

The exciting finding that irLHRH in the nervus terminalis changes during courtship suggests a proximate model for how male-female courtship interactions affect the dramatic changes seen in female sexual receptivity in the rough-skinned newt. When a male first captures a female in amplexus, she is sexually unreceptive. The hind limb contractions provide a tactile stimulus that, along with other possible visual and olfactory stimuli, may cause an increase in irLHRH concentrations in the nervus terminalis. Since this change is so rapid, it is probably mediated through post-translational modification of the pro-hormone for LHRH, and not through de novo synthesis. Alternately, the increase in irLHRH concentration could be the result of a decrease in LHRH release from the nervus terminalis.

As the male continues to court the female, LHRH in the nervus terminalis may be transported to specific, extrahypothalamic brain areas where it would effect the females behavior directly. As well, LHRH from the nervus terminalis could be transported to the olfactory epithelium where it may influence the female's sensitivity to pheromonal stimulation. The pheromones may then activate other neurological pathways and further facilitate receptive behavior.

Once the female has mated, other physiological

changes may be responsible for the decline in receptivity. Changes in estradiol concentrations could interact with progesterone, corticosterone, or any number of neuropeptides to insure that the female becomes, and remains, unreceptive to courtship.

Courtship in any animal is a series of behavioral interactions that often culminate in reproduction. The behavioral interactions induce changes in each member of a courting pair: behavioral changes which are probably mediated by changes in neuroendocrine state. The results of this study demonstrate that courtship mediated changes in female T. granulosa receptive behavior are paralleled by changes in irLHRH concentrations in the brain and specifically in the nervus terminalis. Not only do these results represent the first reported changes in sexual behavior correlated with neuropeptide content in the brain, but this study also provides the first evidence that irLHRH in the nervus terminalis responds to naturally occurring stimuli.

Figure 3.1. Immunoreactive LHRH concentrations in the A) anterior telencephalon (AT) and B) infundibulum (INF) during and after courtship. The ANOVA gave a significant interaction in the AT ($F_{(3, 48)} = 3.5$; $P = 0.02$) and a significant effect of time in the INF ($F_{(3, 48)} = 4.9$; $P < 0.01$). The * indicates a significant difference between mated females at 3 minutes after the initiation of courtship and at sperm transfer and between mated and control treatments 2 weeks after courtship initiation.

Figure 3.1

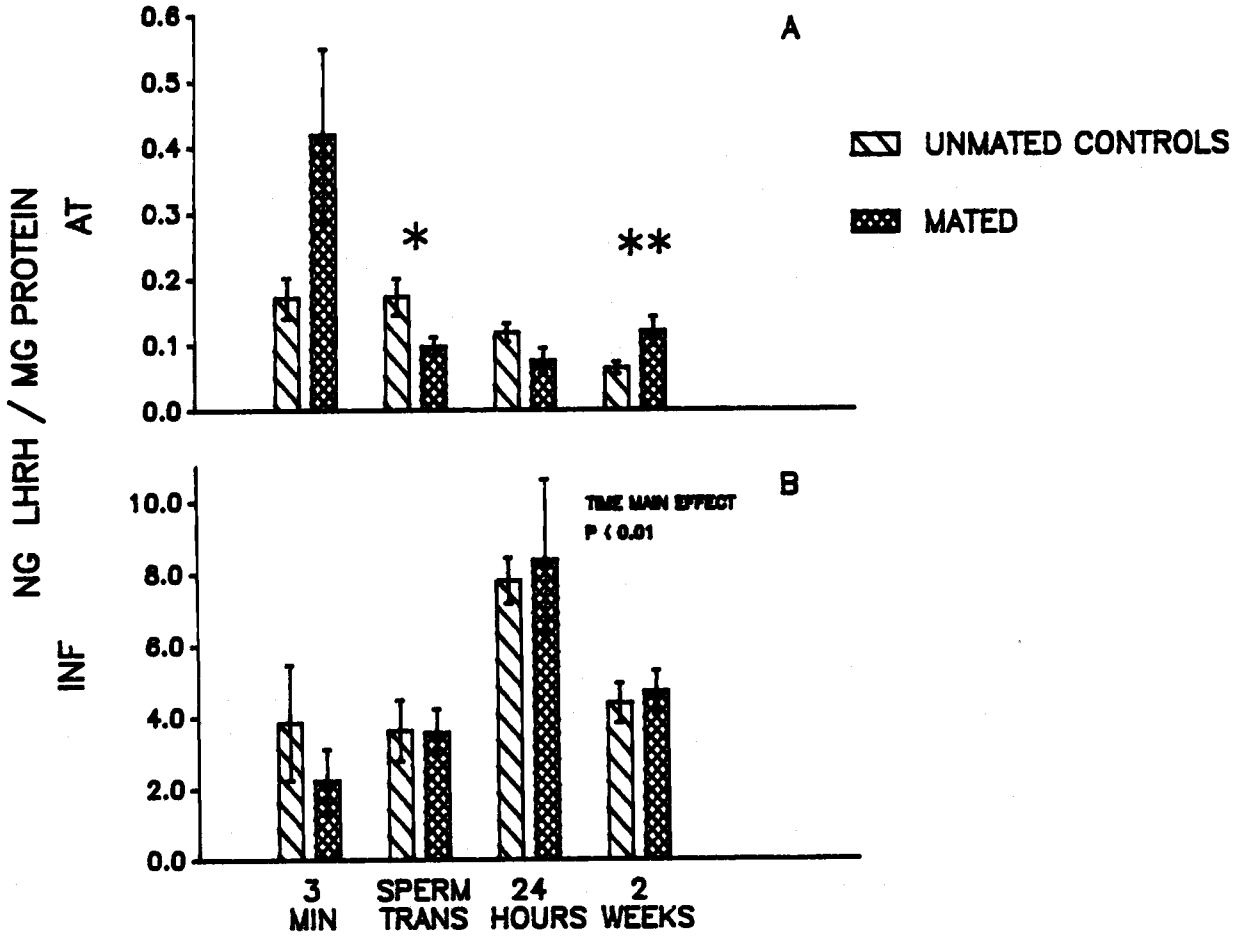

A
B
NG LHRH / MG PROTEIN
AT
INF
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10.0
8.0
6.0
4.0
2.0
0.0
*
**
TIME MAIN EFFECT
P < 0.01
UNMATED CONTROLS
MATED
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS

Figure 3.2. Immunoreactive LHRH concentrations in the A) ventral preoptic area (VPOA) and B) the ventral hypothalamus (VH) during and after courtship. There was no significant effect of mating or time on irLHRH concentrations in either brain region.

Figure 3.2

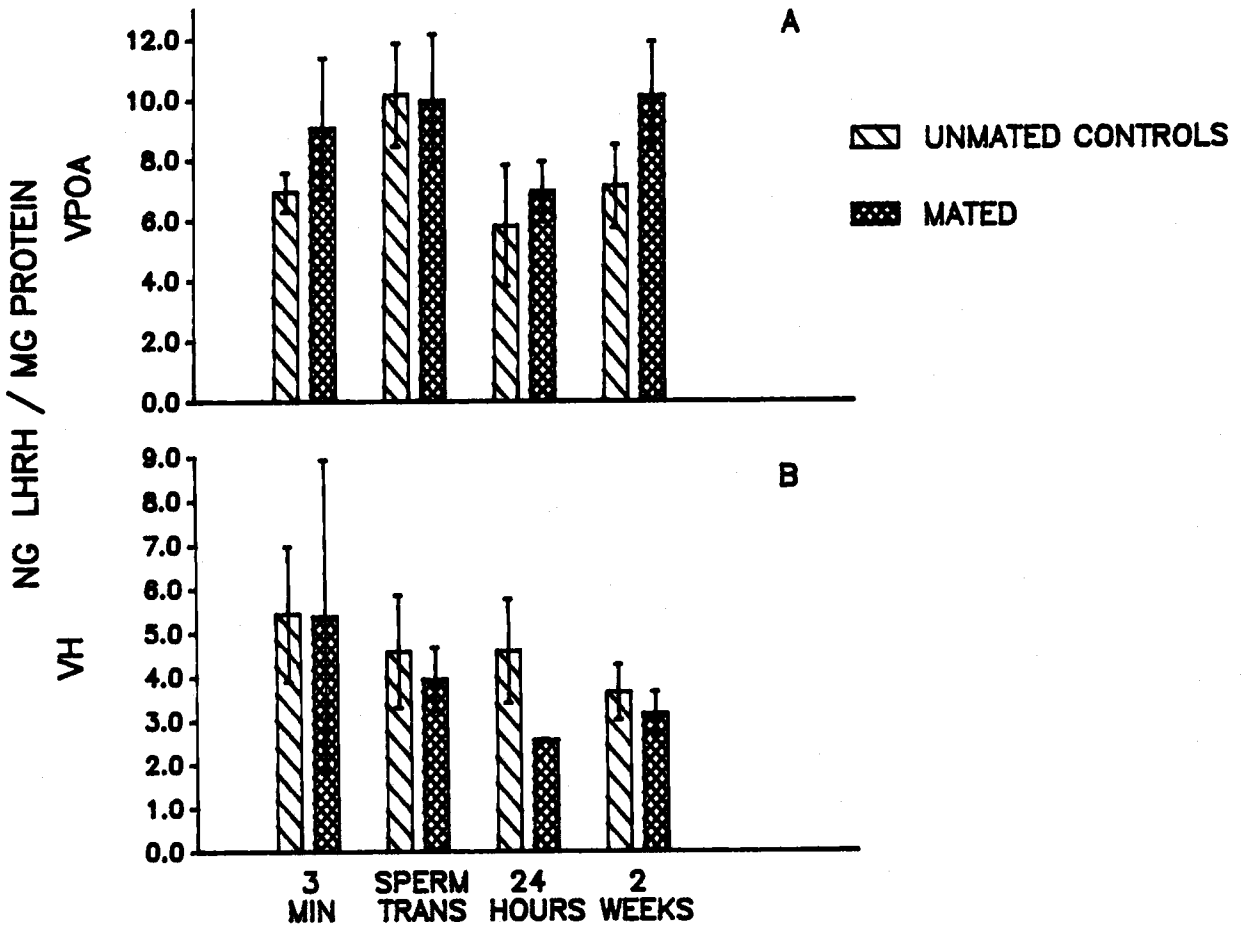

A
B
VPOA
VH
NG LHRH / MG PROTEIN
12.0
10.0
8.0
6.0
4.0
2.0
0.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS
UNMATED CONTROLS
MATED

Figure 3.3. Steroid plasma concentrations during and after courtship. There was a significant interaction for estradiol (3A) concentrations ($F_{(3, 50)} = 7.74$; $P < 0.01$). The * indicates a significant difference between mated and control treatments at sperm transfer and 24 hours after the initiation of the experiment. Also, estradiol concentrations were higher in mated females at sperm transfer than at any other time. There was no significant effect of mating or time on plasma progesterone (3B) or corticosterone (3C) concentrations.

Figure 3.3

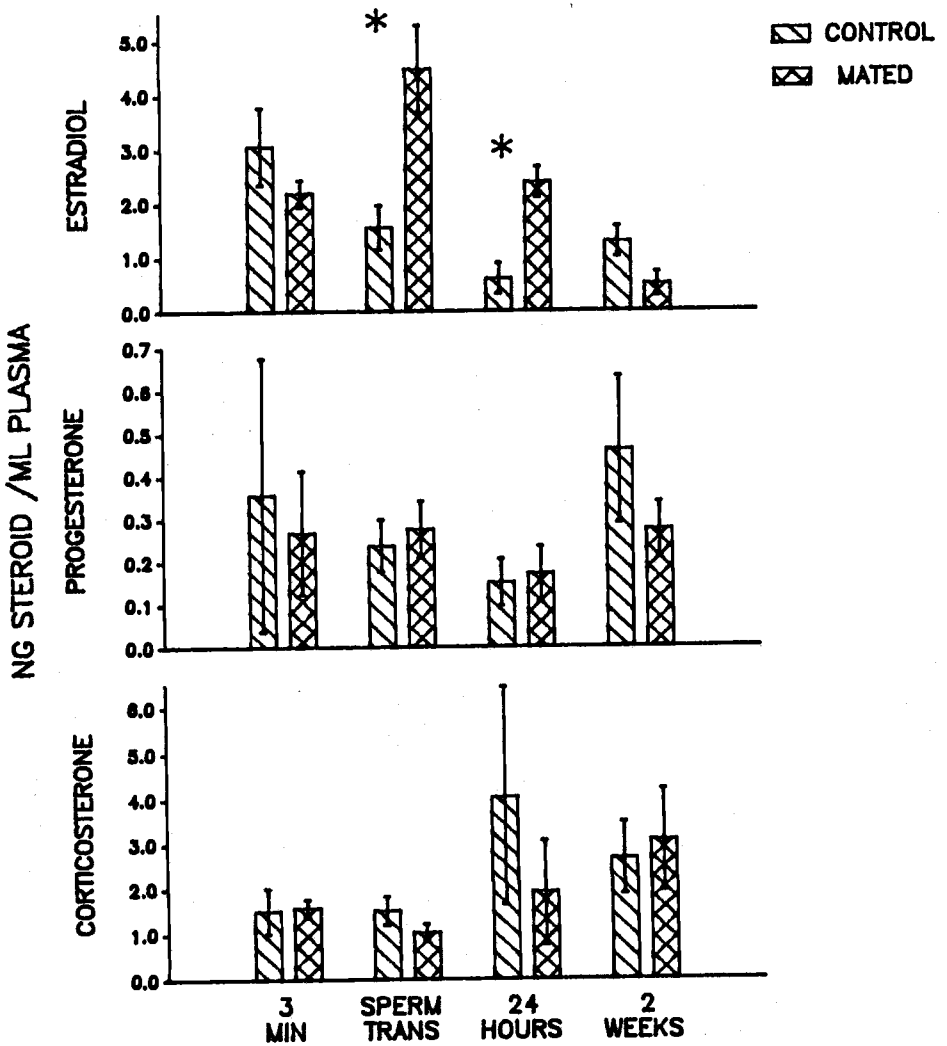

CONTROL
MATED
NG STEROID /ML PLASMA
ESTRADIOL
PROGESTERONE
CORTICOSTERONE
5.0
4.0
3.0
2.0
1.0
0.0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
*
*
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS

Figure 3.4. Immunoreactive LHRH concentrations in the A) nervus terminalis (NT) and B) the INF during the early stages of courtship. The ANOVA indicated a significant mating effect in the NT ($F_{(1, 49)} = 5.52$; $P = 0.02$). There was no significant effect of courtship or time on irLHRH concentrations in the INF.

Figure 3.4

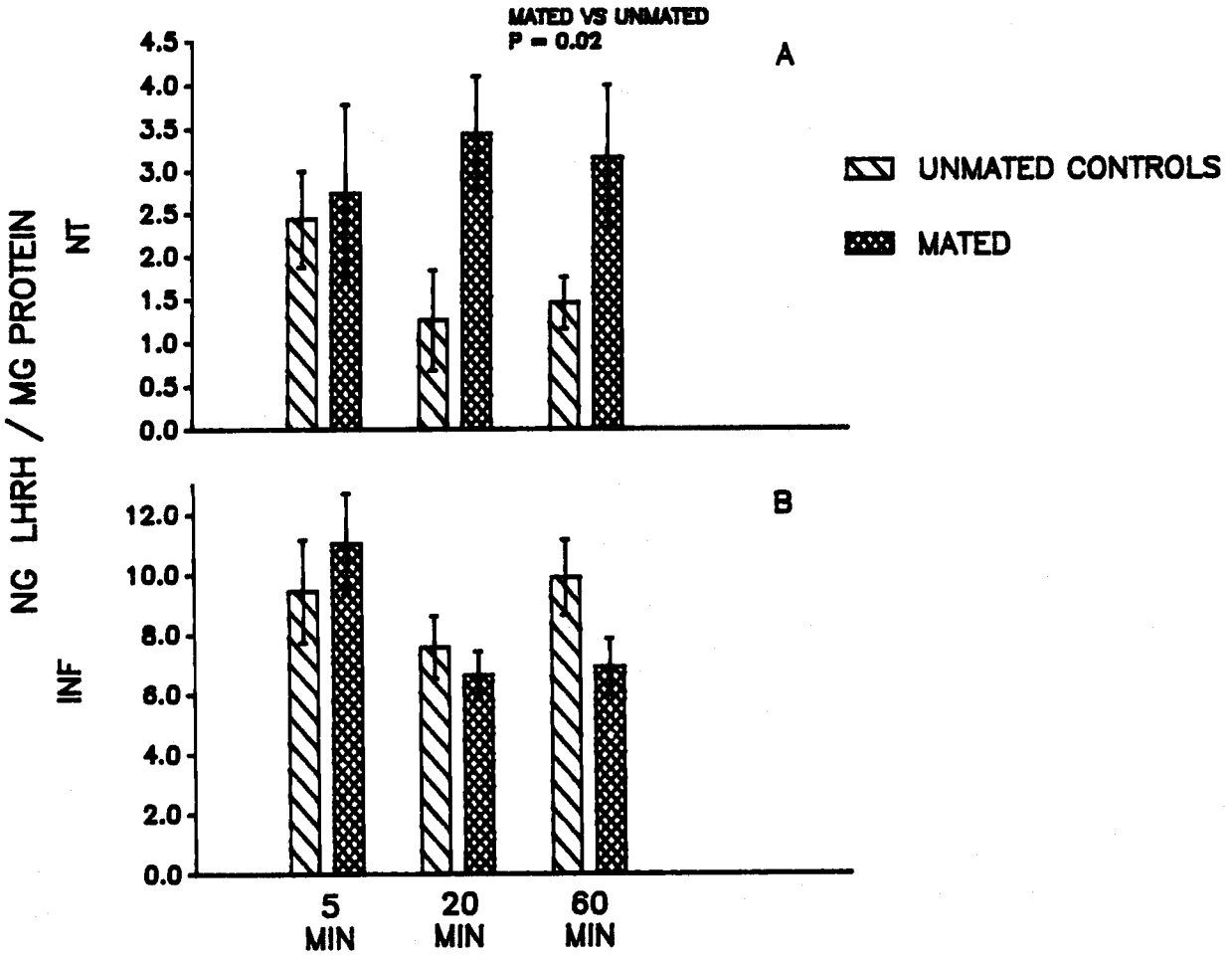
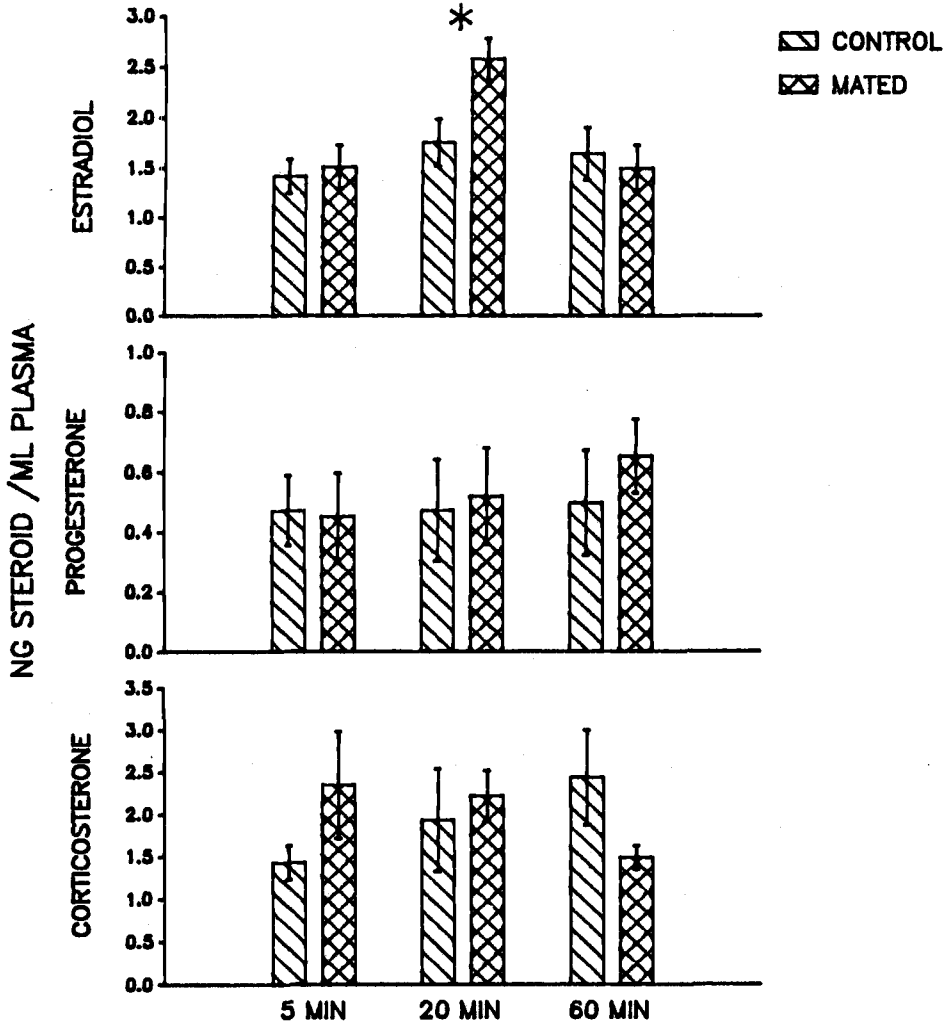

MATED VS UNMATED
P = 0.02
A
B
NT
INF
NG LHRH / MG PROTEIN
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
5 MIN
20 MIN
60 MIN
UNMATED CONTROLS
MATED

Figure 3.5. Plasma steroid concentrations during the early stages of courtship. The ANOVA indicated a significant interaction in plasma estradiol (3A) concentrations ($F_{(2, 53)} = 3.62$; $P = 0.03$). The * indicates a significant difference between mated and control treatments at 20 minutes after the initiation of courtship. There was no significant effect of courtship or time on plasma progesterone or corticosterone concentrations.

Figure 3.5


CONTROL
MATED
ESTRADIOL
PROGESTERONE
CORTICOSTERONE
NG STEROID /ML PLASMA
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
3.5
*
5 MIN
20 MIN
60 MIN

CHAPTER 4: EFFECTS OF ESTRADIOL, PROGESTERONE, AND LUTEINIZING HORMONE-RELEASING HORMONE ON FEMALE SEXUAL RECEPTIVITY IN *TARICHA GRANULOSA*

INTRODUCTION

The activational effects of ovarian steroids on female sexual receptivity in mammals have been well studied in a number of species (for reviews see Feder and Marrone, 1977; Feder, 1984; Arnold and Breedlove, 1985; Pfaff and Schwartz-Giblin, 1988). In general, estradiol stimulates sexual behavior, while progesterone can either facilitate or inhibit sexual receptivity (see Feder and Marrone, 1977).

Ovarian steroids also are known to effect female sexual receptivity in nonmammalian vertebrates. For example, removal of the functional left ovary in the female ring dove (*Streptopelia risoria*) blocks a female's behavioral responses to male courtship until the usually inactive right ovary develops and begins secreting estrogen and progesterone (Cheng, 1973a). Bilateral ovariectomy in *S. risoria* inhibits sexual behavior and treatment with estradiol benzoate restores sexual behavior (Cheng, 1973b). In the lizard *Anolis carolinensis*, ovariectomy abolishes sexual behavior, and treatment with estradiol restores female sexual receptivity (McNicols and Crews, 1979). Furthermore, in this lizard, progesterone facilitates estradiol's effects (Tokarz and Crews, 1980;

Wu et al., 1985), while treatment with the anti-estrogen, CI-628, inhibits sexual receptivity (Tokarz and Crews, 1980). Ovarian steroids, therefore, act as mediators of female sexual behavior in a variety of vertebrate species.

Only a couple of studies have investigated the effects of ovarian steroids in amphibians. In the frog Xenopus laevis, ovariectomy eliminates sexual receptivity; whereas, treatment with both estradiol and progesterone restores female receptive behavior (Kelley, 1982). In Rana pipiens, ovariectomy does not eliminate female sexual receptivity, and treatment with estradiol or progesterone has no enhancing effects on female sexual behavior (Diakow et al., 1978). These results suggest that in anuran amphibians steroidal control of female sexual behavior is species-specific. To my knowledge there are no published reports regarding the endocrine control of female sexual receptivity in urodele amphibians; however, seasonal studies of female endocrine cycles in Pleurodeles waltl (Garnier, 1983) and Euproctus asper (Clergue et al., 1985) have found elevated plasma estradiol concentrations during the breeding season.

Luteinizing hormone-releasing hormone (LHRH) has been found to facilitate female sexual receptivity. In rats, administration of LHRH, including central injections at low concentrations, activates female receptivity independently of the gonads (Moss and McCann, 1973; Pfaff,

1973; Moss et al, 1975; Sakuma and Pfaff, 1980; Rodriguez-Sierra and Komisaruk, 1982). There is also evidence that LHRH can activate female sexual receptivity in several vertebrate taxa including birds (Cheng, 1977), reptiles (Alderete, et al., 1980), and amphibians (Kelley, 1982), indicating that such activation is a widespread phenomenon among vertebrates.

In the rough-skinned newt Taricha granulosa, exposure of females to male courtship behaviors results in concurrent changes in plasma estradiol and brain LHRH concentrations and in female sexual behavior (Chapter 3). Soon after entering ponds, females are captured in amplexus (a sexual clasp) by males. Initially, in response to male courtship behaviors, females do not show receptive behaviors, and frequently they appear to make attempts to escape; at this time plasma estradiol concentrations are low, and LHRH concentrations in the nervus terminalis (a little understood cranial nerve; Demski and Schwanzel-Fukuda, 1987) are elevated. After several hours of exposure to male courtship behaviors, females signal sexual receptivity (Chapter 2), at which time the plasma estradiol concentrations are elevated, and the LHRH concentrations in the nervus terminalis are lower. After females signal sexual receptivity, they are released by the males, and a sperm transfer attempt is made. After an insemination attempt, females are

recaptured in amplexus by males, and pairs remain in amplexus for several more hours until females exhibit unreceptive behaviors. At this time, plasma estradiol concentrations in courted and inseminated females have dropped slightly, but significantly, and LHRH concentrations remain low. Therefore, during courtship, female rough-skinned newts exhibit changes in sexual behavior (from unreceptive, to receptive, and back to unreceptive) that are associated with changes in estradiol and LHRH concentrations (Chapters 2 and 3). These findings suggest that estradiol and LHRH may be activating the changes in female receptive behaviors.

The purpose of this study was to determine whether estrogen, progesterone, or LHRH affect sexual receptivity in T. granulosa. The first experiment investigated whether ovariectomy alone or ovariectomy combined with steroid replacement influences courtship-induced sexual receptivity. The second experiment investigated whether raising plasma estradiol concentrations by short-term exogenous estradiol administration activates sexual receptivity. The last experiment addressed whether central administration of LHRH facilitates female sexual behavior.

METHODS AND MATERIALS

Animals: Females were captured in pit traps at Soap Creek Ponds, 12 miles north of Corvallis, Benton County, Oregon in February and March of 1984 (Experiment 1) and 1987 (Experiments 2 and 3). This method insures that females are captured before they enter ponds to mate. Females were maintained in an artificial pond in an environmentally-controlled room at the natural photoperiod and at a temperature of 8°C. The pond contained leaves and a platform for females to leave the water. Females were regularly fed mealworms and earthworms. Males were captured the day before the experiment at Soap Creek Ponds and placed in 40 liter aquaria containing 10 liters of water. The aquaria were in an experimental observation room that was maintained at 13°C. The day before the experiment, females were moved to and placed in 20 liter buckets containing 0.5 liters of water in the experimental room.

Statistics: Differences between treatment groups in the number of sexually receptive females, at 12 and 24 hours after the initiation of courtship, were determined using a Fisher's Exact Test for each experiment. The significance level was set at $P < 0.05$.

Experiment 1: Effects of ovariectomy and steroid replacement on sexual receptivity.

The purpose of this study was to determine whether ovariectomy and implantation with the steroid hormones estradiol and progesterone effect female sexual receptivity.

Surgery for Ovariectomy: Females were anesthetized by immersion for 25 minutes in dechlorinated water containing 0.0025% benzocaine. Anesthetized females were bilaterally ovariectomized through a short (1 cm) incision made about 5 mm to the right of the ventral midline. Implants of empty (blank) or steroid-filled Silastic capsules (0.5 cm length x 0.147 cm i.d.; 17B-estradiol and progesterone from Sigma) were placed inside of the body cavity of each ovariectomized female (see below). The wound was closed using a 9 mm wound clip (Autoclips, Clay Adams, N.J.), and the females were placed in an antibiotic solution for 2 hours before being placed in a 20 liter bucket containing 14 liters of water which was maintained at about 12°C.

Experimental Design and Methods: From March 6 through March 9, 1984, females were treated as follows: 1) Intact: females were not anesthetized or ovariectomized; 2) Sham operated: females were anesthetized and surgeries were performed as above except the ovaries were not removed,

and no Silastic capsule was implanted; 3) ovariectomy with no steroid: females were ovariectomized and implanted with two empty Silastic capsules; 4) ovariectomy with 17B-estradiol: females were ovariectomized and implanted with two estradiol-filled Silastic capsules, 5) ovariectomy with progesterone: females were ovariectomized and implanted with two progesterone-filled capsules, or 6) ovariectomy with 17B-estradiol and progesterone: females were ovariectomized and implanted with one progesterone- and one estradiol-filled capsule. All the ends of the capsules were sealed.

Twelve days after surgery, females were placed individually in aquaria containing males. Pairs were observed each 15 minutes; courting and mating behaviors were noted for the first 12 hours of courtship. Any female that successfully picked up a sperm cap was considered to be receptive. Pairs were not checked for the next 12 hours (the dark phase of the photoperiod), but since a sperm cap remains visible in a female's cloaca for about 24 hours after insemination, it was possible to determine if a female had become receptive and been inseminated during this 12 hour unobserved period. Unattractive females (females clasped by a male for less than 1 hour) were not included in the analysis. Initial sample size (N) = 10 for all treatment groups.

Experiment 2: Effects of 17B-estradiol and the Anti-estrogen CI-628 on female sexual receptivity.

The purpose of this study was to determine whether short term administration of estradiol or an estradiol antagonist (CI-628) affected female sexual receptivity. The CI-628 antagonist inhibits sexual receptivity in rats by apparently blocking the interaction of estradiol with its receptor in the brain (see Etgen, 1979). This antagonist also blocks sexual receptivity in female A. carolinensis (Tokarz and Crews, 1980).

Experimental Design and Methods: On March 9, 1988, females were injected intraperitoneally with one of the following four treatments: 1) 0.05 ml saline (amphibian's Ringers with 5% Ethanol for all treatments), 2) 1 ug 17B-estradiol in 0.05 ml saline, 3) 10 ug 17B-estradiol in 0.05 ml saline, or 4) 200 ug CI-628 in 0.05 ml saline (CI-628 from Warner-Lambert Co, Pharmaceutical Research Division, Ann Arbor, Michigan).

Two hours after the injection, females were placed one per aquarium into tanks containing 2 males. When one of the males captured a female in amplexus, the other male was removed. Incidence of female sexual receptivity was noted as in experiments 1. The sample size equaled 15 for all treatments.

Experiment 3: Effects of LHRH agonists and potential antagonists on incidence of female sexual receptivity.

The purpose of this study was to determine whether a single intracerebroventricular (icv) injection of mammalian LHRH or potential LHRH antagonists influence sexual receptivity in the female rough-skinned newt. Synthetic mammalian LHRH (Sigma Chemical Co.) was chosen because it is the most abundant form of LHRH in the brain of T. granulosa (Sherwood et al., 1986). The two other analogs were chosen for their potential antagonistic properties (Schally, 1983). The biological activity of mammalian LHRH can be blocked by changing the type of amino acid in the two and three amino acid position. Therefore, when the potential agonist binds to the LHRH receptor, it will not affect a typical LHRH response in the target tissue. The binding affinity of the analog to the receptor can be enhanced with changing the amino acid in the six position. Such a substitution would block binding of endogenous LHRH to its receptor. The potential antagonist analogs were kindly provided by Drs. J. Stepinski and K. Folkers (Institute for Biomedical Research, The University of Texas, Austin, Texas).

Experimental Design and Methods: On March 2 and again on March 4, 1988, 30 females were assigned to 6 different treatment groups (Total N = 10/treatment, or 5/treatment/day). The treatments were as follows: 1) No injection, 2) Saline injection (amphibians Ringer's with 0.1% tartaric acid in all treatments), 3) 10 ng synthetic mammalian LHRH in 1 ul saline, 4) 100 ng synthetic mammalian LHRH in 1 ul saline, 5) 1 ug LHRH analog 1 in 1 ul saline (analog 1 = [N-Ac-D-Thr^{1},D-pClPhe^{2},-D-$Trp^{3,6}$,Pro^{9}]-LHRH, or 6) 1 ug LHRH analog 2 in 1 ul saline (analog 2 = [N-AC-Pro^{1},D-pFPhe^{2},D-$Trp^{3,6}$,$AzaGly^{10}$]-LHRH). All injections were administered (icv) after the method of Moore and Miller (1983).

RESULTS

Experiment 1:

Ovarian steroids did not appear to be necessary for the induction of female receptivity (Table 4.1): ovariectomy did not eliminate courtship-induced receptivity in T. granulosa females, nor did treatment with estradiol and/or progesterone enhance sexual receptivity. However, females treated with progesterone never became sexually receptive when courted by a male. There was no difference in incidence of sexual receptivity between sham operated females and intact controls, indicating that surgery did not affect female sexual behavior.

Experiment 2:

Short-term administration of exogenous estradiol did not enhance female sexual receptivity, and the injection of the anti-estrogen CI-628 did not block receptivity (Table 4.2).

Experiment 3:

Intracerebroventricular injection of LHRH or LHRH analogs did not affect female sexual receptivity (Table 4.3). Furthermore there was no significant difference between uninjected females and saline injected females in incidence of female sexual receptivity, indicating that the icv injection procedure did not affect sexual behavior.

DISCUSSION

The results from these experiments were surprising in that hormones commonly associated with the induction of female sexual receptivity apparently did not influence sexual receptivity in female T. granulosa. Estradiol, progesterone, and LHRH did not enhance receptive behaviors. Ovariectomy, anti-estrogen, and LHRH antagonist analogs did not inhibit receptivity. However, progesterone, a hormone sometimes associated with decreases in receptivity in mammals (see Feder and Marrone, 1977), did inhibit sexual receptivity in this salamander.

Ovariectomy, or treatment with either an anti-estrogen, or estradiol, did not have an effect on female sexual receptivity. The fact that ovariectomy or ovariectomy plus estradiol treatment did not affect sexual receptivity suggests that ovarian estradiol is not necessary for the activation of receptive behavior. However, it is possible that a non-ovarian source of estradiol may have remained after ovariectomy and may have been sufficient to influence sexual receptivity. Furthermore, the 12 days between the ovariectomy and the behavioral testing may not have been a sufficient amount of time to metabolize the endogenous estradiol which could have influenced behavior.

In the second study, 17B-estradiol was injected to induce a short-term elevation in plasma estradiol that might mimic the rise in estradiol seen during courtship (Chapter 3), yet this treatment had no influence on the timing of female receptive behavior. The results from these findings suggest that the courtship-induced rise in plasma estradiol concentrations (Chapter 3) may not be influencing the concomitant change in sexual receptivity.

The anti-estrogen CI-628 appears to inhibit sexual receptivity in rats by blocking estrogen interactions with its receptor in the brain (see Etgen, 1979). For this reason CI-628 was injected to inhibit any central effect the endogenous rise in estradiol has in mediating the courtship-induced female sexual receptivity in T. granulosa. This treatment had no effect on female sexual behaviors. The results of this experiment further support the results seen in the first experiment: estradiol may not be mediating courtship-induced sexual receptivity in female newts.

As in T. granulosa, ovarian hormones do not affect the expression of receptive behaviors Rana pipiens (Diakow et al., 1978). In this frog, female sexual receptivity is not affected by ovariectomy or the administration of estradiol or progesterone. Therefore, the presence of estradiol and progesterone may not always be necessary for the expression of female sexual receptivity.

Central injection of mammalian LHRH or the LHRH antagonists had no effect on female sexual receptivity in T. granulosa. It is possible that the exogenous LHRH and the antagonists analogs did not reach the behaviorally important regions of the brain. For example, a major LHRH-containing system in the brain of T. granulosa is the nervus terminalis which has projections through the olfactory nerve and medial septal region. The injection of LHRH analogs into the third ventricle would not place LHRH in the potential target sites of the nervus terminalis. Therefore, although endogenous LHRH may mediate female sexual receptivity in T. granulosa by influencing specific targets in the brain, exogenous LHRH administration in experiment 3 may not have reached behaviorally important targets.

In T. granulosa, there are changes in LHRH concentrations in the nervus terminalis during courtship that are correlated with changes in female sexual receptivity (Chapter 3). This finding suggests that LHRH and sexual behavior may be associated. It would be most interesting to investigate the behavioral effects of LHRH administration to the nervus terminalis target regions in T. granulosa.

It is also possible that a different form of LHRH may influence female sexual behavior in the rough-skinned newt. The amphibian brain has three forms of LHRH:

mammalian, salmon, and chicken II (King and Millar, 1986; Sherwood et al., 1986). The most abundant form in the brain of T. granulosa is the mammalian type (Sherwood et al., 1986), which was the form of LHRH injected in experiment 3 and the form of LHRH that changes in the nervus terminalis during courtship (Chapter 3). Although females did not exhibit enhanced sexual receptivity in response to this form of LHRH, they may be behaviorally sensitive to other forms of endogenously occurring LHRH.

However, it is also possible that LHRH is not an important mediator of sexual receptivity in the female newt. Injections of LHRH stimulate female sexual receptivity following estradiol and progesterone administration in the frog, X. laevis (Kelley, 1982). The difference in the behavioral responses to LHRH between the X. laevis and T. granulosa may be associated with differences in reproductive tactics: fertilization in X. laevis is external, and female receptive behaviors are temporally associated with ovulation and oviposition. Conversely, fertilization in T. granulosa is internal and females can store sperm for months. This form of reproduction allows T. granulosa females to uncouple temporally sexual behaviors from ovulation and oviposition.

Treatment of female T. granulosa with progesterone inhibited sexual receptivity. This result is consistent

with studies that have found progesterone inhibition of sexual behavior in other species exhibiting post-insemination declines in sexual behavior (see Feder and Marrone, 1977). For example, female golden hamsters, Mesocricetus auratus, exhibit progesterone-facilitated decreases in sexual receptivity after mating (Carter et al., 1976). The lizard A. carolinensis (Crews, 1973) also exhibits a post-copulatory decline in receptivity that may be progesterone-mediated (Valenstein and Crews, 1977). Interestingly, receptivity in female rough-skinned newts also decreases after mating (Chapter 2). The fact that progesterone-treated females do not become receptive when courted suggests that progesterone may influence the mating-induced inhibition of sexual behavior.

In summary, neither estradiol or LHRH enhanced courtship-induced female sexual receptivity in T. granulosa. Since this study represents, to my knowledge, the only investigation into the endocrine control of female sexual receptivity in any female urodele amphibian, it is not yet possible to determine whether this finding is unusual or wide spread among salamanders. Progesterone, on the other hand, was effective in reducing female sexual receptivity and may play a role in the mating-induced decline in receptivity exhibited by this species.

Table 4.1. Effects of ovariectomy and steroid replacement on sexual receptivity 12 and 24 hours after the initiation of courtship. (N) = final sample size.

	% Females Sexually Receptive	
Treatment	12 hours after courtship initiation	24 hours after courtship initiation
Intact (10)	80	100
Sham (7)	50	88
Ovex + (8) Blank Implant	43	57
Ovex + (5) Estradiol Implant	20	40
Ovex + (6) Progesterone Implant	0	17 *
Ovex + (5) Estradiol + Progesterone Implant	20	20 *

*Treatment significantly different from sham operated control.

Table 4.2. Effects of estradiol and CI-628 on the incidence of female sexual receptivity 12 and 24 hours after the initiation of courtship. There were no significant differences between treatments compared to the saline controls.

	% Females Sexually Receptive	
Treatment	12 hours after courtship initiation	24 hours after courtship initiation
Saline	47	73
1 ug Estradiol	53	80
10 ug Estradiol	40	67
200 ug CI-628	33	67

Table 4.3. Effects of LHRH and LHRH analogs on the incidence of female sexual receptivity 12 and 24 hours after the initiation of courtship. There was no significant effect of any treatment when compared to the saline control.

	% Females Sexually Receptive	
Treatment	12 hours after courtship initiation	24 hours after courtship initiation
Uninjected	70	90
Saline	50	70
10 ng LHRH	40	50
100 ng LHRH	60	70
Analog 1	20	60
Analog 2	30	50

CHAPTER 5: BRAIN ARGININE VASOTOCIN AND PLASMA STEROID CONCENTRATIONS IN FEMALE *TARICHA GRANULOSA* AT DIFFERENT REPRODUCTIVE STATES IN NATURE

INTRODUCTION

Reproduction in many amphibians is characterized by several stages including migration to water, pre-mating behaviors (courtship), and laying eggs. Several studies have documented seasonal changes in plasma steroid and brain neuropeptide concentrations in amphibians (d'Istria *et al.*, 1974; Rastogi *et al.*, 1983; Licht *et al.*, 1983; Clergue *et al.*, 1985; Garnier, 1985; Jokura and Urano, 1985; Rastogi *et al.*, 1986; Iela *et al.*, 1986; Specker and Moore, 1978; Zoeller and Moore, 1985; Zoeller and Moore, 1986); however, little is known about physiological states associated with the different reproductive stages. In the frog *Pachymedusa dachnicolor*, females have elevated plasma estradiol, testosterone, and progesterone concentrations during amplexus and oviposition, and concentrations of these hormones decline within two days after mating and egg-laying (Iela *et al.*, 1986). In the bullfrog, *Rana catesbeiana* females have seasonal changes in plasma estradiol, testosterone, and corticosterone concentrations, but plasma progesterone concentrations are detectable only around the time of ovulation (Licht *et al.*, 1983). To my knowledge, there are no similar data available for urodele amphibians. Reproduction in

urodeles is different from that of anurans in that internal fertilization enables females to separate temporally courtship and insemination from fertilization and oviposition. Such a separation of major reproductive events in female salamanders suggests that the endocrine profile associated with mating may be different from that associated with ovulation and oviposition.

During the breeding season, female rough-skinned newts, Taricha granulosa, exhibit distinctive sequential reproductive states. Starting in late winter, females migrate to water to breed (Pimentel, 1952; 1960). Soon after entering the water, females are captured in an amplectic clasp by a male, courted, and, after several hours, inseminated (Smith, 1941; Davis and Twitty, 1964; Chapter 2). After insemination, the pairs resume amplexus and remain together for several more hours before females are released from amplexus by the male. A few weeks after insemination, females ovulate and lay eggs on submerged objects in the pond (McCormack, 1979; Moore et al., 1979).

A few hormones have been found to be associated with reproduction in T. granulosa females (Chapter 3). Laboratory studies have found changes in brain luteinizing hormone-releasing hormone (LHRH) and plasma estradiol concentrations occurring concurrent with changes in female behaviors during courtship and insemination.

Another neuropeptide, arginine vasotocin (AVT), also

plays a role in reproduction. Female T. granulosa oviposit in response to injections of AVT (Boyd et al., pers. comm.). In the frog Rana pipiens, injections of AVT activate female sexual receptivity (Diakow, 1978; Diakow and Ramondi, 1981).

Considering that changes in endogenous hormones may activate each different reproductive state in female urodeles, this study quantified endogenous concentrations of AVT in the brain and steroid hormones in the plasma of female newts at three different stages of reproduction: migration, amplexus (courtship), and oviposition. Because this study found differences in immunoreactive AVT (irAVT) concentrations in the brains of mating and egg laying females, a second study was undertaken to determine whether these differences were simply associated with the reproductive stage or were alsp directly correlated with egg-laying behaviors in female T. granulosa.

METHODS AND MATERIALS

General Methods

Animals: Female T. granulosa were captured at Soap Creek Ponds (experiment 1) and the Corvallis Watershed Reservoir (experiment 2), Benton County, Oregon. Females in different reproductive states (see descriptions for individual experiments) were sacrificed by decapitation in the field within 5 minutes of capture. The heads were immediately heated in 60°C water for 10 minutes and placed on wet ice until transported to the laboratory (within 3 hours). The trunk blood was also collected at the time of sacrifice and placed on wet ice. In the laboratory, the blood was centrifuged at 1200 g for 15 minutes, and the plasma was removed and stored at -80°C. The brains were removed and frozen in O.C.T. Compound (Tissue Tek) and stored at -80°C. After the bodies (without heads) of the females were weighed, ovaries were removed and weighed; oviducts and cloacae were examined; number of eggs per oviduct was determined.

Brain Microdissection: Brains were sliced in a cryostat at 100 um, thaw-mounted onto microscope slides, and stored frozen at -80°C. Several brain regions were removed using the Palkovitz and Brownstein (1982) technique, as modified for the newt brain (Zoeller and Moore, 1985). AVT was

extracted from these regions as described in Zoeller and Moore (1986). See Chapter 3 for details of the procedures.

AVT Radioimmunoassay: The antiserum used for these assays was Anti-vasopressin (No. R70 from D.A. Fisher) at a final dilution of 1:300,000. Protein was measured using a modified Bradford Assay (Bradford, 1976; Chapter 3). For experiment 1, the intra- and interassay coefficients of variation were 12.5% and 19% respectively (over 5 assays). Sensitivity ranged between 2-4 pg/tube. Each assay consisted of all of the samples from any single brain area in each experiment. For experiment 2, irAVT concentrations in all samples were measured in a single assay. The sensitivity of the assay was 10 pg/tube and the intraassay coefficient of variation was 3%.

Steroid Radioimmunoassays: Plasma was extracted and chromatographed on LH-20 columns to separate progesterone, corticosterone, and estradiol. Steroids were assayed by RIA as described in Chapter 3. The intraassay coefficients of variation were 7.5% for estradiol, 16.9% for progesterone, and 12.3% for corticosterone. The sensitivities were 4.09 pg for estradiol (0.05 ng/ml), 11.67 pg for progesterone (0.15 ng/ml), and 4.71 pg for corticosterone (0.062 ng/ml). For each steroid, all samples from both experiments were measured in a single

assay.

Study 1: Brain irAVT and plasma steroid concentrations from female rough-skinned newts at different reproductive states.

On April 3 and 5, 1985, females from three different reproductive states were captured and sacrificed. The "Migration" group consisted of females that were entering ponds for mating (N = 10). These females were captured using a drift fence and pit traps set about 20 meters from the pond's edge. This method of capture insures that the females have not mated previously in the season. The "Amplexus" group consisted of females that had entered the pond and were being actively courted by males (N = 9). The "Oviposition" group consisted of females captured in the pond while exhibiting egg laying behaviors (N = 11). Females were sacrificed between 1 and 5 minutes after capture, and brain tissue and plasma were processed as described in the general methods.

Study 2: Brain irAVT concentrations and plasma steroid concentrations in ovipositing and non-ovipositing females.

This experiment was designed to determine whether the low irAVT concentrations in the dorsal preoptic area (DPOA) found at oviposition in study 1 were correlated directly with the egg-laying behaviors. Females were captured in late spring when there is little courtship and mating activity (Deviche, Propper, and Moore, pers. comm.). Females in the "Oviposition" group were captured while exhibiting egg-laying behaviors (N = 12), and females in the "Non-oviposition" group were captured while swimming, walking or foraging over the bottom of the lake (N = 12). Sampling methods were as in study 1.

Statistics: In study 1, a one-way analysis of variance (ANOVA) followed by Fisher's least squared difference test was used to test for differences among groups in irAVT concentrations, steroid concentrations, body weights, and ovarian weights. A student's t-test was used to make comparisons between groups in study 2. Incidences of oviducal or cloacal eggs were compared between groups with a Fisher's exact test. For all tests, differences were considered to be significant if $P < 0.05$.

RESULTS

Study 1:

Females in different reproductive states had different irAVT concentrations in the brain. Concentrations of irAVT in the DPOA were significantly lower in females that were exhibiting egg-laying behaviors than in the other two groups (Fig. 5.1). There were no differences among groups in irAVT concentrations in the amygdala, cerebral spinal fluid, ventral preoptic area, dorsal and ventral hypothalamic nucleus, pars distalis, optic tectum and interpeduncular region (Fig. 5.1).

Plasma steroid concentrations were different among the three groups (Fig. 5.2). Plasma estradiol concentrations were higher in females in amplexus than in females migrating or ovipositing ($P = 0.002$). Also, plasma corticosterone concentrations were lower in ovipositing females than in females in either of the other two groups ($P < 0.001$). There were no significant differences in progesterone concentrations among the groups.

Table 5.1 shows the results from the morphological and oviducal egg data. Although there were no differences in body weights among females in different reproductive states in study 1, ovarian weights were higher in females that had not yet entered ponds, suggesting that once

females move into the water or begin to mate, some change, possibly related to water balance occurs in the ovary. Only females that were exhibiting oviposition behaviors had oviducal eggs.

Study 2:

There were no significant differences between groups in either DPOA or cerebral spinal fluid concentrations of irAVT (Fig. 5.3). However, there were significant differences in estradiol concentrations between the two groups (Fig. 5.4). Plasma estradiol concentrations were higher in ovipositing females than in non-ovipositing females ($P = 0.012$). There were no significant differences between the two groups in plasma progesterone or corticosterone concentrations.

There were no significant differences in body weight or ovarian weight between the ovipositors and non-ovipositors in study 2 (Table 5.2). However, although many of the non-ovipositors had oviducal eggs, females that were ovipositing had a higher incidence of eggs in the oviduct and cloaca. None of the hormone concentrations were associated with the presence of oviducal eggs.

DISCUSSION

Concentrations of brain irAVT were associated with the stage of reproduction in female T. granulosa. The DPOA has AVT-containing cell bodies in anuran amphibians (Jokura and Urano, 1985). From the DPOA, fibers are sent to the pars nervosa and many extrahypothalamic sites (Vandersande and Dierickx, 1976; Jokura and Urano, 1985; 1987) where AVT is released either into the peripheral circulation or into specific brain regions. Arginine vasotocin not only induces oviducal contractions in many amphibians (Heller, 1970; Guillette et al., 1985), but it also stimulates oviposition in many vertebrates (La Pointe, 1977). Injection of AVT activates egg-laying behaviors in female T. granulosa (Boyd et al., in prep.). Possibly the difference in DPOA irAVT concentrations found among the groups in study 1 is associated with the release of AVT into the periphery or brain where this hormone may activate oviducal contractions and egg-laying behaviors in female T. granulosa.

To determine if irAVT concentrations in the DPOA were correlated with the actual performance of oviposition behavior, study 2 was conducted at the end of the breeding season, when most courtship is over and many females are ovipositing. In this experiment, there was no difference in irAVT concentrations in the DPOA between

females ovipositing and females in the ponds swimming, foraging, or walking over the substrate. This result suggests that changes in irAVT concentrations in the brain are not directly associated with the performance of egg-laying behavior.

In male T. granulosa, corticosterone not only suppresses sexual behavior, but is also elevated in individuals subjected to confinement stress (Moore and Miller, 1984). Furthermore, male newts confined to small boxes before being exposed to a female do not exhibit sexual behaviors. These results suggest that for male rough-skinned newts, corticosterone inhibits sexual behavior. Female rough-skinned newts held in tanks in the laboratory do not usually lay eggs (Propper, pers. obs.). As is the case in male newts, it is possible that the confinement-induced inhibition of reproduction in females is also mediated by corticosterone. If corticosterone does inhibit female ovulation and oviposition, perhaps the low corticosterone concentrations seen in females at the time of oviposition in this study act to facilitate ovulation and oviposition. The role of corticosterone in ovulation and oviposition remains to be investigated.

The finding that females in amplexus had higher estradiol concentrations than did migrating or ovipositing females parallels laboratory findings in which courtship induced a rise in plasma estradiol concentrations (Chapter

3). Estradiol concentrations rose in females during courtship and were higher than unmated controls by the time the time females became sexually receptive. Furthermore, estradiol concentrations remained elevated for at least 12 hours after insemination. The fact that estradiol concentrations were elevated in females in amplexus in natural conditions and in the courted females in the laboratory suggests courtship does induce an endogenous rise in estradiol. The physiological meaning of this rise in estradiol remains to be determined, but results from Chapter 4 suggest that estradiol may not be mediating the courtship-induced receptive behaviors seen in this species.

Females exhibiting egg-laying behaviors in study 2 had lower plasma estradiol concentrations than did females that were not ovipositing. Possibly, this result is associated with the fact that ovipositing females had a higher incidence of both oviducal and cloacal eggs than did non-ovipositing females.

In male rough-skinned newts there are seasonal changes in irAVT concentrations in the optic tectum (Zoeller and Moore, 1986) and corticosterone concentrations in the plasma (Zoeller and Moore, 1985). As well, in male and female R. catesbeiana, there are seasonal changes in plasma corticosterone, estradiol, and progesterone concentrations. (Licht et al., 1983). In P.

dacnicolor, there are elevated estradiol and progesterone concentrations during amplexus and oviposition (Iela et al., 1986). However, considering that in frogs sexual behaviors are temporally associated with ovulation, these changes in plasma steroid concentrations in P. dacnicolor may reflect follicular development and not behavioral changes. Female T. granulosa, have mature follicles by the time they enter the ponds (McCormack, 1979). The courtship-induced rise in estradiol seen in courting females may be necessary for ovulation and oviposition, but this change in estradiol concentration is not sufficient for ovulation the rise in estradiol occurs some time before insemination (Chapter 3). If this change in estradiol concentration stimulated ovulation, the females that were courted, but not inseminated, should ovulate and lay eggs. However, insemination is necessary for the induction of ovulation (McCormack, 1979; Moore et al., 1979). Therefore, other factors associated with insemination may act in concert with the courtship-induced changes in estradiol concentration to activate ovulation and oviposition.

The results of this study indicate that there are distinct changes in brain irAVT and plasma steroid concentrations that are associated with the reproductive state of female rough-skinned newts. The elevated estradiol concentrations found in females in amplexus with

males further support the laboratory finding that courtship induces changes in plasma estradiol concentrations (Chapter 3). Dorsal preoptic area concentrations of irAVT and plasma concentrations of corticosterone decrease some time between insemination and oviposition. However, the physiological significance of these differences in irAVT, corticosterone, and estradiol concentrations among females T. granulosa in different reproductive stages is unknown.

Table 5.1. Morphological information on the females captured while in different reproductive states for study 1. Values for body and ovarian weight represent means with the standard errors in parentheses.

	Migration	Amplexus	Oviposition*
Body Weight (grams)	11.78 (0.46)	11.88 (0.42)	10.85 (0.44)
Total Ovarian Weight (grams)	1.86 [a] (0.13)	1.45 [b] (0.12)	1.35 [b] (0.12)
% Females with Oviducal Eggs	0% [a]	0% [a]	80% [b]

* Values within each parameter that have different superscripts are significantly different from each other.

Table 5.2. Morphological information from ovipositing and non-ovipositing females collected for study 2. Values for body and ovarian weight represent means with the standard errors in parentheses.

	Non-Oviposition	Oviposition*
Body Weight (grams)	8.6 (0.41)	8.5 (0.32)
Total Ovarian Weight (grams)	1.09 (0.09)	1.06 (0.07)
% Females with Oviducal Eggs	66% [a]	100% [b]
% Females with Cloacal Eggs	17% [a]	58% [b]

* Values with each parameter that have different superscripts are significantly different from each other.

Figure 5.1. Immunoreactive AVT concentrations in the brain of rough-skinned newts in three different reproductive states. In the dorsal preoptic area (DPOA), irAVT concentrations are significantly lower in ovipositing females than in females from the other two reproductive stages ($F_{(2, 25)} = 26.83$; $P < 0.001$). Bars that have different letters above them are significantly different from each other. There were no other significant differences between groups in any other brain area. A) VPOA: ventral preoptic area; VHN: ventral hypothalamic nucleus; DHN: dorsal hypothalamic nucleus; B) IP: interpeduncular region; PD: pars distalis; OT: optic tectum; CSF: cerebral spinal fluid; AMYG: amygdala (medial and lateral).

Figure 5.1

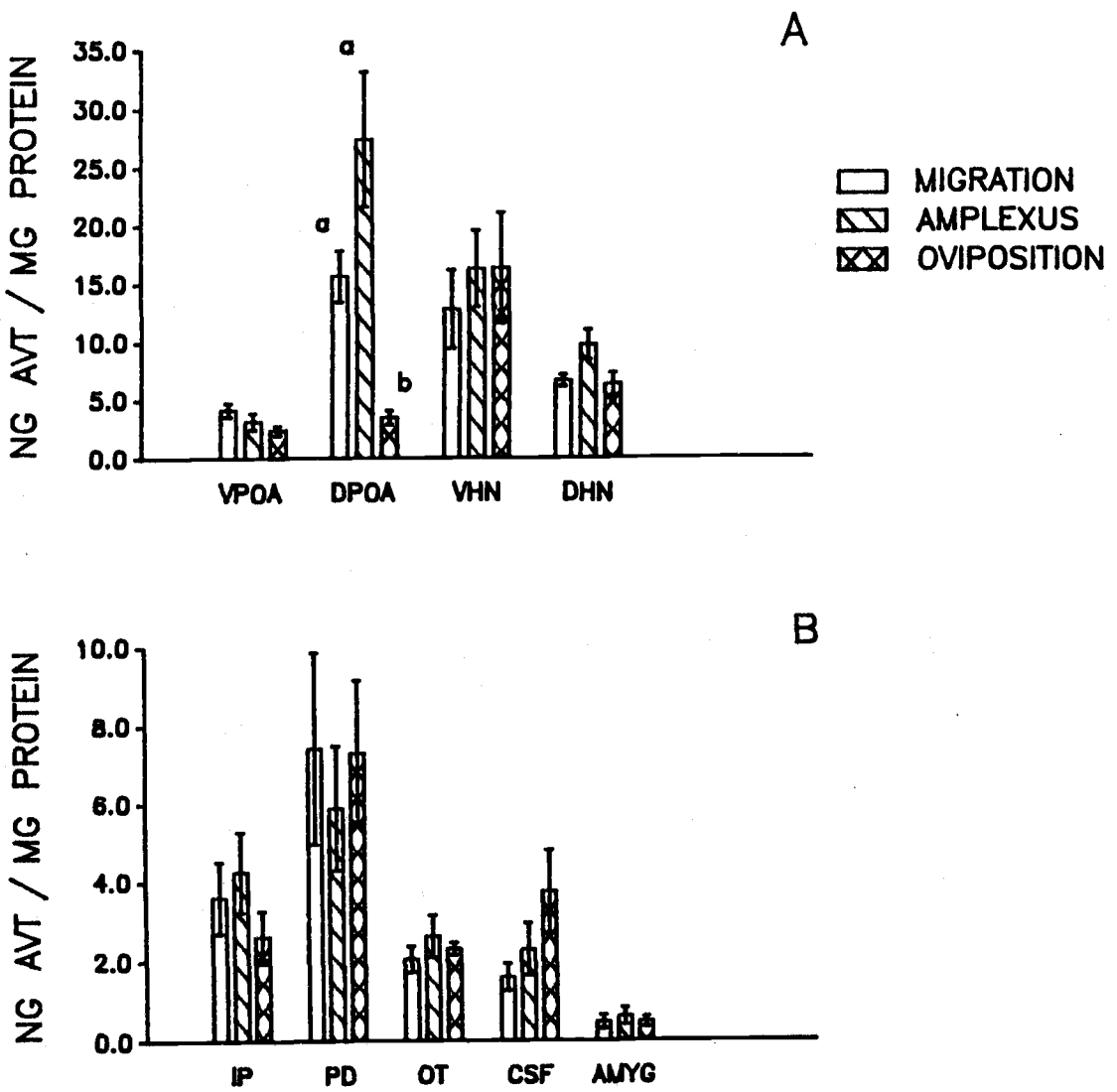

A
NG AVT / MG PROTEIN
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
a
a
b
VPOA
DPOA
VHN
DHN
MIGRATION
AMPLEXUS
OVIPOSITION
B
NG AVT / MG PROTEIN
10.0
8.0
6.0
4.0
2.0
0.0
IP
PD
OT
CSF
AMYG

Figure 5.2. Estradiol, progesterone, and corticosterone concentrations in the plasma of females in three different reproductive stages. Estradiol concentrations are higher in females that are in amplexus than in females migrating to ponds or females ovipositing eggs ($F_{(2, 27)} = 7.63$; $P = 0.002$). Corticosterone concentrations are lower in females that are ovipositing eggs when compared to the other two groups ($F_{(2, 27)} = 45.5$; $P < 0.001$). Bars that have different letters above them are significantly different from each other.

Figure 5.2

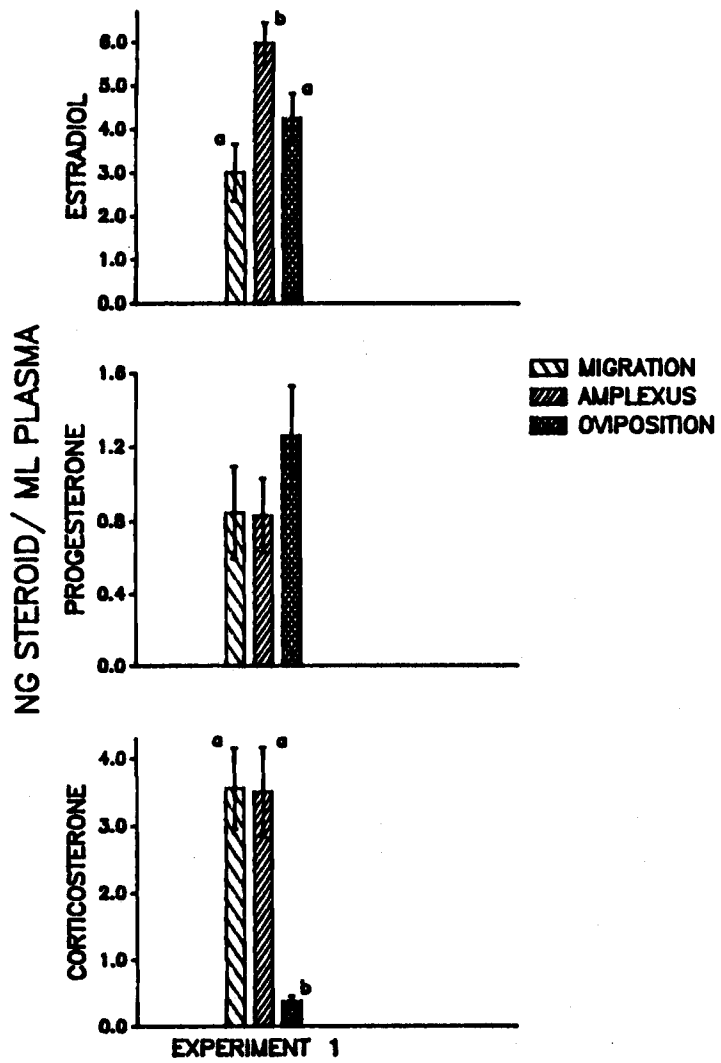

NG STEROID/ ML PLASMA
ESTRADIOL
PROGESTERONE
CORTICOSTERONE
EXPERIMENT 1
MIGRATION
AMPLEXUS
OVIPOSITION

Figure 5.3. Immunoreactive AVT concentrations in the CSF and DPOA in ovipositing and non-ovipositing females. There is no significant difference in irAVT concentrations between the two groups in either brain region.

Figure 5.3

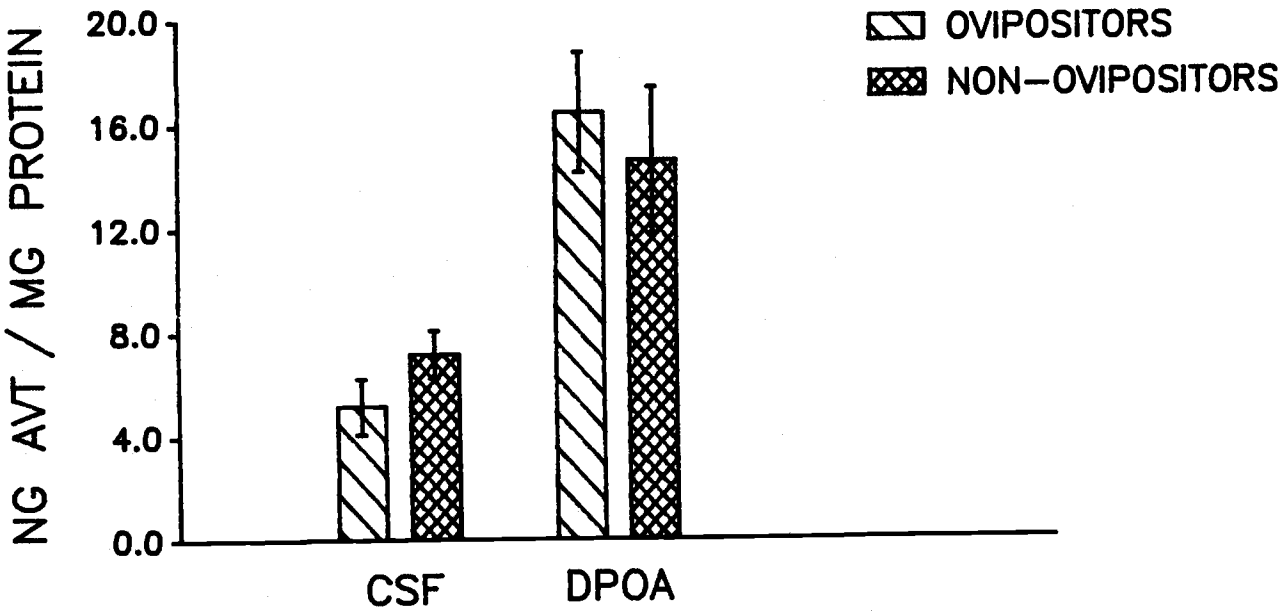
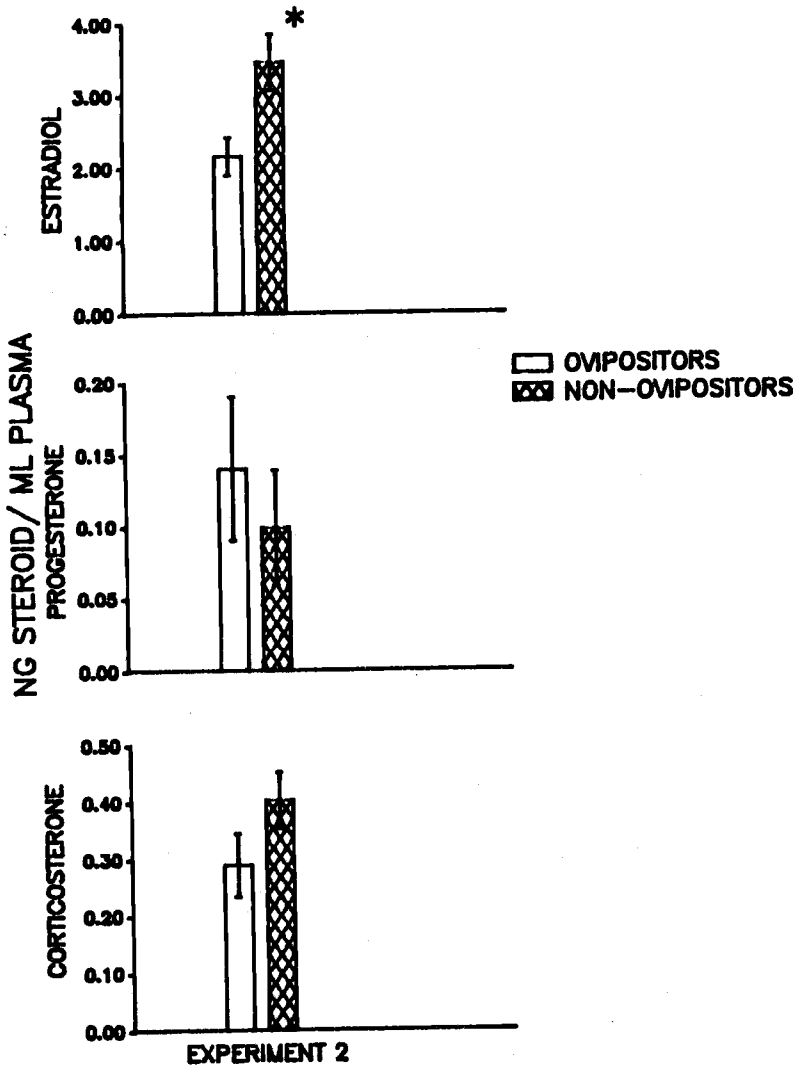

NG AVT / MG PROTEIN
20.0
16.0
12.0
8.0
4.0
0.0
CSF
DPOA
OVIPOSITORS
NON–OVIPOSITORS

Figure 5.4. Estradiol, progesterone, and corticosterone concentrations in the plasma of ovipositing and non-ovipositing females. Estradiol concentrations were significantly higher in non-ovipositors compared to ovipositors ($t = 2.75$; $P = 0.012$). There was no significant difference in progesterone or corticosterone concentrations between the two groups.

Figure 5.4


ESTRADIOL
4.00
3.00
2.00
1.00
0.00
*
NG STEROID/ ML PLASMA
PROGESTERONE
0.20
0.15
0.10
0.05
0.00
CORTICOSTERONE
0.50
0.40
0.30
0.20
0.10
0.00
OVIPOSITORS
NON-OVIPOSITORS
EXPERIMENT 2

CHAPTER 6: GENERAL DISCUSSION

Courtship, as with other behavioral interactions, among individuals, is composed of behavioral signals that are perceived through the senses and are integrated in the nervous system. All of these signals are integrated in the brain to coordinate reproductive physiology and behavior. The studies in this thesis investigated how the neuroendocrine system of female rough-skinned newts (Taricha granulosa) reacts to the behavioral signals of courtship, and whether courtship-induced physiological changes in the brain and periphery of females activate sexual receptivity.

To interpret the significance of physiological changes occurring in response to courtship, it is important to understand the components of the behavioral interactions. The first part of this thesis asked several questions. What are the behavioral interactions between courting males and females? Do the behavioral interactions lead to successful insemination? Do specific behavioral interactions stimulate changes in female sexual behavior?

I found that previous descriptions of courtship in T. granulosa were incomplete. Courtship in this species consists of a series of behavioral changes exhibited by males and females. Females are sexually unreceptive when first captured by a male. During the early stages of

amplexus, the female responds by deflecting her head down and by struggling vigorously as if to escape when the male attempts to rub her his submandibular gland across her nares. As courtship progresses the female no longer moves her head down during a male's chin rub, and she may even raise her head as a signal that she is becoming sexually receptive in response to this stimulus. If a female maintains a raised-head posture, remains motionless, and releases an air bubble, all signals that the female is sexually receptive, the male releases her from amplexus and places a spermatophore on the substrate. The female will usually follow the male and attempt to pick up the sperm cap in her cloaca. After the sperm transfer attempt, a male reclasps a female in amplexus and the pair remains together for several more hours. The post-insemination amplexus facilitates a decrease in female sexual receptivity.

The results of Chapter 2 found that the probability of a sperm transfer attempt leading to successful insemination in T. granulosa is higher than in any other salamander species so far studied (Arnold, 1976; 1977; Halliday, 1977; Verrell, 1983; Massey, 1988). Furthermore, the median amount of time spent in courtship before the male attempts a sperm transfer is longer for T. granulosa than for other known urodeles studied. These results support Arnold's (1977) hypothesis that the amount

of time a male invests in courtship "may affect the probability of sperm transfer during a courtship."

Once insemination is complete, there are several ways that paternity is insured by male newts. First, the sperm cap itself acts as a plug to prevent further insemination. But, since the plug is absorbed by the female after a few hours, it only provides a relatively short-term barrier against future insemination. Male newts have other means that prevent future insemination by a new male once the female absorbs sperm cap. In some females, courtship and insemination inhibit future receptive responses. Also, post-insemination amplexus acts as a physical barrier against new courtship by a competing male. Last, a female that receives post-insemination clasping no longer is receptive to courtship by a new male.

The results of Chapter 2 demonstrated that, during each mating episode for T. granulosa, behavioral signals from the male cause first an activation and then a suppression of female receptivity. The next part of my thesis used these findings and addressed the following question: What are the proximate mechanisms that control these changes in behavioral states? Specifically, what neuroendocrine changes are produced in females by exposure to male courtship behaviors?

Exposure to male courtship behaviors was found to induce changes in brain concentrations of irLHRH and

plasma concentrations of estradiol. Estradiol concentrations are low at the initiation of courtship, but are elevated by the time a female becomes receptive. Furthermore, estradiol concentrations remain elevated for at least 12 hours after insemination. Concentrations of irLHRH in the anterior telencephalon were higher in courted females three minutes after the initiation of courtship than they were at sperm transfer. Because in the telencephalon irLHRH is localized primarily in the nervus terminalis (Muske and Moore, 1987), irLHRH concentrations were determined in this little understood cranial nerve during the early stages of courtship. This second study confirmed that irLHRH concentrations in the nervus terminalis of female newts are higher in courted females then in uncourted controls.

Both LHRH and estradiol influence female sexual receptivity in many other vertebrates. However, although the studies in Chapter 3 found that LHRH and estradiol concentrations changed as females became receptive, follow-up studies (Chapter 4) failed to provide evidence that LHRH or estradiol caused the courtship-induced change in female sexual receptivity in T. granulosa. Blocking the actions of LHRH and estradiol by antagonists or lowering plasma concentrations of estradiol by ovariectomy failed to disrupt courtship-induced receptivity. Injecting LHRH into the cerebral spinal fluid or elevating

estradiol and/or progesterone through injection or implantation failed to enhance female sexual receptivity. Treatment with progesterone, however, inhibited sexual receptivity, suggesting that progesterone may be responsible for the mating-induced reduction in receptivity that occurs after insemination and post-insemination amplexus.

The observed changes in irLHRH may not be directly activating female sexual receptivity in T. granulosa; however, there are other possible functions for the observed changes. One possibility is that LHRH is transported to the median eminence where it is released to act on pituitary release of luteinizing hormone. Luteinizing hormone would then affect gonadal steroidogenesis and release. Indeed, plasma levels of estradiol did change during courtship, suggesting that some activation of the hypothalamic-pituitary-gonad axis occurred during courtship. However, neither hypothalamic nor infundibular LHRH concentrations changed during the course of courtship, suggesting that LHRH from the nervus terminalis was not traveling down nerve fibers that run to the median eminence.

Luteinizing hormone-releasing hormone in the nervus terminalis may also travel through the olfactory nerve, possibly to the olfactory epithelium. In support of this hypothesis are the facts that irLHRH nervus terminalis

fibers terminate in the olfactory epithelium of mice (Jennes, 1987). There are efferent impulses running from the nervus terminalis to the olfactory system in sharks (Bullock and Northcutt, 1984). If LHRH is being transported to and released in the olfactory epithelium, then this neuropeptide could act to sensitize the olfactory system to pheromonal stimulation.

This proposal, that the nervus terminalis controls olfactory sensitivity to pheromones is opposite to the hypothesis proposed by Demski and Northcutt (1983). These investigators suggested that the nervus terminalis is a chemosensory organ responding to pheromonal input. However, the results of two other studies suggest that pheromones do not effect the nervus terminalis. First, in the shark, Squalus acanthias several extracts of chemicals that may be used in prey detection were found to be ineffective in inducing changes in electrical activity in the nervus terminalis (Bullock and Northcutt, 1984). Second, in the goldfish, Carassius auratus application of a known sex pheromone to the nares of males (Dulka et al., 1987) was found to have no effect on nervus terminalis electrical activity (Fujita et al., submitted). Although pheromones failed to stimulate the nervus terminalis in these studies, tactile stimulation did. In S. acanthias, mechanical movement of water near the head caused changes in electrical activity from the nervus terminalis (Bullock

and Northcutt, 1984); in the goldfish, rubbing a glass rod along the side of the body evoked a reduction in the firing rate of the nervus terminalis (Fujita et al., submitted). Interestingly, there is a tactile component to goldfish courtship (Kyle et al., 1987). These results suggest that nervus terminalis activity may be sensitive to tactile rather than pheromonal stimulation.

In T. granulosa, courtship has a strong tactile component. A male contracts his hind limbs against the side of a female and rubs his submandibular gland over the female's nares (tactile stimulation as well as pheromonal; Chapter 2). My hypothesis for how tactile and pheromonal stimulation may act to facilitate courtship-induced receptivity is that tactile stimulation from male hind limb contractions, or from male chin rubs, causes increases in LHRH synthesis and release in the nervus terminalis; the LHRH from the nervus terminalis LHRH then may increase olfactory epithelium sensitivity to a submandibular gland pheromone. This hypothesis not only accounts for the decrease in LHRH concentrations in the nervus terminalis by the time females are receptive, but also explains the female's behavioral change in response to male chin rubs.

The above hypothesis offers a possible proximate mechanism for the induction of sexual receptivity in newts, but it by no means provides an entire picture of

the endocrine, neuroendocrine, and environmental factors involved in these behavioral changes. Certainly, other hormones and neurotransmitters are also involved in evoking sexual receptivity in this amphibian.

Which reproductive pattern of Crews (1984; 1987) is exhibited by female T. granulosa? Upon entering the ponds to mate, females have mature gonads (McCormack, 1979), which suggests that this amphibian fits an associated reproductive pattern. However, although courtship affects changes in plasma estradiol concentrations, this hormone does not appear to be necessary for the expression of female sexual receptivity. These results suggest that reproduction in female rough-skinned newts exhibit a dissociated reproductive pattern. Apparently, the mating system of T. granulosa females fits a reproductive pattern best described as intermediate between those defined by Crews (1984; 1987).

Courtship in any vertebrate involves a series of specialized behavioral exchanges. One member of the pair performs a behavior that is perceived and integrated in the central nervous system of the partner who then responds accordingly. This interchange of behavior continues and, if specific changes in the nervous system occur, culminates in insemination and successful reproduction. If the nervous system does not respond with the specific neuroendocrine and electrophysiological

changes necessary to induce a sexually responsive state, then the interaction does not result in mating.

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APPENDIX

APPENDIX

COURTSHIP-INDUCED CHANGES IN BRAIN AVT CONCENTRATIONS

METHODS AND MATERIALS

Animals and Experimental Design: Please see Chapter 3, Experiments 1 and 2.

AVT Radioimmunoassay: The assay method used is the same as in Zoeller and Moore (1986). The antiserum was Anti-vasopressin (No. R70 from Dr. D.A. Fisher) diluted to 1:75,000 in phosphate-buffered saline with gelatin in a final assay volume of 500 ul. Standards were prepared in triplicate using synthetic AVT (Sigma). Standard concentrations ranged from 2 to 200 pg AVT. Standards and samples were incubated for 24 hours at 4°C in antiserum before 4500 cpm of ^{125}I-AVP (New England Nuclear) was added. All tubes were incubated for another 72 hours at 4°C. Bound from free label was separated by the addition of 1.5 ml of ice cold 95% ethanol to all standards and samples, which were then centrifuged at 1500 g for 10 minutes. The supernatant was decanted, the tubes were dried, and radioactivity was counted in a Beckman 5500 Gamma Counter. The specific binding was 50.8%, the minimum limit of the assays were 2.0 - 4.0 pg. The intra- and interassay coefficients of variation were 12.5% and 19% respectively.

Protein Assay: Please see Chapter 3.

Brain Regions and Peptides Assayed: The following brain regions were assayed for immunoreactive arginine vasotocin (irAVT): amygdala, interpeduncular nucleus, dorsal preoptic area, dorsal hypothalamic nucleus, pars distalis, and the optic tectum.

Statistics: Please see Statistics, Chapter 3.

RESULTS

Experiment 1: Effect of Courtship on Brain irAVT Concentrations

In the cerebral spinal fluid (Fig. A.1A), irAVT concentrations were elevated in courted females. There was an interaction effect between time and courtship in the dorsal preoptic area (DPOA; Fig. A.1B): in mated females, as in controls, irAVT concentrations were higher 3 minutes after the initiation of courtship than they were at spermatophore transfer. These results suggest that DPOA irAVT concentrations dropped within 12 hours of being placed in an aquarium. Mated females had higher irAVT concentrations in the DPOA than controls by two weeks after the initiation of courtship.

Courtship did not affect brain irAVT concentrations in the other regions (Fig. A.2 to A.4). In the dorsal hypothalamic nucleus (Fig. A.3A) irAVT concentrations decreased by 24 hours after the initiation of the experiment regardless of treatment.

Experiment 2: Effect of Courtship on Brain irAVT Concentrations During Early Courtship

During the early stages of courtship, irAVT concentrations in the dorsal preoptic area (Fig. A.5A) of

control females were lower 5 minutes after the initiation of the experiment than they were at 60 minutes after the initiation of the experiment. At 5 minutes after the initiation of courtship, mated females had significantly higher irAVT concentrations than did unmated females, suggesting a rapid change in irAVT concentrations soon after amplexus begins. There were no significant differences among the mated treatment groups. There were no significant effects of courtship or time after the initiation of the experiment on irAVT concentrations in any other brain region (Fig. A.5B to Fig. A.6).

Figure A.1. AVT concentrations in the A) cerebral spinal fluid (CSF) and B) dorsal preoptic area (DPOA) during and after courtship. In the CSF, mated females had higher irAVT concentrations than did unmated ($F_{(3, 41)}$ = 17.90; $P < 0.001$). There was a significant interaction between treatment and time in the DPOA ($F_{(3, 41)}$ = 3.6; P = 0.02). The * indicates a significant difference between mated and control treatments at 2 weeks after the initiation of the experiment.

Figure A.1

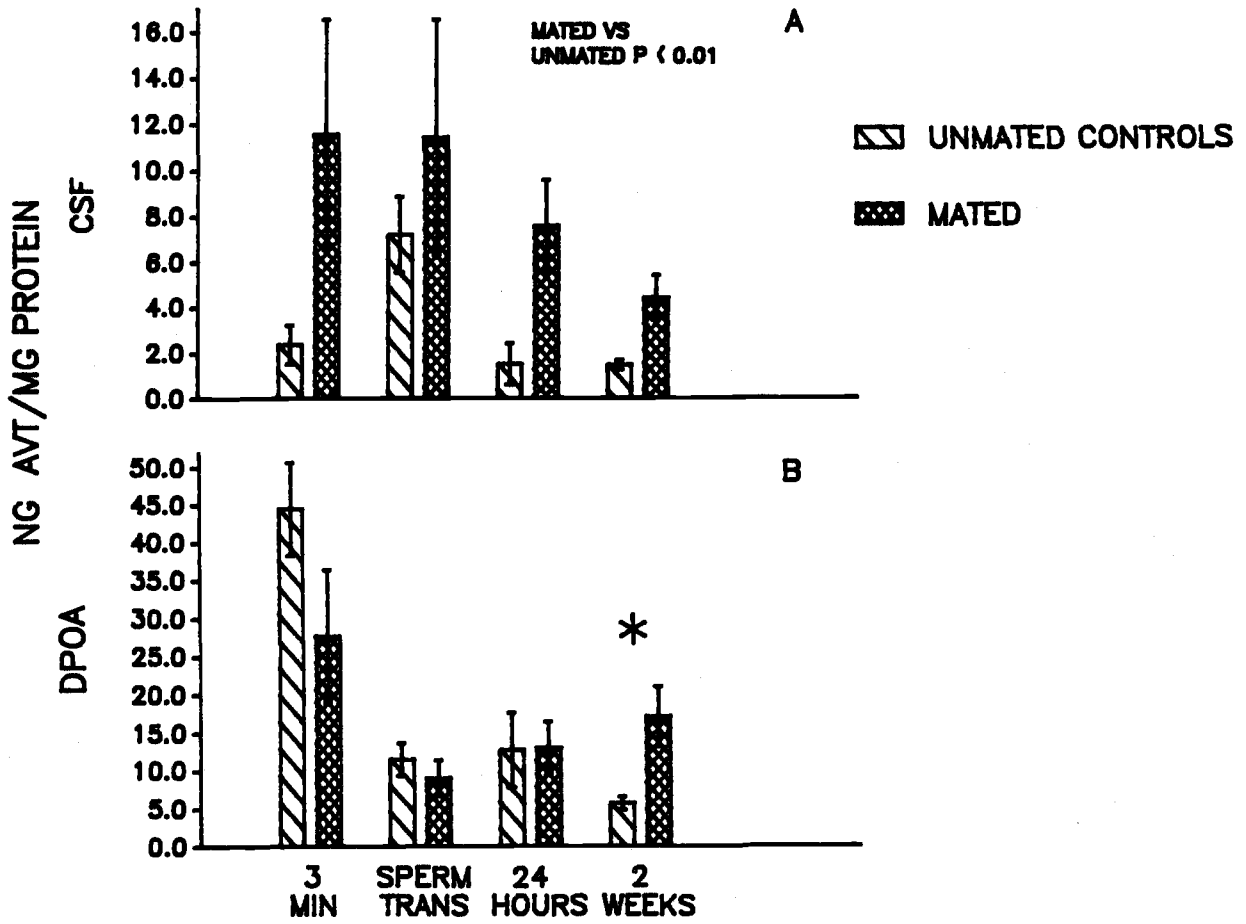

NG AVT/MG PROTEIN
CSF
DPOA
A
B
MATED VS
UNMATED P < 0.01
UNMATED CONTROLS
MATED
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
*
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS

Figure. A.2. AVT concentrations in the A) interpeduncular and B) optic tectum during and after courtship. There was no significant effect of mating or time on AVT concentrations in either brain region.

Figure A.2

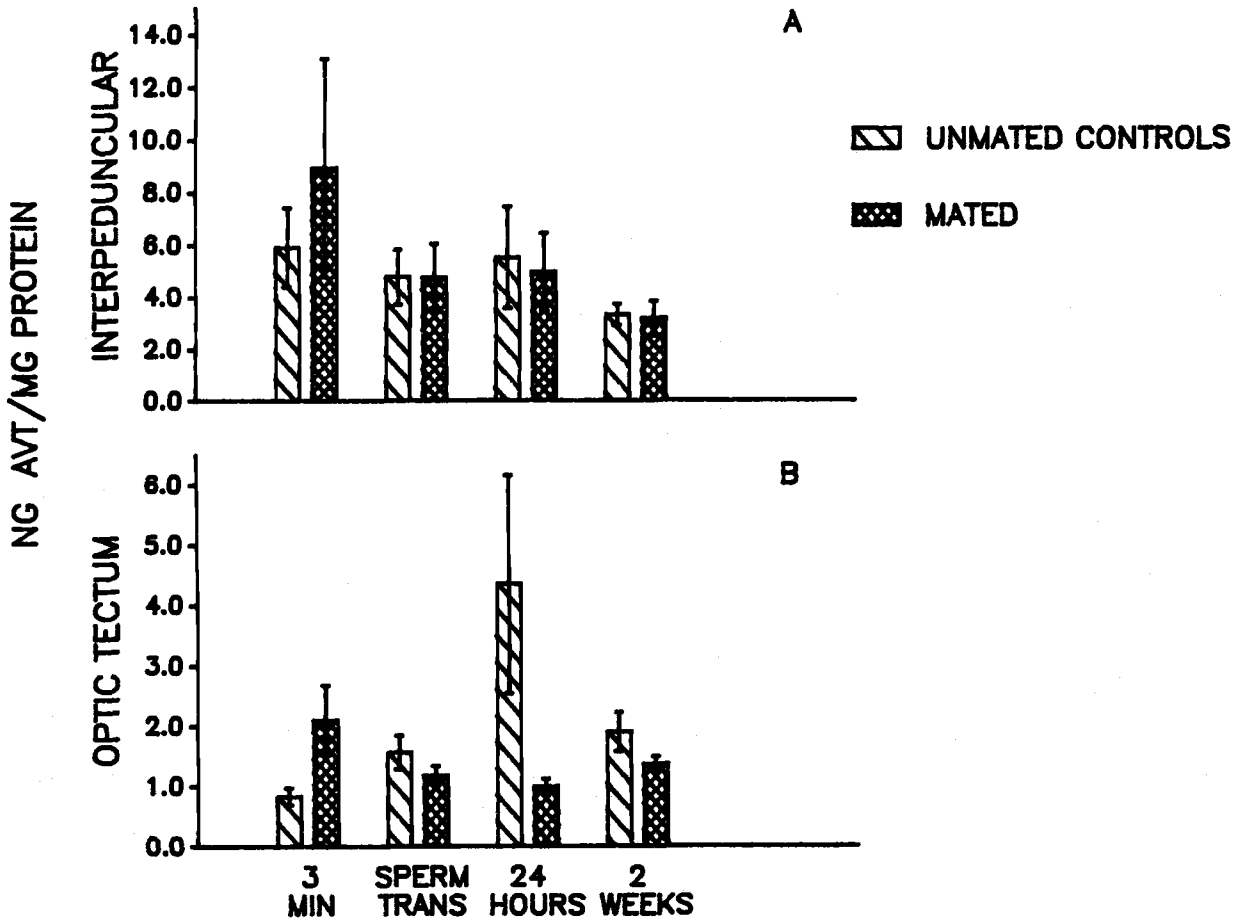

A
B
INTERPEDUNCULAR
OPTIC TECTUM
NG AVT/MG PROTEIN
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
UNMATED CONTROLS
MATED
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS

Figure A.3. AVT concentrations in the A) dorsal hypothalamic nucleus (DHN) and B) pars distalis (PD) during and after courtship. There was a significant effect of time ($F_{(3, 45)} = 2.91$; $P = 0.05$) but not mating in the DHN: irAVT concentrations decreased through time. There was no significant effect of mating or time on AVT concentrations in the PD.

Figure A.3

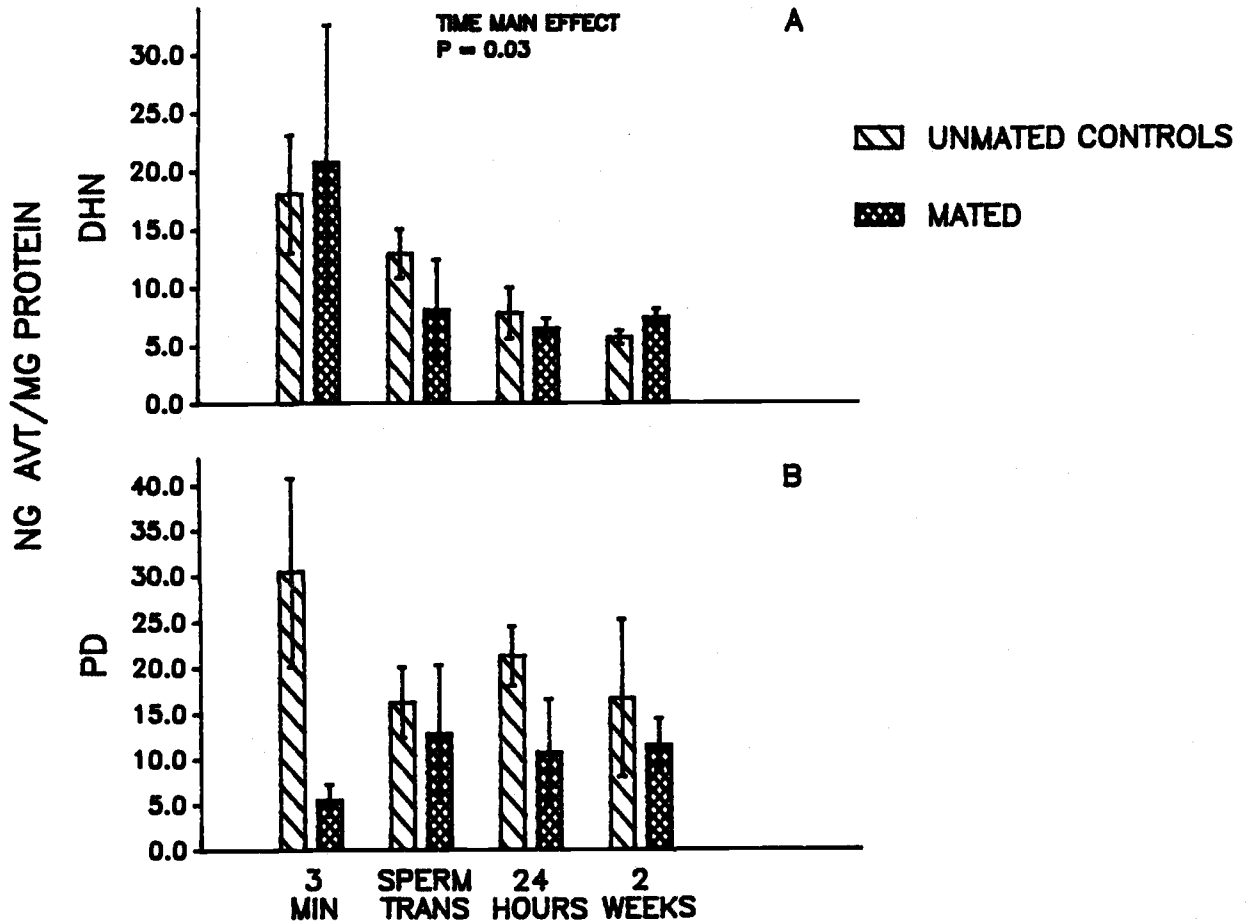

TIME MAIN EFFECT
P = 0.03
A
B
UNMATED CONTROLS
MATED
NG AVT/MG PROTEIN
DHN
PD
30.0
25.0
20.0
15.0
10.0
5.0
0.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS

Figure A.4. AVT concentrations in the amygdala during and after courtship. There was no significant effect of mating or time on AVT concentrations.

Figure A.4

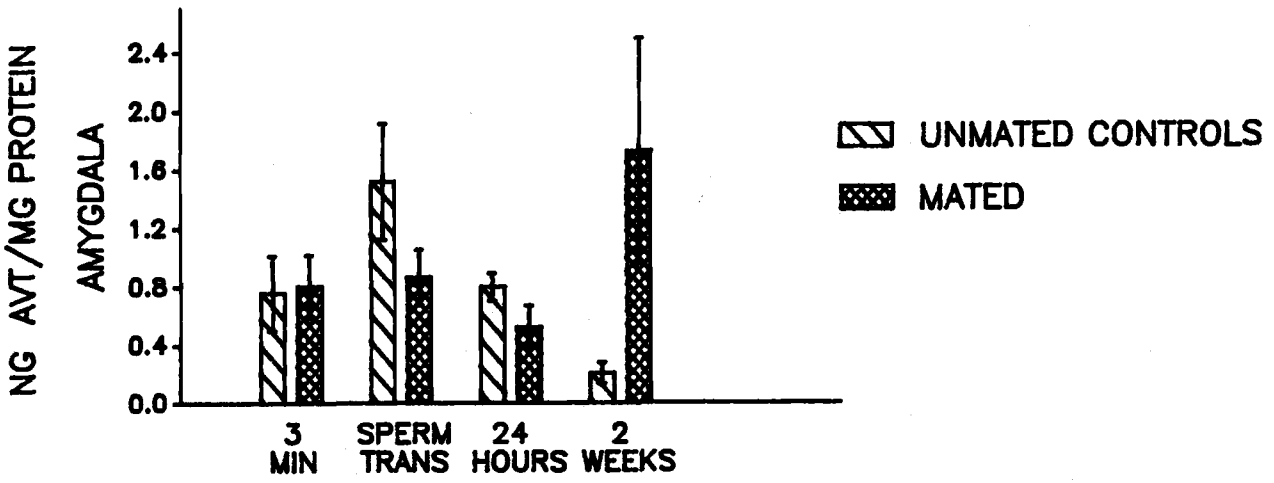

NG AVT/MG PROTEIN
AMYGDALA
2.4
2.0
1.6
1.2
0.8
0.4
0.0
3 MIN
SPERM TRANS
24 HOURS
2 WEEKS
UNMATED CONTROLS
MATED

Figure A.5. AVT concentrations in the A) DPOA and B) CSF during the early stages of courtship. There was a significant interaction between treatment and time in the DPOA ($F_{(2, 52)} = 3.15$; $P = 0.05$). The * indicates a significant difference in AVT concentrations between mated and control treatments at 5 minutes after the initiation of courtship. There was no significant effect of courtship or time on AVT concentrations in the CSF.

Figure A.5

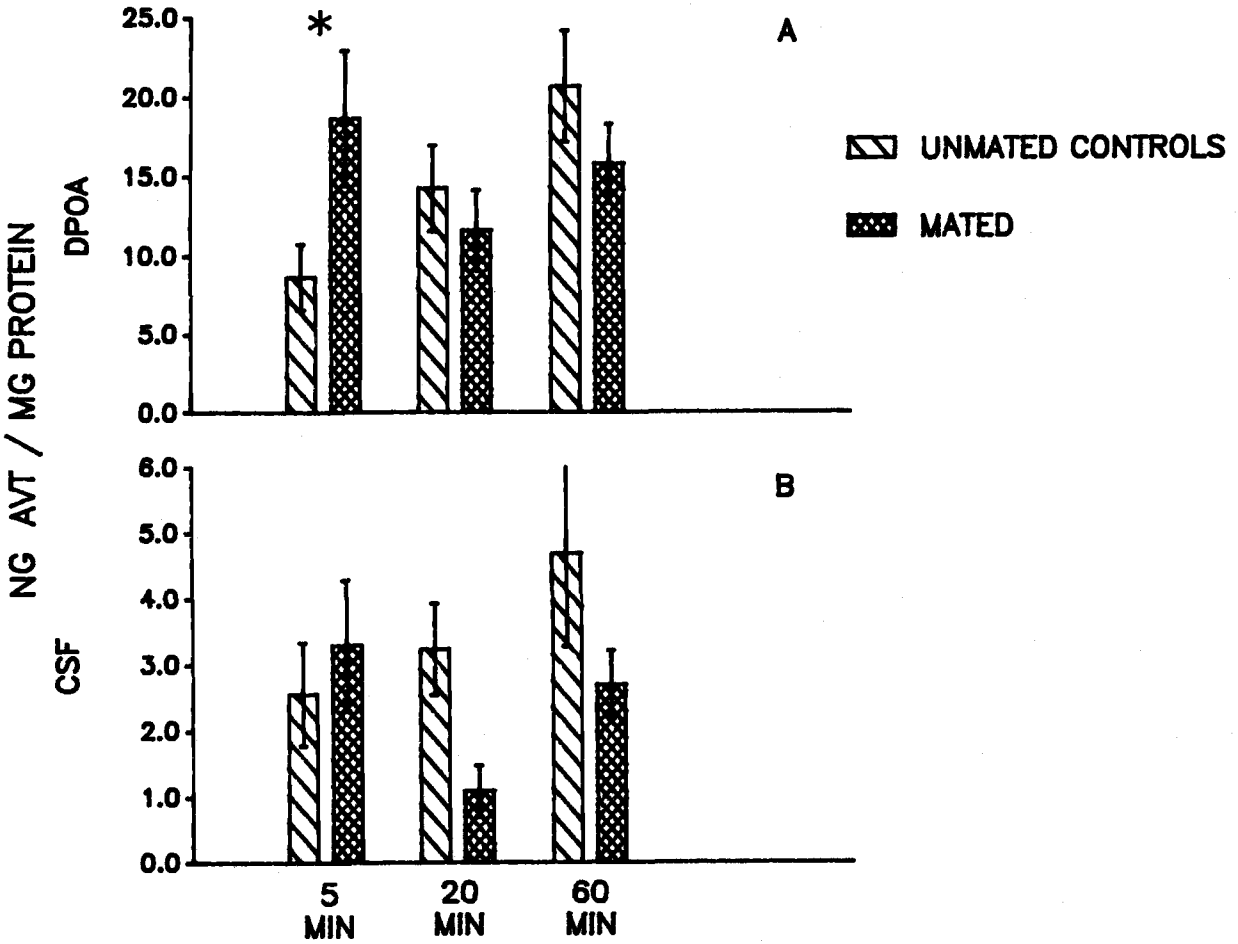
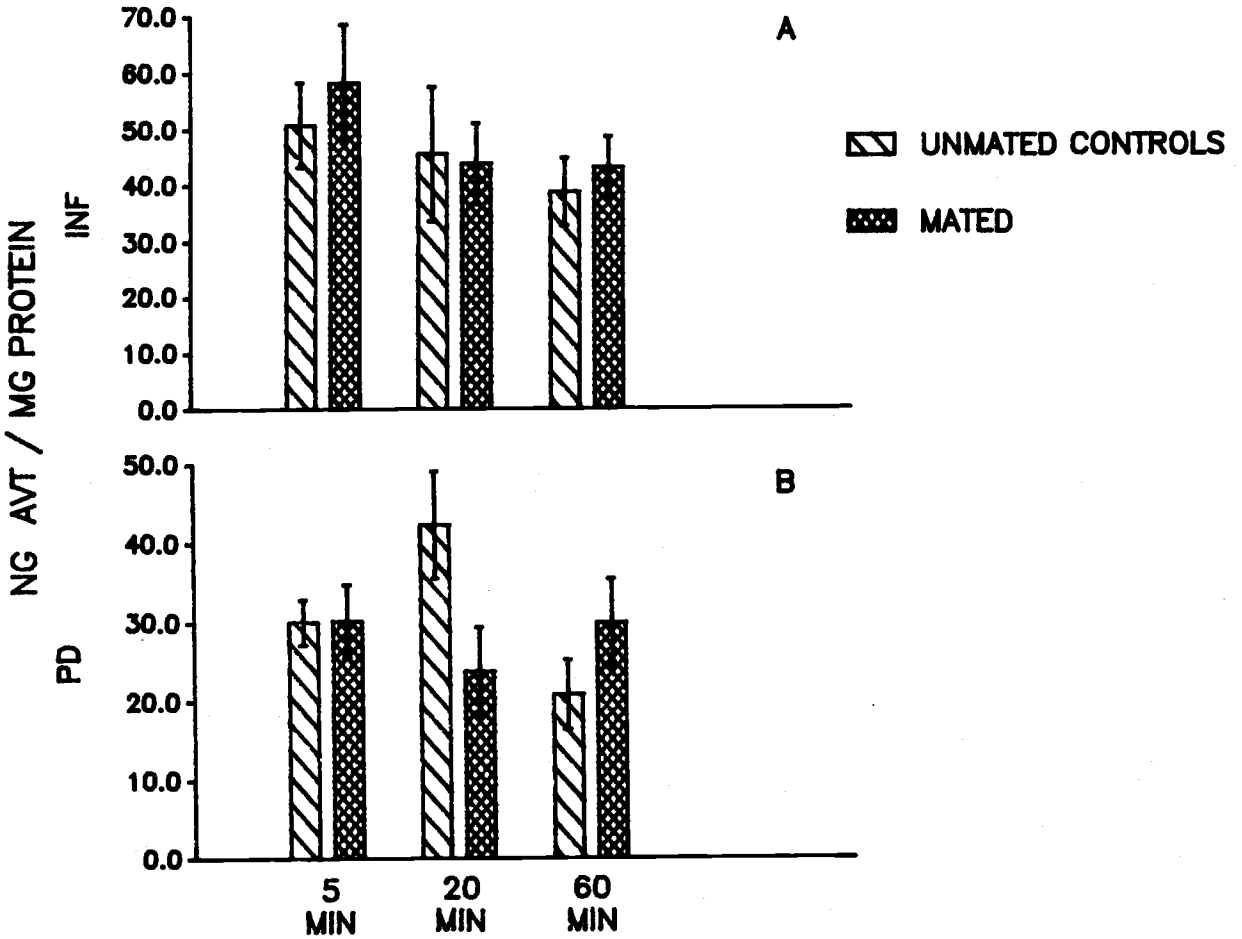

NG AVT / MG PROTEIN
DPOA
CSF
A
B
*
25.0
20.0
15.0
10.0
5.0
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
5 MIN
20 MIN
60 MIN
UNMATED CONTROLS
MATED

Figure A.6. AVT concentrations in the A) INF and B) PD during the early stages of courtship. There was no significant effect of mating or time on AVT concentrations in either brain region.

Figure A.6


NG AVT / MG PROTEIN
INF
PD
A
B
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
50.0
40.0
30.0
20.0
10.0
0.0
5 MIN
20 MIN
60 MIN
UNMATED CONTROLS
MATED