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Courting and Noncourting Male Red-Sided Garter Snakes, *Thamnophis sirtalis parietalis:* Plasma Melatonin Levels and the Effects of Pinealectomy

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Previous studies found that pinealectomy of male Canadian red-sided garter snakes (*Thamnophis sirtalis parietalis*) in the autumn, before prolonged exposure to low temperatures (hibernation), significantly impaired the expression of courtship behavior upon emergence in the spring. Additionally, pinealectomized animals with a disrupted diel cycle of plasma melatonin did not court while those exhibiting a more typical diel pattern did. These results suggested that the pineal gland functions in the transduction of a temperature cue which stimulates courtship. To test this hypothesis, we pinealectomized males in the spring after they had undergone a normal hibernation but were still courting. Pinealectomy of courting males in the spring, in each of the 3 years of study, had no effect on courtship. This result suggests that once the cue is transduced, the pineal gland no longer has a modulatory effect on courtship behavior. Finally, we took advantage of the fact that, in the laboratory, there is always a small percentage of males that do not court upon emergence. Pinealectomy of these noncourters greatly increased the percent of males expressing courtship behavior in each of the study years. Plasma melatonin levels of unmanipulated courting and noncourting males was measured after emergence in successive years. In both years, courters had a typical pattern of melatonin secretion (low in the photophase, high in the scotophase) while persistent noncourters displayed the opposite pattern.

In mammals, a variety of circadian rhythms (e.g., biochemical, physiological, behavioral) are under the control of a master pacemaker, the suprachiasmatic nucleus (SCN) of the hypothalamus (for review see Turek, 1985; Turek and Van Cauter, 1994). However, even the SCN is part of a network of multiple coupled circadian oscillators and, although it may be the master oscillator, studies indicate some modulation of circadian rhythms by melatonin, the product of the pineal gland (Turek, 1985; Rossenwasser and Adler, 1986; Cassone, 1990; Turek and Van Cauter, 1994). Unlike some lower vertebrates, the pineal gland in mammals does not have an autonomous circadian rhythm of melatonin secretion (Menaker, 1982). In several species of birds, reptiles, and fish, however, the pineal gland does play a major role in the organization of the circadian system. Additionally, its product, melatonin, promotes the coupling of multiple subordinate oscillators which may, in turn, be autonomously producing melatonin (e.g., retinae and other extra-pineal tissue; for review, see Rossenwasser and Adler, 1986; Cassone and Menaker, 1984; Cassone, 1990). Feedback among the oscillators is necessary for proper synchronization of circadian rhythms. Pinealectomy in many species of birds and reptiles can cause splitting or abolishment of circadian behavioral rhythms (e.g., locomotory, feeding, drinking, thermoregulatory, migratory restlessness), yet in other species there is no effect, indicating other centers of circadian control (McMillan 1972; Gwinner, 1978; Ralph, Firth, and Turner, 1979; Erskine and Hutchinson, 1981; Underwood, 1981; Underwood, 1983; Beldhuis, Dittami and Gwinner, 1988; Janik and Menaker, 1990; Ralph, 1983; Vivien-Roels, 1985; Foa, 1991; Refinetti and Menaker, 1992).

In addition to modifying activity patterns, the pineal gland is also implicated in the regulation of seasonal gonadal activity, especially in mammals. Many studies have found that it functions as a transducer of photoperiodic information and can thus have anti- or pro-go-

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nadal effects, depending on the species (Reiter, 1981, Cassone, 1990). In birds, the pineal gland does not have strong effect on gonadal development (Binkley, 1989; Cassone, 1990).

In reptiles, it has been shown to have pro- or antigonadal effects, again depending on species or timing of pinealectomy or melatonin administration (Misra and Thapliyal, 1979; Thapliyal and Haldar, 1979; Underwood, 1985; Haldar and Pandey, 1989a, 1989b). However, the central effects of the pineal gland or melatonin on sexual behavior in any vertebrate have been largely unexplored. Studies have indicated effects of melatonin on sexual behavior, but these effects are indirect and mediated through the activation of the hypothalmo-hypophyseal-gonadal axis (Arendt, Symons, Laird, and Pryde, 1983; Haresigan, 1990; Forsberg, Fougner, Hofmo, and Einarsson, 1992).

One species where a central effect of pinealectomy on sexual behavior has been found is the male red-sided garter snake from Manitoba, Canada. These males exhibit a dissociated pattern of reproduction: mating occurs when testes are regressed and androgen levels are basal (Crews, 1990). Males hibernate eight months of the year. Upon emergence, males exhibit vigorous courtship behavior in the presence of attractive females for a transient one to three week period. There is evidence that garter snake males have a second mating season in the fall as animals are returning to the hibernaculum. Females have been found with sperm in their oviducts (Whittier, Mason and Crews, 1987; Mendonça and Crews, 1989) and occasionally animals have been observed copulating at the hibernaculum in the autumn (M. Mendonça and A. Tousignant, personal observation). Extensive studies have indicated that the activation of courtship behavior by males is not under the control of androgens (Crews, 1990). While androgens are important in the long-term maintenance of courtship (Crews, 1991), the immediate cue that activates courtship appears to be the exposure of garter snakes to a prolonged period of cold with subsequent warming (Crews, 1990). In the laboratory, males rarely court unless exposed to this prolonged bout of low temperature. Therefore, any effect of pinealectomy would not be mediated indirectly through the hypothalamo-hypophyseal-gonadal axis but should have direct central nervous system effects.

Pinealectomy in the fall before hibernation inhibits courtship behavior in a majority of male garter snakes the next spring (Nelson, Mason, Krohmer, and Crews, 1987; Crews, Hingorani and Nelson, 1988; Mendonça, Tousignant, and Crews, 1996). This result suggested that pineal might function in the transduction of the period of low temperature information. However, in a 3-year study in which pinealectomy abolished courtship in the majority of males, approximately 40% of the treated animals consistently remained courters (Mendonça *et al.,* 1996). All of the pinealectomized animals had detectable levels of melatonin, indicating an extrapineal source in the garter snake. However, pinealectomized males that continued to court retained a diel melatonin rhythm, whereas the pinealectomized males that ceased courtship exhibited a disrupted cycle. This result suggests that the expression of sexual behavior in male snakes is dependent on the proper coordination of the pineal hierarchy.

In nature, all males apparently exhibit courtship after emergence when exposed to attractive females (although this has not been determined empirically). When males are artificially hibernated in the laboratory, typically 80–90% of the males court. However, in some years, up to 50% of males hibernated have not courted. Such males exhibit no interest in attractive females and are categorized as ‘‘noncourters.’’ Since pinealectomy disrupted the melatonin rhythm in some males and affected their ability to exhibit courtship activity, we postulated that noncourters also had a disrupted melatonin rhythm. We hypothesized the temperature cue of prolonged exposure to low temperatures had not been transduced properly in these noncourting males. We also wanted to further test the role of the pineal in modulating spring courtship *after* emergence from hibernation (thus after the assumed transduction of the hibernation period). That is, is the disruption of the signal only significant before or during hibernation or is its integrity also essential during courtship? The purpose of this paper is to explore what effect pinealectomy in the spring (after the hibernation period) had on courting males, and to determine if noncourting males exhibited a diel melatonin pattern that differed from those that courted.

METHODS

Animals and Housing

Adult male red-sided garter snakes, *Thamnophis sirtalis parietalis,* were collected in mid-September of 1987, 1989, and 1990 from the Interlake region of Manitoba, Canada. Animals were returned to the laboratory, weighed, the snout-vent length measured and abdominal scales clipped to identify individuals. Individuals were kept for 2 to 4 weeks at room temperature (approximately 24°C) and a 10:14 L:D cycle (lights went on at 0500, off at 1900). Two weeks before being placed in hibernation, they experienced a corresponding day:night temperature step-down regimen of 18:13°C for 1 week and then 13:8°C for an additional week. They were then placed in bags with moist sponges and kept in constant dark at 4°C for 17 weeks. They were then placed at room temperature (21–25°C) under a 12:12 L:D cycle (equivalent to the natural photoperiod length of Chatfield, Manitoba at the time of natural emergence in the spring). Lights came on at 0700 and went off at 1900. The housing conditions did not vary among the years. This manipulation is standard protocol in the laboratory to simulate hibernation. This treatment typically results in 80–90% of unmanipulated males courting after being removed from these conditions and placed at room temperature with attractive females (Crews *et al.*, 1984). Before and after the ‘‘step down’’ and hibernation periods, males (approximately 20/cage) were housed in 29 gallon aquaria with ad lib water. Males are aphagic at the lower temperatures and during their courtship period (Morris and Crews, 1990). After the courtship period, animals were fed chopped fish and earthworms supplemented by vitamins three times a week until being killed or returned to the field. Sham and treated males were equally mixed within the aquaria.

Surgery and Blood Collection

Males undergoing surgery were anesthetized by an intramuscular injection of Sodium Brevital (15 mg/kg body mass) in the neck. The skull was then drilled using a. 5-mm circular trephine bit at the juncture of the parietal and frontal scales (superior to the pineal) until reaching the brain meninges . Bone was removed from animals to be pinealectomized but kept in place for the sham animals. The blood sinus containing the pineal was exposed and a small slit made. The pineal was momentarily visible and removed by grasping the deep stalk with 5 Dumont forceps . Bleeding was stopped by placing Gelfoam over the sinus (Nelson *et al.*, 1987).

Plasma was collected from the caudal vein posterior to the vent. We incised the tip of the tail with a razor and let blood drip into a heparinized test tube. Bleeding was stopped by elevating the tail and applying pressure with a compress. Blood was then centrifuged, the plasma pipetted and frozen at −20°C for later analysis of circulating melatonin levels. Blood collection during the night (i.e., after 1900 and before 0700 hr) was done in total darkness. Individual animals were distinguishable by being in individual cages which were marked by different patterns of tape. Level of blood in tube was determined by holding test tube to a crack in the door. After eyes adapted to darkness, enough light penetrated from the darkened, exterior hallway to permit the determination of blood level in tube.

Behavior Testing

Recently emerged females, classified as ‘‘very attractive’’ from previous testing with intact, courting males, were placed with treated and sham males (two females/cage). Males were given as much as 15 min to court. Generally males court in the first minute of the females being placed in the cage and certainly upon first encountering the females. Males that had not courted by the end of the testing period had females placed directly in front of them and then allowed an extra 5 min to court. If there was no courtship in this interval, these males were given a 0 courtship score.

The intensity of male courtship was judged on the 0–2.5 scale using the criteria outlined in Camazine *et al.*, (1980). In brief, 2.5 indicated actual intromission while 0 indicates no reaction to the female. Males were classified as ‘‘courting’’ when they exhibited intense courtship, e.g., 2 or 2.5 on the scale on at least five consecutive days. This behavior consists of paralleling and closely following the female, while the male is ‘‘chin-rubbing’’ the female’s back and displaying rhythmic muscular contractions along the length of his body. Only animals that reached at least a 2 out of the 0–2.5 scale were classified as engaged in courtship. Only rarely are there intermediate values in scoring this behavior; males either court intensely or ignore the female. Intermediate values occur when females are unattractive or the breeding period is finishing (in the laboratory, approximately 3 weeks after emergence). This criteria resulted in an extremely conservative measure of courtship. Animals were tested for a 3-week period in 1989 and for 2 week periods in 1990 and 1991 when it became evident that courtship began to naturally decline in the third week after emergence.

Melatonin Assay

Assay protocol followed that of Heideman and Bronson (1990). Each plasma sample (50–100 μl) was extracted with 1.25 ml chloroform. An 1-ml aliquot of the extract was evaporated under nitrogen gas and resuspended in a TRIS buffer solution. An aliquot of a standard diluent (i.e., a 60% TRIS buffer and 40% charcoal-stripped rat plasma mixture) was also added to reduce nonspecific binding. Trial assays were previously conducted testing the efficacy of stripped rat plasma and stripped garter snake plasma. There was no significant difference in the binding curves or the accuracies obtained between the stripped rat and snake plasma (3 trials). Therefore, since rat plasma was more readily obtainable (and to prevent the sacrifice of garter snakes), stripped rat plasma was used for the standard diluent mixture. Melatonin antibody, obtained from Dr. J. Arendt, University of Surrey, Guilford, Surrey, United Kingdom, was added to the resuspended samples and to a melatonin standard curve at a 1:3500 dilution yielding approximately 30–35% binding. After 15 min, tritium labeled melatonin (Amersham) was added at a dilution that resulted in 14,000 cpm / 50 μl. The sample was vortexed and stored at 4°C for 12–15 hr. A charcoal TRIS buffer-gelatin solution was then added, incubated at 4°C for 15 min. Test tubes were centrifuged at 3000 rpm for 15 min. The supernate was poured off into liquid scintillation vial, scintillation fluid was added, vortexed, allowed to reach equilibrium for 6 hr, and then counted on a Beckman beta counter. Intra-assay variation was 5.9%; inter-assay variation was 15.1%. Sensitivity varied between years. In 1990 the assay was sensitive to 5 pg./ml. In 1991, sensitivity dropped to 10-12 pg./ml. Validation of the melatonin assay is described in Mendonça, Tousignant and Crews (1995). Briefly, parallelism was found among dilution curves of a known amount of melatonin and snake plasma extract whether a standard diluent of stripped snake plasma or stripped rat plasma was used. Additionally samples were sent to another laboratory and similar values found for individual samples (Mendonça *et al.,* 1995).

Histology

At the end of the behavior testing in 1990, a subsample of treated males were given a lethal injection of Sodium Brevital and perfused with reptilian Ringer's solution. Animals were decapitated and heads placed in Kolhmer's solution (a preservative and decalcifier). At the end of 2 weeks, heads were removed from the solution and most of the skull surrounding the brain was trimmed except for the bone around the surgery area. These brains were then embedded in paraffin and sectioned at 20 μ. Sections were stained with cresyl violet (Humason, 1972).

Statistical Analysis

The difference in number of courters vs noncourters in different treatment groups was analyzed using a Fisher's exact test. The continuity corrected significance statistic was used in cases of small sample size. Circulating melatonin plasma values were tested for heterogeneity of variances within groups. The variances were heterogeneous so all melatonin values were log-transformed to correct for this. A paired Students t test was used to compare the melatonin values for courters vs noncourters between sample periods and an unpaired Student's t test used to compare values between two treatments.

EXPERIMENT 1

Males were hibernated in Fall, 1987 for 17 weeks. When they emerged in Spring, 1988, they were tested for a number of days and categorized as courters (exhibited vigorous courtship daily for five days) vs noncourters (tested daily for either 10 or 15 days and never reached courtship criterion in any of their test days). Courters (n = 12), were either pinealectomized (PINX, n = 6) or sham-operated (SHAM, n = 6). On the same day, noncourters (n = 14) were also either PINX (n = 7) or SHAM (n = 7). The initial courter groups were then tested daily for courtship behavior for 16 additional days while the initial noncourter groups were tested daily for 13 days.

EXPERIMENT 2

Males were hibernated in Fall, 1989 for 17 weeks. Upon emergence, they were tested daily for 7 days and either categorized as courters (n = 25) or non-courters (n = 26) using the same criteria Experiment 1. These groups then underwent either a PINX and SHAM treatment (10 and 15 for courters and 10 and 16 for noncourters, respectively). The treated animals were then tested daily for courtship for an additional 14 days. A subset of unmanipulated courters and non-courters were bled at noon and midnight on the 10th day after emergence.

EXPERIMENT 3

Males were hibernated in Fall, 1990 for 17 weeks. Upon emergence, they were tested daily for 2 days. They were then categorized as courters and noncourters and bled at 0000 and 1200 hr. We continued to test them daily for 12 days after emergence, when courting males were pinealectomized (n = 6) or sham-operated (n = 7) as were noncourting males (PINX, n = 3; SHAM, n = 3). There were few non-courting males in the 1991 emergence. All males were tested daily for 10 additional days and then bled 2 weeks after their surgery.

RESULTS

Experiment 1

After surgery, a high proportion of courters (80–100%) continued to exhibit vigorous courtship behavior, regardless of treatment (Fig. 1A). In fact, PINX courters exhibited courtship for days after the SHAM animals experienced the natural decline in courtship seen in unmanipulated males, Of the males that had not exhibited courtship activity toward attractive females for 17 days, few animals courted within 2 days of surgery. However, by Day 4 after surgery (Day 22 post-emergence), 85.6% (6/7) males were courting (Fig. 1B). For the next 6 days 3–6 of these 7 males courted females vigorously. No more than 2/6 SHAM operated noncourting males ever exhibited courtship and this maximum number occurred within the first 3 days of surgery.

Experiment 2

In 1990, males were again ranked as courters and noncourters after 5 days of testing. They were either pinealectomized or sham-operated and again there was no decline in courtship activity in animals that were already courting but the frequency of the expression of courtship by the initial noncourters increased significantly (Fisher's Exact Test, $P = 0.045$; Fig. 2).

At 10 days after emergence, unmanipulated courters had low melatonin levels in mid-photophase and high melatonin levels in mid-scotophase, a typical pattern (paired t test: $t = 5.67$, $P = 0.002$, $df = 5$; Fig. 3). Noncourting males had low levels at both sample periods. These levels did not differ significantly from one another but night levels averaged lower than those observed in the day (paired t test: $t = -2.32$, $P = 0.07$, $df = 5$; Fig. 3). Mean melatonin levels for courting males at the 2000 sample were significantly higher than those of the noncourters ($t = 6.4$, $P = 0.0001$, $df = 10$), while samples obtained in the levels obtained from the 1200 sample did not differ significantly ($t = -0.61$, $P = 0.55$, $df = 10$).

The brains of a subsample of courting and noncourting PINX males were inspected to determine efficiency of the pinealectomy surgery. No PINX males, regardless of subsequent behavior, had a pineal gland. We did not detect the presence of a pineal stalk. Some males had damage to their choroid plexus but the presence or extent of choroid plexus damage did not appear to correlate to the presence or absence of courtship behavior.

Experiment 3

Pinealectomizing courting males had no significant effect on courtship (5/7 of PINX courted vs 4/6 of SHAMs). Pinealectomized non-courting males became courters (2/3 of PINX vs 0/3 of SHAM) but the actual behavioral response was not as robust as the previous year. Although these results are consistent with the previous years, caution must be used due to the low sample sizes. Melatonin levels before pinealectomy and shortly after emergence (2 days) again revealed that noncourters appeared to have a disrupted cycle (Fig. 4). At this time (shortly after emergence), melatonin levels were higher during the day than at night in noncourters, though this difference was not significant (paired t value $= -1.51$, $df = 7$, $P = 0.17$, $n = 8$), while the courters displayed the opposite pattern (paired t value $= 2.05$, $df = 7$, $P = 0.08$, $n = 8$). Mean melatonin values in courters were significantly higher than in noncourters at the 0000-hr sample ($t = -2.58$, $df = 14$, $P = 0.02$) but not at the 1200 sample ($t = -1.8$, $df = 14$, $P = 0.09$). Melatonin values after surgery were very low and many were below the sensitivity of the assay for 1991 (see Mendonça *et al.*, 1995 for discussion). Samples sizes, already small, were thus further reduced. There were night/day values for only one of the PINX noncourter males which became courters. This animal exhibited a normal (i.e., higher in the night sample than the day sample) pattern as did the three PINX males that remained courters for which we have values. All other samples were under the sensitivity of the assay.

DISCUSSION

The majority of male garter snakes do not exhibit courtship upon emergence from hibernation if they had been pinealectomized in the autumn (Nelson *et al.*, 1987; Crews *et al.*, 1988; Mendonça *et al.*, 1996). It has been suggested that the pineal acts as a transducer of temperature. Since the only manipulation known to stimulate the male to court is exposure to prolonged periods of low temperatures followed by subsequent warming, it has been proposed that the pineal is somehow involved with the transduction of this cue. The hypothesis that pinealectomy in the spring (when the cue had already been transduced) would not affect behavior was supported by the continued vigorous courtship of males pinealectomized in Experiments 1 and 2 after hibernation.

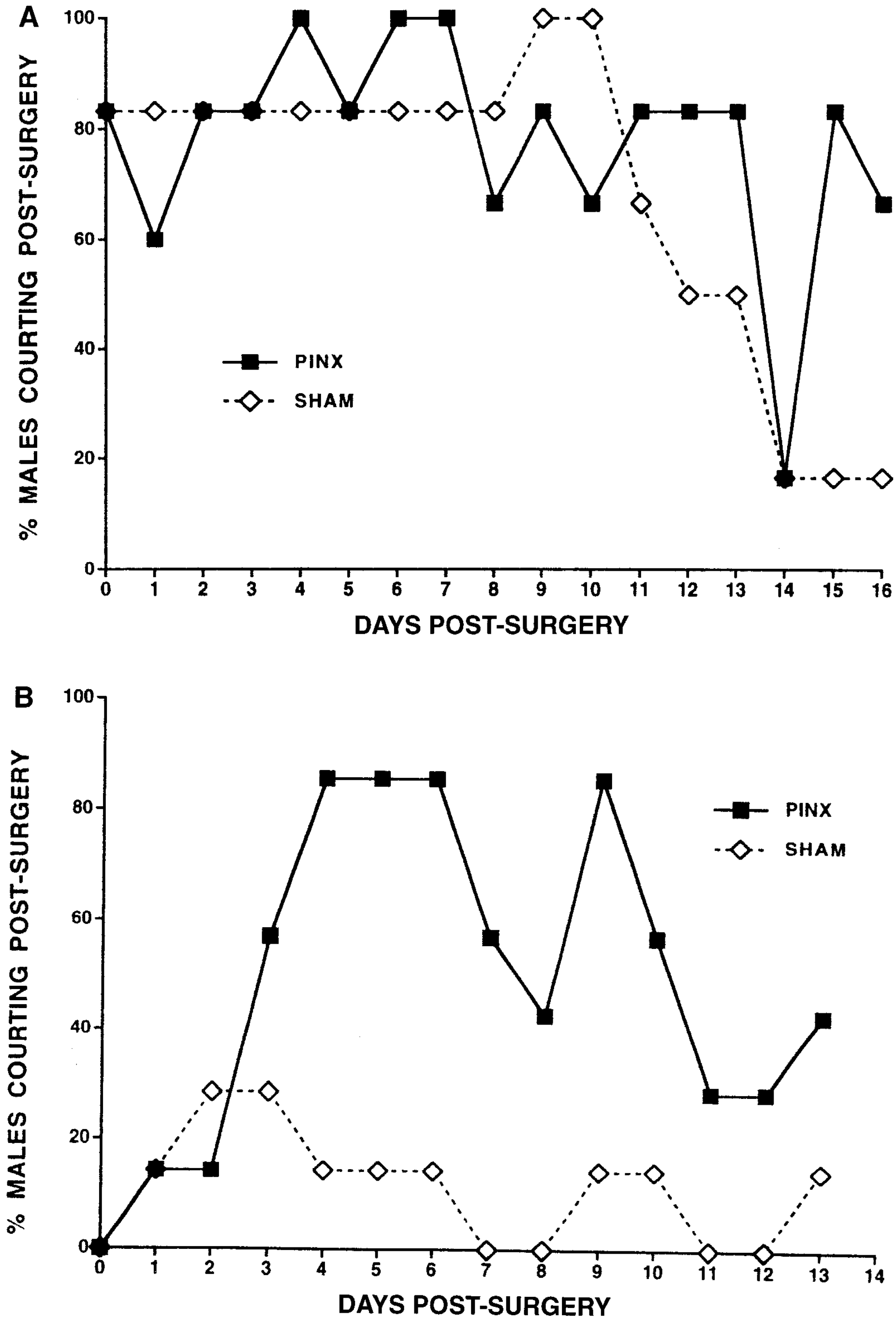

FIG. 1. Effect of pinealectomy on courtship behavior in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Panel A depicts the percentage of initial courters who courted each day after surgery (pinealecomized or sham-operated) in Spring, 1988. Panel B depicts the percentage of initial noncourters who courted each day after surgery (pinealecomized or sham-operated), Spring, 1988.

What was surprising was the effect of pinealectomy on noncourting males. In each of the 3 years of the experiment, pinealectomy of noncourting males increased their courtship activity; that is, they suddenly began to court frequently and vigorously. This is the first experimental manipulation besides altering temperature regimens that stimulated a noncourting male garter snake to exhibit courtship behavior (Gartska and

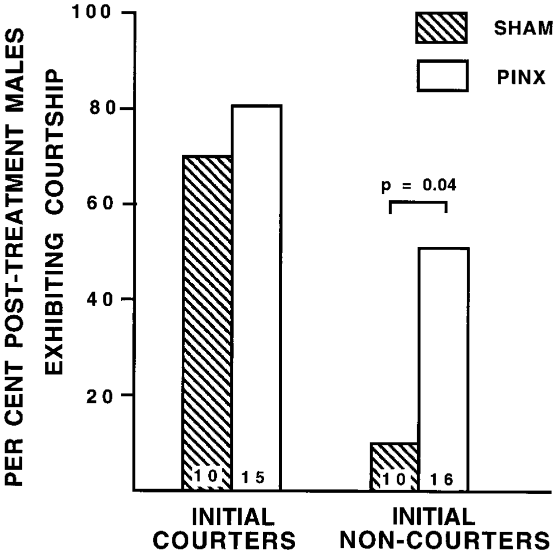

FIG. 2. Effect of pinealectomy on the behavior of courting and noncourting male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Depicted is the percentage of inital courters and noncourters who were classified as courters after surgery (pinealecomized or sham-operated) (Spring, 1990).

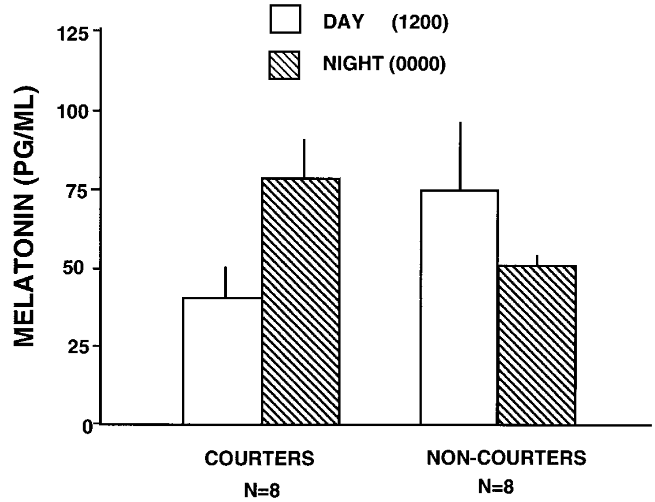

FIG. 4. Circulating concentrations of melatonin during the day and night in courting and noncourting male red-sided garter snakes (*Thamnophis sirtalis parietalis*) 3 days after emergence (Spring, 1991).

Crews, 1982; Khromer and Crews, 1988). Pinealectomy in the ruin lizard, *Podocaris sicula,* also induced a sudden transition in a behavior pattern (Innocenti, Minutini and Foa, 1994). In this case, pinealectomy of free-running lizards kept in constant darkness caused them to switch from a bimodal activity pattern displayed in the summer to a unimodal pattern usually seen in the autumn and spring (Innocenti *et al.,* 1994). It appears that, in this lizard, the pineal gland plays a central role in seasonal reorganization of the circadian system and melatonin may function in coupling of multiple circadian oscillators to insure the proper seasonal pattern of locomotor behavior (Underwood, 1981, 1983; Innocenti *et al.,* 1994). The pineal gland in garter snakes may also function in modulating the seasonal expression of courtship behavior.

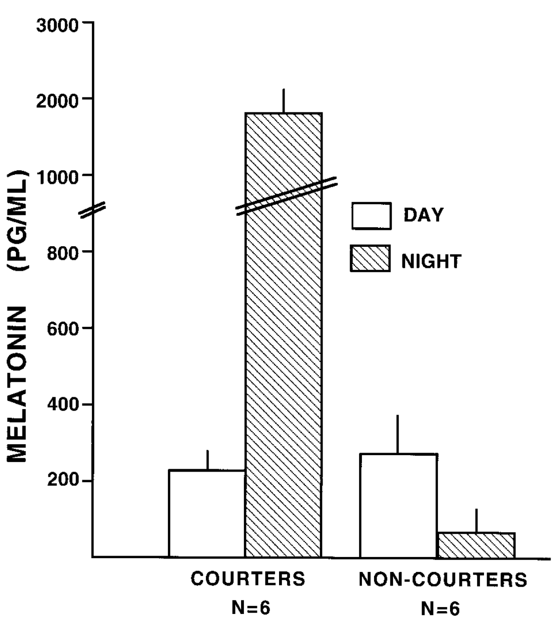

FIG. 3. Circulating concentrations of melatonin during the day and night in courting and noncourting male red-sided garter snakes (*Thamnophis sirtalis parietalis*) 10 days after emergence, Spring, 1990.

Noncourting males can become courting males naturally in the laboratory. In some years, for the first few days, males do not court when placed with attractive females (Mendonça *et al.,* 1995). Some males never court in the entire (3 week) mating period but most eventually exhibit courtship behavior. The percentage of males that court varies among years as does the rate they express courtship. For example, in 1990 and 1991, on the day of emergence, 37% vs 77% of the males courted. It was not until the third day after emergence that percentages did not differ significantly (86% vs 96%, in 1990 vs 1991 (Mendonça *et al.,* 1995). In 1990, the diel melatonin pattern was disrupted upon emergence, while in 1991, males exhibited a typical pattern (Mendonça *et al.,* 1995). Therefore, it appears that some males respond differently to the change in environment. Garter snakes appear to have an extra-pineal source of melatonin. When pinealectomized in the autumn and then sampled in the spring, some pinealectomized animals do not court while others do so vigorously. Pinealectomized courters exhibit a more typical diel pattern of circulating melatonin levels, higher in the scotophase than in the photophase while pinealectomized noncourters do not (Mendonça *et al.*, 1996). We did not test the source of the extrapineal melatonin but the percent of pinealectomized males exhibiting courtship increased significantly when they were kept in constant darkness (by covering their eyes, Mendonça, Tousignant, and Crews, unpublished data) suggesting the retina plays a role. It may be that different oscillators have different rhythms of melatonin production but the pineal gland is the prime synchronizer. If animals have different rates of acclimation to the temperature change (or each individual oscillators has a different rate of acclimation), this may affect the diel pattern of melatonin secretion. Alternatively, there may be genetic differences among individuals. Again it appears if melatonin levels, whatever the source, have the proper pattern, males will court. It may be that the pineal gland synchronizes all oscillators and allows the proper pattern to be expressed.

Naturally noncourting males lacked a normal diel pattern of melatonin when sampled either just a few days or 10 days after emergence (when the mating period is about half over). Only two other studies have found a reversal of the diel pattern of melatonin (higher in the photophase than in the scotophase). Trout retinae were found to have higher levels of melatonin in the day than at night as does the pineal gland of pigs kept at natural photoperiods (Gern, Owens, and Ralph, 1978a; Reiter, Britt, and Armstrong, 1987). We are unaware of an equivalent study to determine if animals with behavioral polymorphisms (or dysfunctions) have differences in their diel melatonin cycle.

Individuals of this species appear to vary in their ability to exhibit a normal diel pattern of melatonin and this variation appears to translate into whether or not there will be expression of sexual behavior. It may be that these animals are not transducing the cue from prolonged exposure to temperature properly or these animals may be the individuals that exhibit courtship behavior in the autumn rather than the spring and thus react to either environmental stimuli.

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