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Pinealectomy, Melatonin, and Courtship Behavior in Male Red-Sided Garter Snakes (*Thamnophis sirtalis parietalis*)

MARY T. MENDONÇA, ALAN J. TOUSIGNANT, AND DAVID CREWS
Institute of Reproductive Biology, Department of Zoology (M.T.M., A.J.T., D.C.) and Department of Psychology (D.C.), University of Texas at Austin, Austin, Texas 78712

ABSTRACT Activation of courtship behavior in male red-sided garter snakes is independent of androgens. Only exposure to extended periods of low temperature with subsequent warming stimulates courtship in males. The pineal gland is thought to transduce temperature as well as photoperiodic information in reptiles. Therefore, we explored the relationship of the pineal and melatonin to sexual behavior in this species.

Pinealectomy of male garter snakes disrupted sexual behavior upon emergence from a 17-week period of low temperature in approximately 60% of treated individuals in each of the 3 years of study. However, 40% of the males were unaffected by the pinealectomy, engaging in vigorous courtship. Administration of exogenous, chronic melatonin did not significantly modulate the effect of pinealectomy. Upon pinealectomy in the autumn (before hibernation), plasma levels of melatonin fell. However, upon emergence from hibernation, melatonin levels in pinealectomized (PINX) and sham-treated (SHAM) animals were equivalent, indicating extrapineal source(s) of melatonin. However, PINX males did not exhibit a diel cycle in melatonin levels upon emergence. Instead, melatonin remained elevated through the subsequent 24-hr period. SHAMs did exhibit a diel cycle. Ten days after emergence, PINX animals either had a disrupted/abnormal melatonin cycle and were non-courters or had a cycle similar to SHAM males and courted. Therefore, a normal diel cycle of melatonin appeared necessary for the proper expression of courtship behavior. These results suggest that the pineal in snakes 1) is part of a complex, multi-oscillator system as it is in birds and lizards and 2) may play a role in maintaining polymorphism in timing of reproductive behavior.

The pineal gland, via its hormone melatonin, is a major neuroendocrine transducer of photoperiod in birds and mammals, affecting the circadian rhythmicity of various locomotor, feeding, and drinking activities, as well as seasonal reproductive cycles (see Reiter, '81; Binkley, '88, for reviews). In ectotherms, such as reptiles, the pineal transduces changes in both temperature and photoperiod. The amplitude of the diel melatonin rhythm is increased by high temperatures and attenuated by low temperatures (see Underwood, '92, for review). In these animals the pineal has been implicated in modulating thermoregulatory behaviors as well as affecting activity periods and reproductive cycles.

The effects of melatonin on reproduction in all of the studied species have focused on its role in gonadal function. Melatonin can be antigonadal or progonadal depending on the time of year the manipulation was done, the age of the animal, or the species (Reiter, '81; Binkley, '88). These species all have associated patterns of reproduction: mating occurs at peak gonadal activity (Crews et al., '84). Melatonin thus affects reproductive behavior per se indirectly: If gonads fail to recrudesce because of high melatonin levels, sex steroids remain low and mating is not activated. Very few studies have examined melatonin's direct effect on sexual behavior in species with either an associated or dissociated (mating occurs when gonads are regressed and sex steroids basal) pattern of reproduction (Baum, '68; Nelson et al., '87; Crews et al., '88).

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Mary T. Mendonça is now at Department of Zoology and Wildlife Science, Auburn University, Auburn, AL 36849. Address reprint requests there.

Red-sided garter snakes from Manitoba, Canada exhibit a dissociated reproduction pattern. Since they are in hibernation from late September to early May, they have a short breeding season. Males emerge from hibernation with regressed testes and low levels of testosterone and stay at the hibernaculum awaiting the emergence of females. Females emerge soon after. Upon encountering a female, several to many males will court her vigorously and one of them will be allowed to mate. Males only exhibit courtship behavior upon encountering females for the 3-week period after emergence. Extensive research has been conducted to determine the factors responsible for stimulating courtship behavior in male red-sided garter snakes. Numerous studies have demonstrated that gonadal steroids are *not* the activating factors (Camazine et al., '80; Gartska et al., '82) although testosterone is necessary for long-term maintenance of the behavior (Crews, '91).

Numerous factors have been tested to determine what can activate or inhibit courtship (Gartska et al., '82). The only successful factor to date in stimulating courtship behavior has been exposure of the males to low temperatures (4°C) for a minimum of 12 weeks and then exposure to higher temperatures (i.e., 20–25°C, Whittier et al., '87a). The only treatment that has inhibited courtship after such a regimen has been pinealectomy. Pinealectomy of males in the spring or fall *before* hibernation disrupts the expression of courtship behavior upon emergence the next spring (Nelson et al., '87; Crews et al., '88). It was suggested that the pineal gland may be transducing temperature cues rather than photoperiod cues in these animals, and this transduction initiated courtship behavior. This study explores further what role the pineal gland and its secretory product, melatonin, may be playing in the control of courtship behavior in male red-sided garter snakes.

MATERIALS AND METHODS

Animals and housing

Adult males were collected from Chatfield, Manitoba, Canada in mid-September of 1989 and 1990. In both years, they were transported to our laboratory, weighed, the snout-vent length was measured, and they were individually marked by scale clipping. Males were housed in 29-gallon aquaria (20/aquarium) for 2–4 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 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 returned to the aquaria 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 between years. This protocol has been used in previous studies of reproduction in this species and has proven successful in mimicking natural conditions in stimulating the normal expression of sexual behavior (i.e., 80–95% of unmanipulated males court upon emergence, Crews et al., '84).

Males in aquaria had ad libitum water but are aphagic at the lower temperatures before hibernation and during their courtship period. After the courtship period, animals were fed chopped fish and earthworms supplemented by vitamins three times a week. Sham and treated males were equally mixed within the aquaria.

Surgery and blood collection

Males undergoing surgery were anesthetized by an intramuscular injection in the neck of sodium brevital (15 mg/kg body mass). 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 was made. The pineal was momentarily visible and was removed by grasping the deep stalk with #5 Dumont forceps. Bleeding was stopped by placing Gelfoam over the sinus (Nelson et al., '87).

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. Blood was then centrifuged, and 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 0600 samples) 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 the test tube to a crack in the door. After visual adaptation 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/aquaria and ten males/aquaria). Males were given as much as 15 minutes to court. Generally males court in the 1st min of 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 were allowed an extra 5 min to court. The intensity of male courtship was judged on the 0–2.5 scale using the criteria outlined in Camazine et al. ('80). 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). This behavior consists of paralleling and closely following the female, while the male is "chin-rubbing" the female's back (male's chin is closely pressed to female dorsum) and displaying rhythmic muscular contractions along the length of his body. A "courter" was a male that had at least 5 consecutive days of a score of 2.0 or above in the 14 or 21 consecutive days of behavior testing. There are rarely intermediate values in scoring this behavior; males either court intensely or ignore the female. Intermediate values occur when females are unattractive or the courtship period is finishing (in the laboratory, approximately 3 weeks after emergence). These criteria resulted in an extremely conservative measure of courtship. In 1989, males were tested every day for 21 days. However, it became clear that courtship diminished in all groups after 2 weeks. Therefore, in 1990 and 1991, animals were consecutively tested for a 14-day period. Animals were always tested between 0900 and 1100.

Histology

At the end of the behavior testing in 1989, 1990, and 1991, 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 were 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 μm. Sections were stained with cresyl violet (Humason, '72).

Melatonin assay

Assay protocol followed that of Heideman and Bronson ('90). Each plasma sample (50–100 μl) was extracted with 1.25 ml chloroform. A 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 (three 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 1:3,500 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 minutes. Test tubes were centrifuged at 3,000 rpm for 15 min. The supernate was poured off into liquid scintillation vial, and scintillation fluid was added, vortexed, allowed to reach equilibrium for 6 hours and then counted on a Beckman beta counter. Intra-assay variation was 5.9%, and 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 was based on the following parameters. Garter snake plasma was charcoal stripped following the protocol of Heidemann and Bronson ('90) and then measured for melatonin. Melatonin was below detectable levels in the stripped plasma using the Arendt antibody. We added known amounts of melatonin to the stripped snake plasma and stripped rat plasma and accurately measured (within 5%) the amount that was added. Five serial dilutions were made of extracted snake plasma+stripped snake plasma:TRIS buffer, ex-

tracted snake plasma+stripped rat plasma:TRIS buffer, and a melatonin standard+stripped rat plasma:TRIS and obtained parallelism (Fig. 1). Serial dilutions of the pools of stripped snake plasma+added melatonin and stripped rat+added melatonin also yielded parallel lines.

The relative levels of plasma melatonin in males sampled in the springs of 1990 and 1991 differed from one another. Males in 1990 had levels approximately five times higher than those recorded in 1991. Melatonin values from 1990 were reassayed in 1991 to determine if assay parameters had changed. Values were within 90% of one another. To further validate the perceived difference in years and the absolute levels themselves, the plasma samples (n = 8) from both years were sent to Dr. Andrew Loudon (University of Virginia) to be retested by a radioimmunoassay technique which employed iodinated melatonin, another antibody (R1055), and followed the procedure detailed in Rollag and Niswender ('76). Values of the two laboratories had a mean coefficient of variance ((standard deviation × 100)/mean) of 12.4% ± 6.1 (Mendonça et al., 1995). Therefore, the difference between the years in absolute levels appears valid.

Statistical analysis

The difference in number of courters vs. non-courters in different treatment groups was analyzed using a χ^2 or Fisher's exact test depending on the number of treatments. 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 t-test was used to compare courters vs. non-courters within a treatment group, and Student's t-test was used to compare values between two treatments. A two-way repeated-measures analysis of variance (ANOVA) was used to compare melatonin values in the 24-hour bleed treatments (Sokal and Rolhf, '81).

Experiment 1: Effects of melatonin implants on courtship behavior

In October 1988, we pinealectomized (PINX) or sham-operated (SHAM) males (n = 120, 70 respectively). They were divided into six different treatment groups. PINX males received either one blank silastic capsule (PINX/BLANK) or one 1/2 capsule (same sized capsule but only half-filled

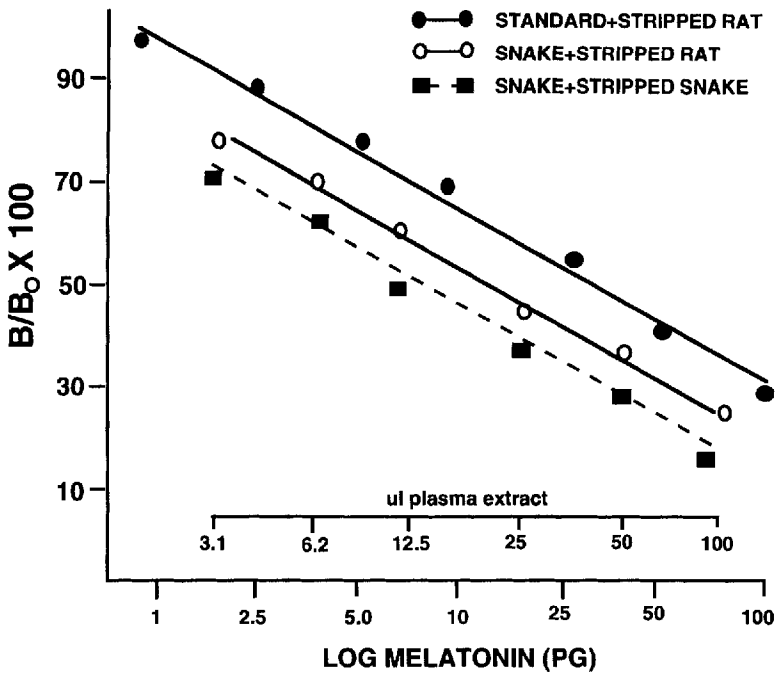

Fig. 1. Parallel dilution curves of known amount of melatonin plus stripped rat plasma: TRIS buffer, extracted snake plasma diluted with stripped snake plasma plus TRIS buffer, and extracted snake plasma diluted with stripped rat plasma plus TRIS buffer.

with melatonin, PINX/1/2 MEL), or one filled capsule (PINX/MEL), or three filled capsules (PINX/3 MEL) (n = 40/group). SHAM males immediately received either a single blank (SHAM/BLANK) or melatonin-filled (PINX/MEL) capsule (n = 35/group). Males were hibernated and tested daily for courtship behavior for 21 days after emergence in February 1989. Blood was collected in mid-afternoon 10 days after emergence. At the end of the overall testing regimen (21 days), a subsample of the males' brains were taken to determine the efficacy of the pinealectomy surgery.

Experiment 2. Endogenous night/day melatonin levels of pinealectomized courters and non-courters 10 days after emergence

In October 1989, additional males were PINX (n = 30) or SHAM (n = 16). A subsample of animals were bled at 0000 of the night before surgery and then two nights after surgery (again at 0000). Manipulated animals were hibernated and tested for courtship behavior upon emergence in February 1990. At the end of 10 days of behavior testing, blood was collected from courting SHAM males (n = 8) and courting and non-courting PINX males (n = 8/group) at 2100 and 0900 hr. Again, the brains of a subsample of males were taken after the behavior testing ended.

Experiment 3. Endogenous melatonin cycle upon emergence and after 10 days for pinealectomized and sham courters and non-courters

In October 1990, males were PINX (n = 40) or SHAM (n = 25). Males were hibernated and tested for courtship behavior upon emergence in February 1991. Upon emergence (1500 hr), a subsample of PINX and SHAM males (n = 8/group) were bled every 4 hours from 1600 to 1600. Another set of PINX and SHAM animals emerged, were behavior tested daily for 10 days, scored as courters vs. non courters, and then bled on the 10th day at 1200, 0000, 0400, 1200, 1600, and 2000 hr. Brains were again taken from a subset of courting and noncourting PINX males.

RESULTS

Experiment 1: Effects of melatonin implants on courtship behavior

Figure 2 indicates the percentage of males classified as courters in each treatment group after 21 consecutive days of testing. In each of the SHAM treatments (SHAM/BLANK and SHAM/MEL), 80% of the males courted. This is the average frequency of courtship in intact males. These two sham frequencies did not differ significantly from one another (P = .71). The frequencies of males from the PINX groups courting did differ significantly from those of the SHAM groups (P = .0001) but not from one another (P = .14). Although the PINX groups did not differ significantly from one another, there appeared to be a very slight dose effect of the melatonin implants (Fig. 2).

Subsequent melatonin analysis of plasma from animals receiving melatonin implants showed levels well over 100 ng/ml, whereas animals receiving blank capsules had low levels of melatonin when bled in the mid-afternoon 10 days after emergence (SHAM BLANK x = 10.7 pg/ml, n = 7).

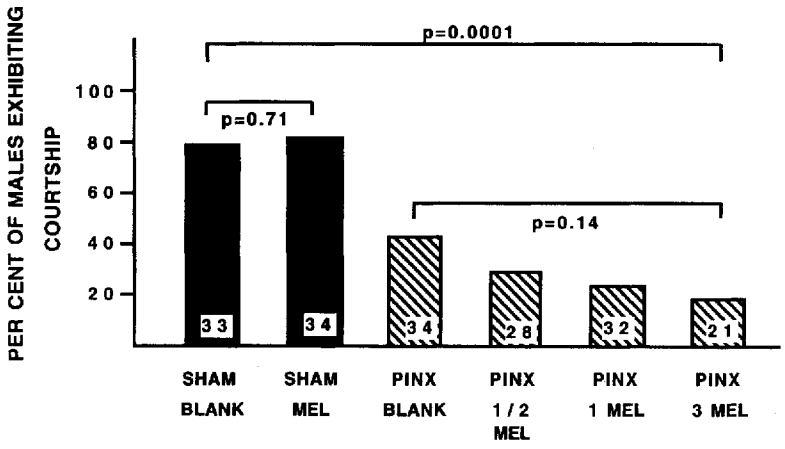

Fig. 2. Effects of different doses of melatonin implants on frequency of intact, sham, and pinealectomized male red-sided garter snakes exhibiting courtship behavior in spring 1989. Sample sizes are indicated at the base of each bar.

The brains of courting and non-courting 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 2: Endogenous night/day melatonin levels of pinealectomized courters and non-courters 10 days after emergence

Because of the significant proportion of apparently pinealectomized males exhibiting courtship behavior in the PINX/BLANK capsule group in spring 1989 (experiment 1), the Nelson et al. ('87) protocol (which did not include capsule implants) was repeated in fall 1989/spring 1990. Again, 42.8% of PINX males emerging in the spring courted vigorously (9/21 PINX males courted vs. 14/16 SHAM; P = .006). None of the PINX animals (n = 11) subsampled for histology had an obvious remnant of the pineal gland, nor was there any relation between choroid damage and the expression of courtship.

The night after the surgery, PINX males had significantly lower melatonin values when sampled at a single point (Fig. 3). However, when sampled months later, after emergence, melatonin was again detectable in males of this treatment group. Single sample night/day melatonin levels indicated that PINX courters exhibited high levels of melatonin at "night" (the 2100 sample) and low day levels (0900), and they differed significantly (P = .05). Melatonin levels of PINX noncourters, on the other hand, did not differ between the night and day samples (P = .37; Fig. 4). Courting SHAM males in this sample also demonstrated a day/night rhythm in melatonin (Fig. 4). However, due to large standard deviation, the difference in night/day levels was not statistically significant (P = .07). From other measures of intact and SHAM animals the normal finding has been that males with pineals do show a difference in diel melatonin levels (Mendonça et al., 1995). Therefore, PINX courting males are more like SHAMs than their noncourting counterparts. It appeared that PINX noncourters did not cycle or, at the very least, were not exhibiting the same cycle as PINX courters.

Experiment 3: Endogenous melatonin cycle upon emergence for pinealectomized and sham courters and noncourters

Again a substantial portion of PINX males courted (6/13, 46.1%), close to the extremely consistent 40% value. The subset of sampled brains (n = 11) again indicated that none of the PINX

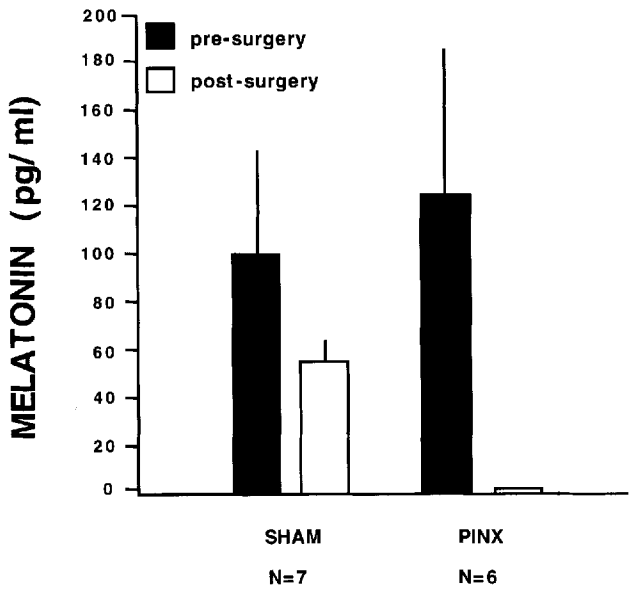

Fig. 3. Mean plasma melatonin levels before and one night after pinealectomy.

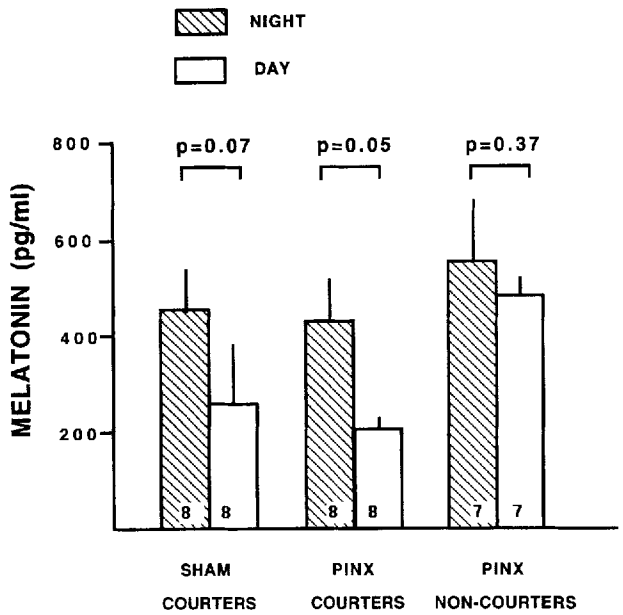

Fig. 4. Mean night and day levels of plasma melatonin in sham courting males, pinealectomized courting males, and pinealectomized non-courting males (±1 standard error), in spring 1990. Sample sizes are indicated at the base of each bar.

animals had obvious pineals nor was there an apparent correlation between choroid damage and courtship behavior.

Bloods taken every 4 hr 24 hr after emergence indicated that PINX animals did not exhibit a diel cycle upon emergence while SHAM animals did (Fig. 5). At the first blood sample, taken 1 hr after emergence, PINX animals had significantly higher melatonin levels than the SHAMs ($P = .04$). However, the SHAMs eventually had their highest levels of melatonin at the 0000 and 0400 sample period. These values were significantly higher than those at other sample times ($P = .05$) of the SHAM group. The PINX animals exhibited no cycle; the values were not significantly different from one another ($P = .59$). A two-factor repeated-measure ANOVA found that the pattern for the PINX group and the SHAM group differed significantly from one another ($P = .05$).

We had a second SHAM/PINX group that was allowed to emerge, tested with females for 10 days, and categorized as courters or noncourters. We then bled these animals at 4-hr intervals from midnight until 2000 the next evening. This more rigorous collection regime seemed to exhibit a different pattern than the same treatment groups in 1990. Instead of PINX noncourters having similar day/night levels, they exhibited a pattern that was 180 degrees out of phase with that displayed by the SHAMs. PINX courters, on the other hand, more closely followed the pattern of SHAM courting males (Fig. 6). It must be strongly noted that sample sizes were very low and none of the treatment groups differed significantly from one another. It did appear, however, that PINX non-courting males again had a disrupted daily melatonin rhythm.

DISCUSSION

There is a lack of studies of the effects of the pineal gland and melatonin levels in reptiles in general and in snakes in particular (Underwood, '92). Endogenous levels of serum melatonin have been described for only one other species of snake, the diamondback water snake, *Nerodia rhombifera* (Tilden and Hutchinson, '93). The work that has been done in reptiles (the vast majority of which deals with lizards and turtles) had focused on the pineal's effects on circadian locomotor activity or on thermoregulation (Ralph et al., '79; Erskine and Hutchinson, '81; Ralph, '83; Vivien-Roels, '85; Vivien-Roels et al., '88; Foa, '91; Refinetti and Menaker, '92; Foa et al., '92a,b; Innocente et al., '94). The presence of a pineal

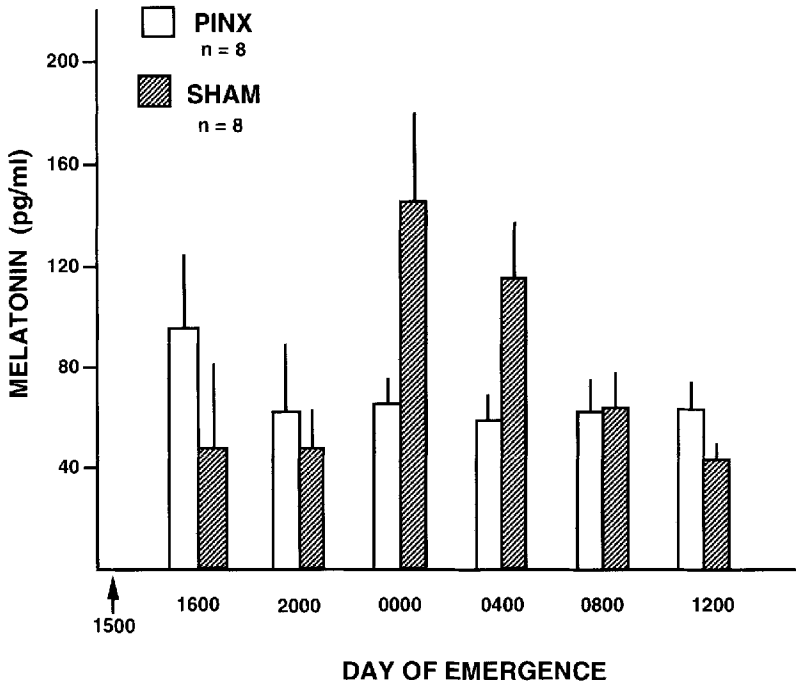

Fig. 5. Diel cycle of mean plasma melatonin in sham and pinealectomized males (±1 standard error) upon emergence from hibernation conditions, spring 1991. Arrow indicates actual emergence time.

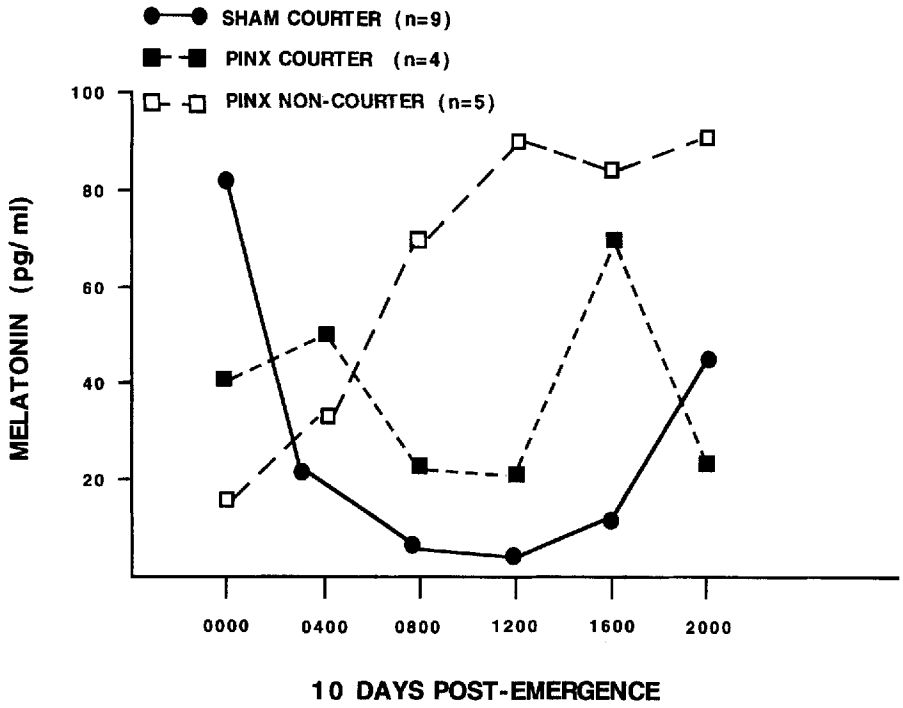

Fig. 6. Diel cycle of plasma melatonin in sham courting males, pinealectomized courting males, and pinealectomized non-courting males. Owing to small sample size and large variation in plasma levels, none of the points are different from one another. Error bars were excluded to clarify trends.

gland and melatonin has been shown to affect gonadal condition in two species of lizards (Misra and Thapliyal, '79; Thapliyal and Haldar, '79; Underwood, '85a,b) and a snake (Haldar and Pandey, '89a,b). These effects, just as in higher vertebrates, can be pro- or anti-gonadal depending on the time of year surgery is done or melatonin implants are given and the species involved. Although melatonin's effects on gonadal growth has been documented to some degree in reptiles and extensively in mammals and birds (Reiter, '81; Binkley, '88; Pevet, '88), very little work has been done on the pineal's direct effect on reproductive behavior in any vertebrate. This lack of information is due to the fact that most species studied exhibit an associated pattern of reproduction. Sexual behavior in these species is influenced by amounts of sex steroids in circulation. Since melatonin influences gonadal activity, it also has an indirect effect on sex steroid secretion and thus the occurrence of reproductive behavior. In the red-sided garter snake, sexual behavior in males is not dependent on gonadal state. Thus, this species is a logical model system to test melatonin's central effect on reproductive behavior.

Although it is well documented that the presence of the pineal and/or melatonin affects other types of behaviors (e.g., locomotory, feeding) independent of sex steroids, to date, few studies have attempted to demonstrate a central effect on sexual behavior. Baum ('68) demonstrated that pinealectomy of neonate male mice accelerated the onset and increased the frequency of copulation, but these changes did not persist into adulthood. Nelson et al. ('87) and Crews et al. ('88) demonstrated that pinealectomy of male red-sided garter snakes in the fall can disrupt the ability to exhibit courtship in the subsequent spring. None of the pinealectomized males courted in the Nelson et al. ('87) study, whereas only 15% (4/29) of the PINX males exhibited vigorous albeit sporadic courtship in the Crews et al. ('88) study.

In our series of experiments on male red-sided garter snakes, pinealectomy in the fall also disrupted the expression of courtship behavior in the spring but not as completely as in the Nelson et al. ('87) and Crews et al. ('88) experiments. Year after year (1989–1991), approximately 40% of the treated pinealectomized garter snake males did not court (60% of PINXs did not court but 20% of the SHAMs also did not court, therefore 40% is the conservative estimate of the effect of pineal-

ectomy). The sample sizes in our experiments were considerably larger than in the previous experiments by Nelson et al. ('87) and Crews et al. ('88). Although it is clear from the three sets of experiments that pinealectomy affects sexual behavior in these male snakes, it is not clear *how* this is being mediated. The problem arises because, in the same 3-year period of experiments, pinealectomy had absolutely *no* effect on courtship behavior in a consistent percentage (40%) of males. This result occurred despite the fact that there was a complete removal of the pineal as confirmed by subsequent histology. Thus it seems that the presence of a pineal gland does not, by itself, play a role in stimulating courtship behavior but rather appears to modulate its occurrence.

Continuous implants of melatonin have proven effective in stimulating mating behavior in some animals with associated patterns of reproduction (e.g., sheep, Waller et al., '88; Haresigan, '92; foxes, Forsberg et al., '90; golden hamster, Reiter, '81). However, the most commonly reported effect of chronic melatonin implants is changing the period of free-running activity periods (either shortening, lengthening, or abolishing the rhythm (Turek et al., '76; Gwinner and Bezinger, '78; Underwood and Harless, '85; Beldhuis et al., '88; Underwood, '79, '81). In male garter snakes, however, melatonin implants failed to modulate the presence or absence of courtship behavior in either SHAM or PINX individuals (Fig. 2).

Given the dichotomy in behavioral response among the PINX males, endogenous levels of melatonin were measured to try to reconcile the above findings and determine melatonin's relationship, if any, to the expression of sexual behavior in SHAM vs. PINX courters and non-courters. Pinealectomy did, in fact, decrease melatonin to nondetectable levels when examined by a single sample soon after surgery (Fig. 3) as it does in most animals. However, melatonin did not remain low in PINX animals. PINX males had detectable levels of melatonin upon emergence from low-temperature conditions (Fig. 4) but did not exhibit a normal diel pattern. Instead, melatonin was elevated in all the sample periods. Levels of melatonin in PINX males equivalent to those of SHAMs were also apparent at the 10-day sample in both 1990 and 1991 (albeit with a differing time course). To date, studies of other species have found that pinealectomy either reduces melatonin levels to basal, abolishing its rhythm, or greatly reduces the amplitude of the rhythm. However, other species, especially lower vertebrates, are known to produce retinal (or extrapineal) sources of melatonin rhythmically which can contribute substantially to circulating plasma levels (Gern et al., '78; Gern and Norris, '79; Gern and Karn, '83; Underwood et al., '84; Pang et al., '85; Foa and Menaker, '88; Delgado and Vivien-Roels, '89). Our findings differ in that pinealectomized males had levels equivalent to those of sham-operated animals. This occurred in both years of pinealectomy although mean levels of melatonin differed between the years (Mendonça et al., '95). At first, we were concerned that the antibody cross-reacted with another substance, but plasma samples were sent to another laboratory which used different antibodies and levels were similar to ours (Mendonça et al., '95). Close histological examination of the brains of PINX males with elevated melatonin revealed no remnant of pineal gland or stalk. Therefore, the data suggest that garter snakes have an extra-pineal source of melatonin synthesis and secretion, presumably located in the retinae as in other species.

The data further suggest that the extra-pineal source secretes melatonin at a different rhythm than that of the pineal. For example, in 1990, when single day and night blood samples were taken 10 days after emergence, PINX males that did not court had elevated day (0800) and night (2000) melatonin levels, whereas courting PINX males exhibited a pattern similar to that of SHAM courting males (Fig. 3). In the more extensive 1991 sampling, PINX males, 10 days after emergence, again had elevated levels of melatonin. However, owing to the low sample sizes and great variation among the values, these data did not differ significantly from one another and can only illustrate the trend that PINX non-courters had disrupted melatonin cycles. These results argue that the pineal in snakes 1) is not the sole source of melatonin production and secretion and 2) is, as proposed in birds and lizards, part of a multi-oscillator system (Cassone and Menaker, '84; Underwood, '92).

In mammals, the rhythmicity of melatonin synthesis is controlled by a master oscillator, the suprachiasmatic nucleus (SCN) (Ralph and Menaker, '88). Studies conducted on several species of birds and lower vertebrates, in contrast, indicate that these animals possess a multi-oscillator system. In birds, the pineal is itself light sensitive and produces an endogenous, oscillatory pattern of melatonin secretion which can respond to changes in light/dark cycles. The retina is also a light-sensitive oscillator, producing melatonin

rhythmically and independently of the pineal (Cassone and Menaker, '84; Binkley, '88). The SCN is important in establishing circadian rhythms in birds (Takahashi and Menaker, '79) but may feedback to the pineal gland to achieve synchronicity among the differing oscillators and their photoreceptive components (Cassone and Menaker, '84).

In reptiles, most pineal studies have centered on lizards (especially the green anole, *Anolis carolinensis*). It is thought that lizards, like birds, have a multi-oscillator system controlling circadian cycles (Underwood, '92; Foa et al., '92a,b). For example, the lizard pineal is, as in birds, an endogenous oscillator (Menaker and Wisner, '83). The melatonin pulse is thought to entrain other extrapineal oscillators. Although their location is unknown, it is hypothesized that, as in birds, they are located at the retinas and the SCN (Underwood, '92). Melatonin has been located immunohistochemically in these locations (as well as the Harderian gland) for a number of reptiles (including a snake, *Natrix tessalata*) although it is unknown whether the melatonin was produced at these sites or was just binding there (Vivien-Roels et al., '81).

Very little is known about the production or role of melatonin in snakes. The snake pineal is not thought to have photoreceptive type cells, making it more similar in structure to mammals than to birds and lizards (Petit, '71). This suggests that response to photoperiod is mediated through a retinohypothalamic pathway as in mammals, but there have been no experimental data to test this. The one other study on melatonin levels in snakes found that serum melatonin amplitude varies directly with temperature as in other reptiles and is entrained by photoperiod (the water snake, *Nerodia rhombifera,* Tilden and Hutchinson, '93). However, retinal melatonin levels in mid-scotophase were not detectable in this species.

The red-sided garter snake, however, appears to have an extrapineal source of melatonin. Melatonin levels 36 hr after pinealectomy in the autumn were undetectable. However, since the measurement was at a single time point, it cannot be discounted that pinealectomy uncoupled a melatonin-producing oscillator from its synchronizer (in this case, the pineal). In fact, this pattern is what was suggested by the more intensive (every 4 hr) spring samples. Melatonin in PINX animals remained elevated or became asynchronous with the day/night cycle, suggesting an uncoupling of oscillators. The PINX males exhibited a split in their behavioral and melatonin response to the surgery. Males whose oscillators could apparently resynchronize exhibited melatonin levels that approximated a normal diel pattern and remained vigorous courters. Males whose oscillators apparently remained uncoupled (asynchronous?) had abnormal circulating melatonin patterns and demonstrated no courtship activity. A small percent of unmanipulated males also do not court upon emergence in the laboratory. They, too, exhibit a disrupted pattern diel melatonin (Mendonça, unpublished observation).

The polymorphism in the response is not unusual. Other animals demonstrate population polymorphism in normal circadian rhythms (Johnston and Zucker, '83) and in response to pinealectomy (Underwood, '81, '83; Pevet, '88; Binkley, '88). Snakes, which switch from diel to nocturnal activity at different times of year, should be especially labile in their circadian rhythmicity. Additionally, red-sided garter snakes apparently mate in spring and fall just before entering the hibernaculum (Whittier et al., '87b). Perhaps the differential response to pinealectomy reveals a population polymorphism in timing of seasonal mating. The present data suggest that courtship behavior in the garter snake is modulated by a normal pattern of melatonin secretion and that this effect is mediated centrally and not through the gonadal axis.

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