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Seasonal Changes and Annual Variability in Daily Plasma Melatonin in the Red-Sided Garter Snake (*Thamnophis sirtalis parietalis*)

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We report seasonal and annual variation in the daily cycle of plasma melatonin levels in male red-sided garter snakes, *Thamnophis sirtalis parietalis*. In autumn of 1989 and 1990, levels averaged a maximum of 210 pg/ml during scotophase and a minimum of 45 pg/ml during photophase and had a similar diel pattern. Under hibernation conditions (4°, 0:24 L:D), melatonin was undetectable and a diel cycle could not be determined. In Spring 1990, melatonin levels rose rapidly and precipitously within an hour of emergence (while in photophase), peaked at levels significantly higher than those seen in the autumn (approximately 900 pg/ml) and remained significantly high for 24 hr after emergence (though the majority of animals did have decreased levels at the 0400 sample). By the 10th day after emergence, a diel cycle was reestablished and absolute melatonin levels had decreased. The next spring (1991), melatonin again rose within an hour after emergence, while in photophase, but not as high as the previous year. Also unlike the previous year, a diel cycle was observed within 24 hr of emergence. Melatonin levels at emergence were significantly higher than those observed 10 days later. Disruption of a diel rhythm of plasma melatonin (by pinealectomy the previous autumn) inhibits courtship behavior by males the next spring, implying a role for melatonin in the stimulation of sexual behavior. Males in 1991 (with quickly established melatonin cycles) courted much sooner after emergence than did males in 1990. Therefore, the initial day/night difference in melatonin levels at emergence (*i.e.*, establishment of a normal diel cycle) may function in synchronizing and modulating reproductive behavior in male red-sided garter snakes.

The pineal gland, through its cyclic secretion of melatonin, transmits signals concerning photoperiod to a central neuroendocrine network that controls circadian and seasonal rhythms in all vertebrates (Reiter, 1981; Binkley, 1988). In poikilothermic vertebrates, such as reptiles, studies have shown that although the phase of melatonin secretion is not significantly affected by temperature, the amplitude of the circulating level of melatonin is (Menaker and Wisner, 1983; Underwood, 1985*a*; Vivien-Roels, 1985; Vivien-Roels *et al.*, 1988; Tilden and Hutchinson, 1993). Therefore, in reptiles, the pineal can be a transducer of temperature as well as photoperiodic information.

The pineal has been studied extensively in birds and mammals but relatively little is known about this gland in reptiles (Underwood, 1992). The work that has been done in reptiles has focused on the effects of pineal gland removal or of exogenous melatonin administration on circadian locomotor activity and thermoregulation (Ralph *et al.*, 1979; Ralph, 1983; Vivien-Roels, 1985). The presence of a pineal gland and melatonin has been shown to affect gonadal condition in two species of lizards (Misra and Thapliyal, 1979; Thapliyal and Haldar, 1979; Underwood, 1985*b*) and a species of snake (Haldar and Pandey, 1989*a*,*b*). These effects, just as in homeothermic vertebrates, can be pro- or anti-gonadal depending on the time of year surgery was done or melatonin implants were given and the species involved. Endogenous levels of plasma and/or pineal melatonin have been measured in a few species of lizards and turtles and a single species of crocodilian and snake (Underwood, 1985*a*; Underwood and

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Calaban, 1987*a,b*; Firth *et al.*, 1989; Vivien-Roels *et al.*, 1979, 1988; Roth *et al.*, 1980; Janik and Menaker, 1990; Tilden and Hutchinson, 1993).

Very few studies have focused on the role of the pineal gland or of its secretory product, melatonin, in snakes (Underwood, 1992). The snake pineal gland is composed of pineolocytes or pineal parenchymal cells. These cells are more similar in appearance to those of birds and mammals than to the pineal cells of other groups of reptiles (Underwood, 1992). Although apparently not photoreceptive themselves, the pinealocytes belong to the same cell line as the photoreceptive cells found in other reptilian pineals (Petit, 1971; Collin and Oksche, 1981). Pinealectomy had seasonal effects on the testicular cycle of the checkered water snake, *Natrix piscator,* inhibiting gonadal growth during testicular recrudescence and retarding testicular regression or maintaining testes during a time when they would normally be inactive (Haldar and Pandey, 1989*a,b*). Pinealectomy before hibernation abolished courtship behavior upon emergence from low temperature conditions in male red-sided garter snakes, *Thamnophis sirtalis parietalis* (Nelson *et al.*, 1987; Crews *et al.*, 1988). Pinealectomy disrupts the normal pattern of melatonin secretion in this subspecies; males with disrupted patterns do not court (Mendonça *et al.*, submitted). Immunohistochemical study has shown the presence of melatonin in the pineal, retinas, and Hardarian glands of a water snake, *Natrix tesselata* (Vivien-Roels *et al.*, 1981). To date, endogenous levels of serum, pineal, and retinal melatonin have been measured in only one species, the diamondback water snake, *Nerodia rhombifera* (Tilden and Hutchinson, 1993). Serum melatonin ranged from 50 to 500 pg/ml and photoperiod affected the phase while temperature affected the amplitude of the diel melatonin cycle of this species. Pineal but not retinal melatonin was detectable in midscotophase (Tilden and Hutchinson, 1993).

This paper documents the seasonal changes and variation between years in the diel plasma melatonin rhythm in the male red-sided garter snake, *T. sirtalis parietalis,* at ecologically relevant points of this species' annual cycle, as it is about to enter the hibernaculum, in hibernation, and at spring emergence, when courtship behavior occurs.

MATERIALS AND METHODS

Animals. Adult males were collected from Chatfield, Manitoba, Canada, in mid-September of 1989 and 1990. In both years, they were transported to our laboratory, kept in aquaria for 2 to 4 weeks at room temperature (approximately 24°) 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° for 1 week and then 13/8° for an additional week. They were then placed in bags with moist sponges and kept in constant dark at 4° for 17 weeks. They were then placed in aquaria at room temperature (21–25°) 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 upon emergence (Crews *et al.*, 1984).

Blood collection. Males were bled every 4 hr for 24 hr in the fall (early October of the respective year) while in the laboratory under the room temperature, 10:14 L:D regimen in both 1989 ($n = 8$) and 1990 ($n = 8$). Another group of males ($n = 8$) were bled every 4 hr for 24 hr while in their 8th week of hibernation (4°) (January of 1990). Males in Spring 1990 ($n = 8$) and 1991 ($n = 8$) were bled 1 hr after emergence from hibernation (at 1600 hr) and then bled every 4 hr for 24 hr after that, encompassing the first night and day after hibernation. In Spring 1990 and 1991, males ($n = 8$) were bled 10 days after emergence for every 4 hr for a 24-hr span.

Blood 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 was pipetted and frozen at -20° for later analysis of circulating melatonin levels. Blood collection during the night (*i.e.*, after 1900- and before 0600-hr 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 test tube to crack in door. After visual adaptation to darkness, enough light penetrated from the darkened, exterior hallway to permit the determination of blood level in tube.

Melatonin assay. Assay protocol followed that of Heideman and Bronson (1990). Each plasma sample was ex-

tracted with 1.25 ml chloroform. An aliquot (1 ml) of the extract was evaporated under nitrogen gas and resuspended in a Tris buffer solution. An aliquot of a standard diluent (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 when using the stripped rat and snake plasma (three trials, lines were linear and parallel). 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, UK) 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° for 12–15 hr. A charcoal Tris buffer–gelatin solution was then added, incubated at 4° for 15 min. Test tubes were centrifuged at 3500 rpm for 15 min. The supernate was poured off into a liquid scintillation vial and scintillation fluid was added, vortexed, allowed to reach equilibrium for 6 hr, and then counted on a Beckman beta counter. Intraassay variation was 5.9%, interassay variation was 15.1%. Sensitivity varied. In 1990, the assay was sensitive to 5 pg/ml, but in 1991 it dropped to 10–12 pg/ml. Extraction efficiency averaged 79% ± 3 for both years.

Validation of the melatonin assay was based on the following parameters. Garter snake plasma was charcoal stripped following the protocol of Heidemann and Bronson (1990) 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, extracted 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. Finally, plasma samples ($n = 8$) were sent to Dr. Andrew Loudon (University of Virginia), to be retested by a radioimmunoassay technique which employed iodinated melatonin and another antibody (R1055) and followed the procedure detailed in Rollag and Niswender (1976). Values had a mean coefficient of variance of 12.4% + 6.1 (Table 1).

Behavior testing. After emergence in 1990 and 1991, males were tested for courtship behavior from Day 0 (emergence) to Day 14. These males were used in another set of experiments and were not bled for the emergence sample. They were collected in the same areas and treated to the same "step-down" and hibernation regimens as the animals that were bled at emergence. A subsample of this group was bled 10 days after emergence to constitute the second group (+10 days) of this study.

The males ($n = 65$ in 1990 and $n = 26$ in 1991) were

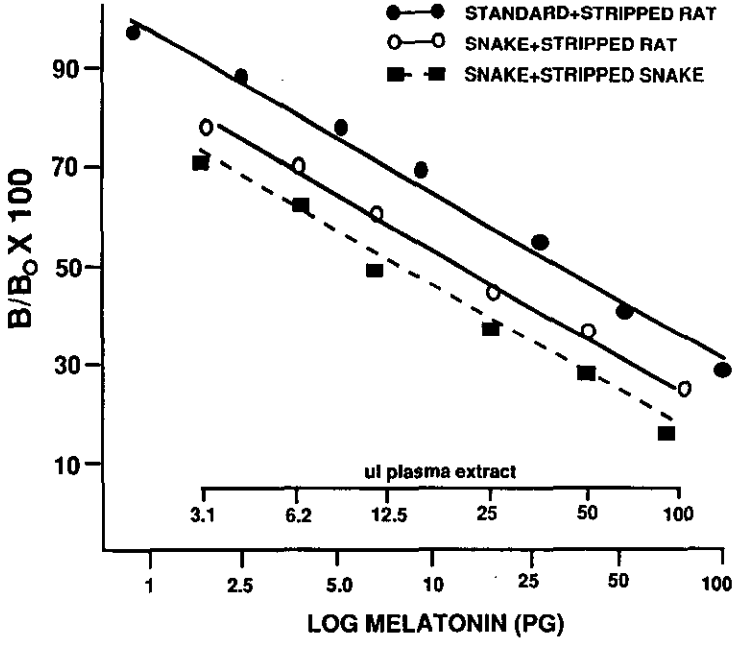

FIG. 1. Parallel dilution curves of standard, extracted snake plasma diluted with stripped snake plasma plus Tris buffer, and extracted snake plasma diluted with stripped rat plasma plus Tris buffer.

TABLE 1
COMPARISON OF MELATONIN VALUES FOR SAME PLASMA SAMPLES WHEN ASSAYED USING DIFFERENT RADIOIMMUNOASSAY TECHNIQUES

	Mendonça *et al.* assay (pg/ml)	Laudon assay (pg/ml)	Coefficient of variation (%)
Sample 1	1050	1381	17.3
Sample 2	1202	910	19.5
Sample 3	1437	1903	16.0
Sample 4	1279	1624	16.8
Sample 5	780	748	3.0
Sample 6	318	384	13.3
Sample 7	450	504	8.0
Sample 8	300	325	5.6
			$X = 12.4$
			SD = 6.1

placed in 29-gallon aquaria (at least 8 males/aquarium) with recently emerged, "attractive" females (2 females/aquarium; females' attractivity determined by previous testing with actively courting males). Males were given up to 15 min to court. Generally males court in the 1st min of the females being placed in the cage and certainly upon first encountering the females. 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 indicates 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 and displaying muscular contractions along the length of his body. 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).

Statistical analysis. Circulating plasma melatonin mean values were tested for heterogeneity of variances within groups. The variances were heterogeneous so all melatonin values were log-transformed to correct for this phenomenon. To determine if there were significant differences in mean melatonin levels among the sample times (*e.g.*, 8 PM, midnight, *etc.*) within a sample period (*e.g.*, Fall 1989), a one-way repeated measures analysis of variance (ANOVA) was used. To determine significant differences in mean levels of melatonin of samples taken in a 24-hr period between years (*e.g.*, Fall 1989 and Fall 1990) or between the emergence and + 10 day sample within a year, a two-way repeated measures ANOVA was used to compare melatonin values. Scheffe's F test was done to determine post hoc comparisons (Sokal and Rohlf, 1981). To determine if frequency of courting males varied between the 2 years upon emergence, the nonparametric Fisher's Exact test was performed.

RESULTS

Fall (1989 and 1990)

The changes in circulating concentrations of plasma melatonin in October 1989 and 1990 exhibited a diel cycle (Fig. 2). There was a significant difference in daily melatonin levels in both years {1989, one-way repeated measures ANOVA, $P = 0.01$; $F = 3.38$; $df = 7$ [*i.e.*, subjects-1], 4 [*i.e.*, treatments-1], 28 [*i.e.*, residual (subjects-1) × (treatments-1)]; 1990, $P = 0.0001$; $F = 19.4$; $df = 7, 4, 28$}. When the first blood sample was taken at 2000 hr (1 hr after lights off), levels were already elevated in the majority of animals, remained high at the 0000 sample period, and then began to fall at the 0400 sample (before lights came on at 0700). In both 1989 and 1990, melatonin levels were significantly higher at 8 PM and at midnight than the other sample times (Scheffe's F test, $P = 0.05$), though they did not differ from one another. There was no significant difference in the daily pattern of mean levels of melatonin between the years (two-way repeated measures ANOVA, $P = 0.62$; $F = 0.254$, $df = 1, 4$; interaction term, year × repeated measure, $P = 0.56$). In both years, melatonin had declined significantly at the 0400 sample, in advance of light onset at 0700.

Hibernation (January 1990)

Only one animal (of the eight sampled) in only one of the sample periods (of a total of six, one every 4 hr) had a detectable level of melatonin (13 pg/ml). All other samples had melatonin levels below the sensitivity of the assay. There was no discernible diel pattern.

Spring Emergence (1990 and 1991)

In 1990, melatonin levels were detectable within 1 hr (1600) after emergence from hibernation conditions. The mean level ($x = 292.7 \pm$

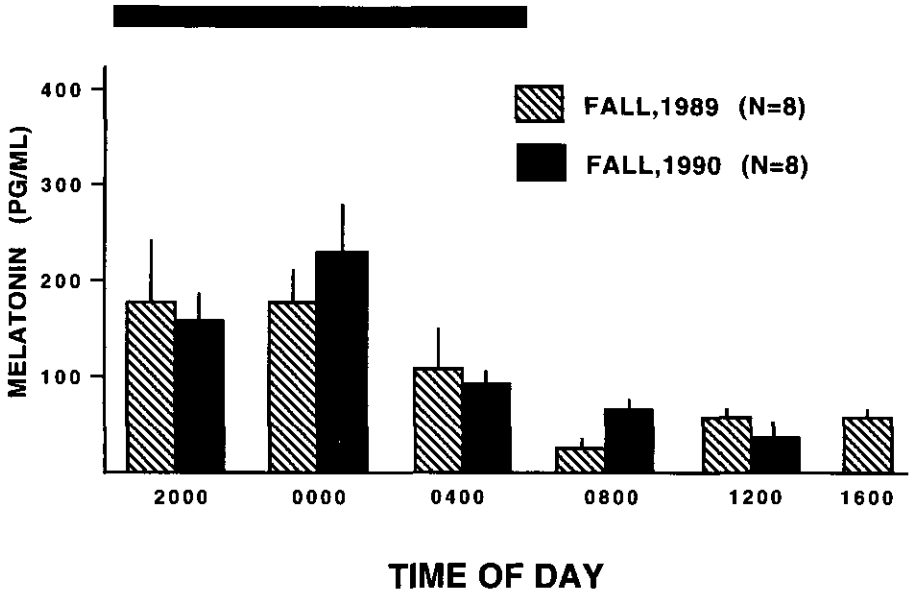

FIG. 2. Diel cycle of plasma melatonin in Autumn 1989 and 1990. Vertical bars indicate 1 SE.

45.7 pg/ml, $n = 8$) was equivalent to maximum mean levels detected in the fall sampling periods. Melatonin levels varied greatly the first night after emergences. Overall, however, individuals underwent a large surge in melatonin, some peaking in the 1–2 ng range. Surprisingly, even daylight samples had very high levels of melatonin although there was great variation among individuals (Fig. 3A).

Only one of the eight males exhibited the normal melatonin pattern: lower in the photophase, highest in the early to midscotophase (male 4; Table 2). The other males had high levels of melatonin during the photophase. Interestingly, there appeared to be a drop in melatonin at the 0400 sample in four of the males followed by a sharp increase upon entering photophase (males 2, 5, 7, 8; Table 2). Males 6 and 1 had the increase at the 1200 bleed (Table 2). Melatonin levels in all individuals at all the sample periods were higher than most of the levels seen during the fall samples. Some individuals, however, were consistently much higher (*e.g.*, males 2,3, and 7; Table 2).

This large variation in the timing and levels displayed by these individuals resulted in the mean melatonin levels taken at 4-hr intervals not differing significantly from one another (one-way repeated measures ANOVA, $F = 1.66$; $df = 7, 5, 47$; $P = 0.17$). There was no indication of a diel cycle the first complete day after emergence (except in one individual).

Ten days after this emergence, another sample of males was bled. A diel pattern of circulating melatonin was now evident (Fig. 3A). The midnight level was significantly higher than the other values (one-way repeated measures ANOVA, $F = 2.94$, $P = 0.03$, $df = 7, 5, 33$; Scheffe's F test, $P < 0.05$). The mean values of the +10 day sampling period did not differ from those at emergence when compared by two-way repeated measures ANOVA ($F = 3.76$, $P = 0.08$, $df = 1, 5, 50$). This result, however, may be due to the high variance seen in the emergence samples.

The following spring, despite identical housing and temperature conditions, the melatonin values of males at emergence exhibited a very different pattern (Fig. 3B). Animals did not exhibit the extremely elevated melatonin levels of the previous year. Levels were almost an order of magnitude lower (two-way ANOVA, $F = 33.3$; $df = 1, 4, 139$; $P = 0.0001$; Fig. 3B). In addition, there was evidence of a significant diel

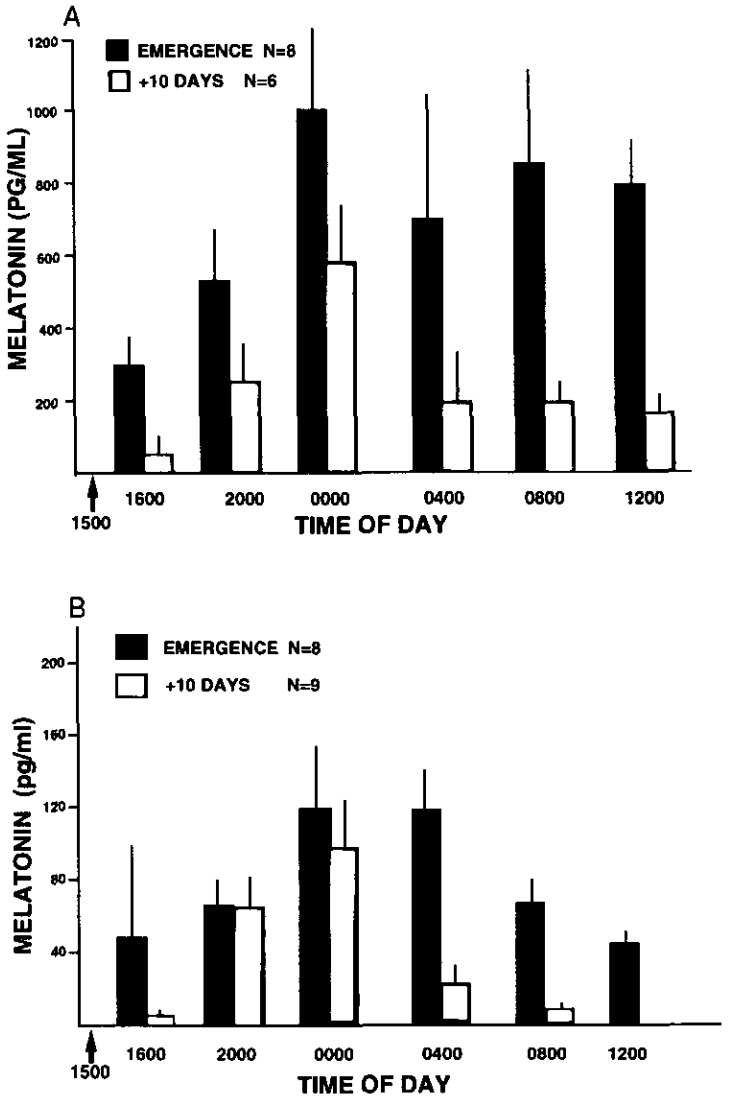

FIG. 3. Diel cycle of plasma melatonin upon emergence from hibernation and 10 days after emergence. (A) Spring 1990; (B) Spring 1991. Males were removed from hibernation at 1500, indicated by arrow. Vertical bars indicate 1 SE.

cycle in this second emergence ($F = 4.9$; $df = 7$, 4, 39, $P = 0.003$). The value at Time 0000 was significantly higher than all other sample periods except the one taken at Time 0400 (Fig. 3B; $P = 0.05$). The sample values did not differ significantly from the 10th day after emergence samples taken in 1990 ($P = 0.49$).

Mean melatonin levels of males sampled 2 weeks after emergence in 1991 did not differ significantly from those sampled at emergence (two-way repeated measures ANOVA; $F = 0.656$; $df = 1$, 4, 108; $P = 0.42$). These melatonin levels were lower than those observed during the same time period in 1990 but not significantly so ($P = 0.31$, Fig. 3). There was a diel cycle with a peak at 0000 and melatonin levels declining significantly by 0400. The 0000 level was significantly higher than all other levels

TABLE 2
MELATONIN VALUES (pg/ml) OF INDIVIDUAL MALES BLED AT 4-hr INTERVALS THE 1ST 24 hr AFTER EMERGENCE FROM 17 WEEKS AT 4°, 0 L:24 D CONDITIONS

	1600 (1 hr postemergence)	2000	0000	0400	0800	1200
Male 1	143	372	897	175	128	433
Male 2	260	625	1468	586	638	1140
Male 3	346	592	2384	2900	2418	1050
Male 4	269	305	400	559	312	341
Male 5	335	602	658	313	1437	926
Male 6	562	399	413	417	417	780
Male 7	170	1185	1558	376	832	1055
Male 8	256	458	697	325	720	705

Note. Animals were placed in 12L:12D at 24°. Lights went off at 1900.

except the 2000 sample. The 2000 mean value was significantly higher than that of the 1600 sample but not that of the 0400 sample.

Courtship Behavior

In 1990, 65 males that had been exposed to the same conditions as the sampled emergence males were tested daily for courtship behavior for 2 weeks with attractive females. On day of emergence (Day 0), 36.9% of those males were classified as courters (Fig. 4). The percentage of courting males increased and reached 90% or above on Day 5 (Fig. 4). This percentage remained stable for the rest of the 2-week testing period. In 1991, 25 males, again exposed to the same conditions as their blood-sampled counterparts, were tested for courtship behavior. In the 2nd year, the percentage of males exhibiting courtship on Day 0 was significantly higher ($P = 0.001$) than the previous year (*i.e.*, 76.9 *vs* 36.9%). The 1st year's percentages of courting males remained significantly lower than 1991 until Day 3 (*e.g.*, Day 1, 49 *vs* 80.7%, $P = 0.005$; Day 2, 64.6 *vs* 96.1%, $P = 0.001$; Day 3, 86.1 *vs* 96.1%, $P = 0.15$).

DISCUSSION

The role of the pineal and its secretory product, melatonin, has only recently been examined

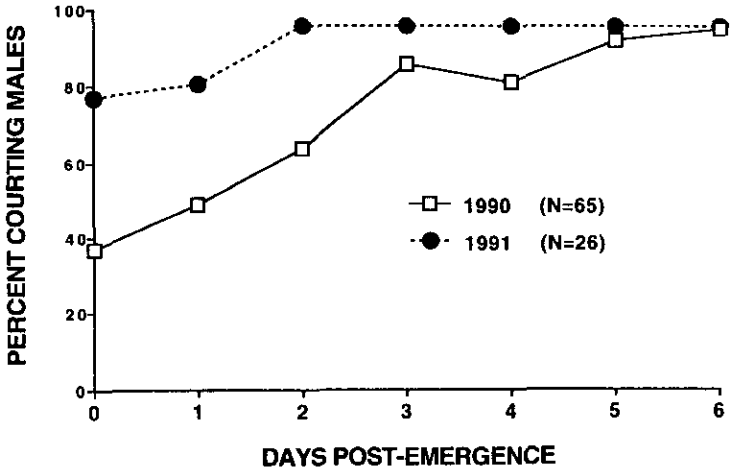

FIG. 4. Percentage of males exhibiting courtship behavior following emergence from hibernation conditions.

for ophidians (Petit, 1971; Nelson *et al.*, 1987; Crews *et al.*, 1988; Haldar and Pandey, 1989*a,b*; Kaslow *et al.*, 1991). Only one other study has measured circulating levels of melatonin in a snake (Tilden and Hutchinson, 1993). The values for the diamondback water snake, *N. rhombifera,* were obtained at a single time of year (midsummer) and ranged between daylight levels of 80 and night levels of 500 pg/ml. Experimental manipulations (different temperature regimens and reversed cycle photoperiod) determined that photoperiod affected the phase of the diel cycle while temperature affected the amplitude as is the case in other ectotherms (Gern *et al.*, 1978; Gern and Norris, 1979; Underwood, 1985*a*; Firth and Kennaway, 1987; Viven-Roels *et al.*, 1988; Firth *et al.*, 1989; Tilden and Hutchinson, 1993). Almost all of these studies examined the cycle at a single time of year. There are fewer studies in either heterotherms or homeotherms that look at seasonal variation or yearly variation in the cycle (Arendt *et al.*, 1981; Brainard *et al.*, 1982; Bubenik and Smith, 1987; Vivien-Roels *et al.*, 1979, 1988; Delgado and Viven-Roels, 1989).

The diel cycle of plasma melatonin levels in male red-sided garter snakes varied seasonally and, at the time period of emergence, between years. In the autumn of 1990 and 1991, after animals had been in the laboratory approximately 1 month, there was no difference in diel patterns or absolute levels of circulating melatonin between the years. Values ranged from 20 to 300 pg/ml, somewhat lower than the values found in the diamondback water snake in midsummer (Tilden and Hutchinson, 1993). Melatonin was significantly elevated by 2000 hr (1 hr after onset of scotophase), remained at this level or higher at 0000 and declined significantly at 0400 hr, anticipating the onset of the photophase. This anticipatory decline was also seen in the spring of both 1990 and 1991 *after* males had been out of hibernation for 10 days. Melatonin has been found to decline in anticipation of the onset of light in both endotherms and ectotherms (Underwood, 1985*a*; Firth and Kennaway, 1987; Binkley, 1988). In hamsters, there is little variation as to when the melatonin peak occurs when animals are housed at short photoperiods *vs* long photoperiods, but, in sheep, the melatonin peak under short photoperiods shifts closer to dawn (Reiter, 1981; Arendt *et al.*, 1981, 1983). The difference in the laboratory photoperiod between the fall and spring conditions was, however, minimal (10:14 L:D vs 12:12 L:D) and may account for the lack of variability in the timing of scotophase pattern.

Melatonin could not be detected in the plasma of hibernating animals. At 10°, the diamondback water snake still had detectable levels of melatonin though they had decreased significantly ($x = 50$ pg/ml) and did not differ between midscotophase and photophase (Tilden and Hutchinson, 1993). This drop in melatonin levels and abolishment of diel pattern at low temperatures is seen in many ectotherms (Vivien-Roels, 1985; Firth *et al.*, 1989) as well as hibernating homeotherms (Florant *et al.*, 1984; Vanececk *et al.*, 1984).

Even though they were still in photophase, melatonin levels rose significantly above baseline within an hour of the garter snakes' being exposed to spring-like temperatures (22–23°). This result differs from the condition of hibernating marmots when aroused; in this species, melatonin stayed at essentially basal levels until the dark portion of the light cycle began (Florant *et al.*, 1984). The response of the garter snake was like that found in other ectotherms: higher ambient temperatures result in higher melatonin levels (Vivien-Roels *et al.*, 1979, 1988; Firth and Kennaway, 1987; Firth *et al.*, 1989; but see Underwood, 1985a). In these studies, however, daylight melatonin levels were always lower than those at night. Most of these studies have been on animals acclimated to constant temperature and photoperiod regimes. The few that have looked at responses of species to fluctuating temperatures indicate a more robust response in melatonin changes (Vivien-Roels *et al.*, 1979; Underwood, 1985*a*, Firth and Kennaway, 1987). No study has previously explored how quickly melatonin rises when ectotherms experience rapid increases in their body temperatures as would occur when males emerge from the hibernaculum in Mani-

toba (where body temperatures can increase from 10 to 30° within minutes).

Plasma melatonin levels at 1600 (an hour after the temperature change) were equal to or higher than peak levels observed in the fall. They continued to rise, peaking at 0000 hr. They remained high throughout the next 12 hr (into the next photophase) though there was great variability among the sampled individuals. Only one male exhibited a typical pattern. The majority of the other males had elevated levels early in the scotophase (2000 and 0000 samples), which declined at the 0400 sample, but then became elevated again during photophase (0800 and 1200). This split in the pattern may be individual variation or it may be evidence that the rhythm of melatonin secretion in the garter snake is controlled by two distinct circadian oscillators. Similar split patterns of wheel-running activity and luteinizing hormone surges are seen when ovariectomized hamsters are exposed to constant light (Swann and Turek, 1985). If there are multiple oscillators in the garter snake, they may have different rates of acclimation to change in conditions before they become recoupled or synchronized. Pinealectomized garter snakes have elevated levels of melatonin (indicating an extrapineal melatonin source) but the pattern of melatonin secretion is disrupted (Mendonça *et al.*, submitted). It may be the pineal plays a key role in regulating or synchronizing a multioscillatory system. A diel pattern was evident, however, at the next sample period (10 days postemergence) even though the ambient temperature and photoperiod remained constant. This may indicate the oscillators were again coupled. In 1991, melatonin levels again rose significantly within 1 hr of the temperature change but to much lower absolute levels. They again peaked at 0000, remained significantly high at the 0400 sample, but became basal during the photophase, establishing a typical melatonin cycle within 24 hr of emergence. The same low levels and cycle was evident at the next sample period (10 days post emergence).

This extreme variation in melatonin levels and their daily cycle between the years occurred despite an equivalent hibernation length and temperature, same emergence hour, and fairly equivalent maintenance temperatures. The only difference in maintenance was that animals were checked slightly more frequently during the 1991 hibernation. Checks were, however, done in darkness without removal of the animals from the 4° walk-in cold room. Melatonin values from 1990 were reassayed in 1991 to determine if assay parameters had changed. Values were within 90% of one another. Samples were sent to another laboratory to test the validity of the assay: values differed by a coefficient of variance of 12% (Table 1). Therefore, the difference between the years in absolute levels and pattern appears real. Few studies have followed animals across years though those that have been done have not found the variability which we observed (Brainard *et al.*, 1982; Bubenik *et al.*, 1987). In our study, animals were collected each year from the same general area of Manitoba but from different small dens which are geographically isolated from one another. There may be possible genetic differences in garter snakes in the neuroendocrine transduction of low temperatures. There was extreme variability in melatonin levels in response to emergence in 1990. Some males had very high levels (for most of the sample periods), while others had a more moderate response. Some males were exhibiting a typical rhythm within that first day, while others seemed to be having two peaks. There are phenotypes within mammalian species that are photoresponsive and nonphotoresponsive. This polymorphism is expressed by differences in circadian behavior and gonadal development in responsiveness to photoperiod and has been shown to have a genetic basis (Eskes and Zucker, 1978; Desjardins, 1981; Desjardins *et al.*, 1986; Puchalski and Lynch, 1991; Margraf and Lynch, 1993). Variation in melatonin production also has a genetic basis (Ebihara *et al.*, 1986).

A similar phenomenon may be occurring in these snakes and this variation may have consequences on the expression of sexual behavior. For example, in the 1990 emergence (when the melatonin levels varied widely between individ-

uals and the pattern appeared almost bimodal), the percentage of courting males was significantly reduced the first 3 days when compared to the 1991 males (which displayed lower levels and a more typical pattern). Therefore, elevated daytime levels or a disrupted cycle may delay expression of courtship behavior by males. Although this study only provides correlative evidence of this effect, more experimental data support this hypothesis. Courtship behavior by males was significantly decreased by pinealectomy (Nelson *et al.*, 1987; Crews *et al.*, 1988; Mendonça *et al.*, submitted) and animals with a disrupted pattern of melatonin (*i.e.*, constantly high or reversed levels) did not court (Mendonça *et al.*, submitted). Chronic elevation of circulating melatonin by silastic implants when males were pinealectomized also tended to supress courtship behavior (Mendonça *et al.*, submitted). Additionally, under laboratory conditions, there is a small proportion of males that never exhibit courtship in the 2-week testing period after emergence. Therefore, it may be that some males may be able to either modulate melatonin levels or regulate diel rhythms better than other males or are genetically unresponsive to these particular conditions. It does appear, however, that the establishment of a diel cycle of melatonin is critical in modulating the expression of courtship behavior in males of this species. Animals exhibit individual variability in how quickly they can reestablish this normal rhythm and this ability may have some genetic basis.

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