Period and Phase Control in a Multioscillatory Circadian System (*Iguana iguana*)

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Abstract The circadian system of the lizard *Iguana iguana* is composed of several independent pacemakers that work in concert: the pineal gland, retinae of the lateral eyes, and a fourth oscillator presumed to be located in the hypothalamus. These pacemakers govern the circadian expression of multiple behaviors and physiological processes, including rhythms in locomotor activity, endogenous body temperature, electroretinogram, and melatonin synthesis. The numerous, easily measurable rhythmic outputs make the iguana an ideal organism for examining the contributions of individual oscillators and their interactions in governing the expression of overt circadian rhythms. The authors have examined the effects of pinealectomy and enucleation on the endogenous body temperature rhythm (BTR) and locomotor activity rhythm (LAR) of juvenile iguanas at constant temperature both in LD cycles and in constant darkness (DD). They measured the periods (τ) of the circadian rhythms of LAR and BTR, the phase relationships between them in DD (ψ_{AT}), and the phase relationship between each rhythm and the light cycle (ψ_{RL}). Pinealectomy lengthened τ of locomotor activity in all animals tested and abolished the BTR in two-thirds of the animals. In those animals in which the BTR did persist following pinealectomy, τ lengthened to the same extent as that of locomotor activity. Pinealectomy also delayed the onset of activity with respect to its normal phase relationship with body temperature in DD. Enucleation alone had no significant effect on τ of LAR or BTR; however, after enucleation, BTR became 180 ° out of phase from LAR in DD. After both pinealectomy and enucleation, 4 of 16 animals became arrhythmic in both activity and body temperature. Their data suggest that rhythmicity, period, and phase of overt circadian behaviors are regulated through the combined output of multiple endogenous circadian oscillators.

Key words circadian organization, pineal gland, eyes, body temperature, locomotor activity, iguana

Circadian systems composed of multiple autonomous circadian oscillators have been described in several classes of vertebrates: birds (Ebihara and Kawamura, 1981; Cassone and Menaker, 1984; Ebihara et al., 1984; Underwood and Siopes, 1984), mammals (Tosini and Menaker, 1996a; Yamazaki

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et al., 2000), fish (Tabata et al., 1988), amphibians (Harada et al., 1998), and reptiles (Underwood, 1981, 1983; Tosini and Menaker, 1998; Tosini et al., 2001). Only by understanding interactions within multioscillator systems and with environmental cycles can we begin to understand the adaptive significance of circadian organization.

The pineal gland has been the focus of much research on circadian rhythmicity in reptiles. It is photosensitive and contains autonomous circadian oscillators that, with a few notable exceptions, control the rhythmic production of melatonin. Pinealectomy has been shown to abolish circadian rhythms of locomotor activity in the lacertid *Gallotia galloti* (Molina-Borja, 1996) and of locomotor activity and visual sensitivity in the lizard *Anolis carolinensis* (Underwood, 1983; Fowlkes et al., 1984). In the lizards *Sceloporus occidentalis*, *Sceloporus olivaceus*, and *Podarcis sicula*, pinealectomy changes the length of the free-running period (τ) of locomotor activity and the duration of activity in each cycle (α). It can also induce "splitting" of activity into two bouts (Underwood, 1977, 1981; Foà, 1991; Innocenti et al., 1996). On the other hand, pinealectomy has no measurable effect on circadian locomotor activity in the lizard *Dipsosaurus dorsalis* (Janik and Menaker, 1990a). The lack of any effect of pinealectomy may be related to the fact that the pineal of *D. dorsalis* does not synthesize melatonin rhythmically in vitro and therefore may not possess an autonomous clock. Finally, the transplantation of a pineal gland into a pinealectomized *P. sicula* causes a change in τ; however, unlike its effect in house sparrows (Zimmerman and Menaker, 1979), pineal transplantation did not confer the phase of the donor animal on the host (Foà et al., 1997).

Pineal melatonin rapidly diffuses into the bloodstream and therefore can affect the function of distant tissues and organs. Pinealectomy either abolishes or greatly reduces the amplitude of the circadian rhythm of melatonin in the blood of all lizards tested to date. It is likely that the behavioral effects of pinealectomy are due to the consequent lack of rhythmicity of circulating melatonin, although most of the evidence in reptiles is only correlative. The importance of melatonin as a neuroendocrine signal in nonmammalian vertebrates is supported by the fact that the lizard *A. carolinensis* has the highest concentration of melatonin receptors of any vertebrate in its brain known thus far (Rivkees et al., 1989) and by experiments showing that exogenous melatonin applied rhythmically through infusions, through injections, or in the drinking water can restore rhythmicity to arrhythmic pinealectomized birds (Gwinner and Benzinger, 1978; Chabot and Menaker, 1994; Heigl and Gwinner, 1995; Gwinner et al., 1997). In the only direct test in reptiles, daily infusions of melatonin were shown to entrain the locomotor rhythms of both pinealectomized and intact *S. occidentalis* (Hyde and Underwood, 1995).

While the SCN of the ventral hypothalamus is the dominant pacemaker in the circadian system of mammals, the role played by this structure in the reptilian circadian system is poorly understood. Recent experiments have shown that hypothalamic lesions produce arrhythmicity in the locomotor activity of several species of lizards (Janik and Menaker, 1990b; Minutini et al., 1995). Bertolucci and Foà (1998) have extended these findings by showing that while intact lizards entrain to daily injections of melatonin, SCN lesioned lizards do not, suggesting that the SCN is a target of pineal melatonin.

Circadian oscillators have been described in the eyes of several classes of vertebrates (Underwood et al., 1990; Tosini and Menaker, 1996a, 1998). Like the pineal gland, the lateral eyes synthesize melatonin rhythmically. In some birds (e.g., quail and pigeon), the eyes also contribute a significant fraction of the circulating rhythmic melatonin (Underwood and Siopes, 1984; Ebihara et al., 1984). As with the pineal, the role of the eyes in the circadian organization of lizards varies among species. Bilateral enucleation changes τ of locomotor activity or produces arrhythmicity in *S. occidentalis*, *S. olivaceus*, and *P. sicula* (Underwood and Menaker, 1976; Underwood, 1981; Foà, 1991), while in *D. dorsalis* (Janik and Menaker, 1990a), it has little effect. The effects of bilateral enucleation may result from the loss of ocular melatonin, but other explanations are also possible.

The circadian system of the green iguana is composed of several circadian oscillators that regulate a panoply of individual circadian rhythms. The circadian rhythm of locomotor activity is thought to be controlled primarily by the SCN, while circadian rhythms of behavioral thermoregulation and of endogenously produced body temperature are regulated primarily by the pineal gland (Tosini and Menaker, 1996b). Iguanas also have measurable circadian rhythms of heart rate (unpublished data from Bartell et al.), melatonin synthesis (Tosini and Menaker, 1998), and retinal sensitivity to light. In the latter case, it is the b-wave of the electroretinogram that is most obviously rhythmic, and its amplitude is reduced by pinealectomy (Miranda-Anaya et al., 2000). The circadian system of the green iguana receives light input through several sets of retinal and extraretinal photoreceptors (pineal, parietal, and encephalic), which may integrate photic information from the complex photic environment (Tosini and Menaker, 1998).

The available data indicate that circadian oscillators in both the pineal gland and the lateral eyes influence the period and robustness of the free-running circadian rhythms of locomotor activity and body temperature and the phase of their entrainment to LD cycles. We exploited the ability to simultaneously measure both locomotor activity rhythm (LAR) and body temperature rhythm (BTR) of the iguana to explore the coupling among individual circadian oscillators that control these rhythms. We investigated whether the free-running period (τ), the phase relationship with an entraining light cycle (ψ_{RL}), and the phase relationship between activity and temperature rhythms (ψ_{AT}) were modulated by the lateral eyes and/or the pineal gland. We employed lesioning techniques to study the individual contributions of each pacemaker and their interactions within this complex system. In our experiments, we used each animal as its own control, thereby enhancing the validity of comparisons among conditions.

METHOD

Animals

Juvenile green iguanas (< 6 months of age, < 200 g) were obtained from Glades Herpetological Supply (Ft. Meyers, FL) and housed in plastic cages under a 12:12 LD cycle. A constant heat source was provided by placing a thermal pad directly under the animal’s cage; the air temperature was 28 °C (±0.2 °). Iguanas were given water ad libitum and fed a mixture of turnip greens and Zu-Preem marmoset food three times weekly.

Surgery

All surgeries were performed during the animals’ day or subjective day. Paraffin-coated radio transmitters were inserted in the peritoneal cavity through a small incision in the lateral abdomen of animals anesthetized with Ketamine HCL (10 mg/kg) and the wound closed with surgical staples.

Pinealectomies and sham pinealectomies were performed on iguanas anesthetized by imbedding in crushed ice. Procaine (10%) was administered subcutaneously to the parietal area as a local anesthetic. A dental drill was used to bore a 3-mm hole in the skull. The exposed dura was perforated using a pair of fine forceps, and the pineal gland was then grasped and removed; during sham operations, the pineal was grasped but not removed. Norepinepherine (4 mM) was applied to the wound to restrict bleeding. The opening was packed with Gelfoam and sealed with Nexband cyanoacrylate adhesive. The animal was allowed to recover and warmed to room temperature on a thermal pad before being placed in the environmental test chamber.

Enucleations were performed on iguanas anesthetized with Ketamine HCL (10 mg/kg). A drop of procaine solution (10%) was administered to the eye as an additional analgesic. The connective tissue around the eye and then the optic nerves were cut; the eye was grasped with a pair of forceps and lifted out of the orbit; Gelfoam was packed into the orbit to retard bleeding, and the eyelids were closed and sutured. One group of lizards ($n = 8$) was first subjected to pinealectomy followed by enucleation. A second group of lizards ($n = 8$) was lesioned in the opposite order (enucleation followed by pinealectomy). After behavioral recordings, the animals were euthanized with an overdose of Ketamine and perfused for histological inspection of the surgery sites. Only animals determined to have complete lesions of the pineal and lateral eyes were included in the data analysis.

Telemetry

Locomotor activity and body temperature data were collected using the VitalView data acquisition system (Mini Mitter; Sunriver, OR). After the implantation of the radio transmitter (Model VM-FH; Mini Mitter), the animal, in its plastic cage, was placed on a radio receiver base (Model RA-1010), which sends body temperature and positional information to a microcomputer for analysis and storage.

Experimental recordings were made while the animals were housed in an environmental chamber (Percival; Boone, IA) at a constant temperature of 27

°C (±0.2 °) and either constant darkness or a 12:12 LD cycle (L ≈ 150 μW/cm^2). Stability of environmental temperature was tested in DD and by placing a transmitter inside a cage contained within the environmental chamber.

Protocol

Lizards were transferred from group enclosures to individual cages and placed in the environmental test chamber in LD for approximately 10 days; they were then allowed to free run in constant darkness until τ stabilized (approximately 2 weeks). At this time, either pinealectomy, enucleation, or sham surgery was performed during the subjective day; they were returned to their cages in DD, and locomotor activity and body temperature were recorded for 1 to 3 weeks. Lizards were returned to LD for at least a week before being subjected to the alternate procedure (pinealectomy for enucleated animals or enucleation for pinealectomized animals) or were euthanized for inspection of the lesion site. While in constant darkness, the animals were fed once a week, during the subjective day, using an infrared viewer (FJW Optical Systems; Elgin, IL). In LD conditions, the animals were fed once a week during the light period.

Data Analysis

Data were analyzed for both rhythmicity and τ using the chi-square periodogram feature in the software program Tau (MiniMitter, Inc.). For each periodogram, at least 5 consecutive days' worth of data from a stable rhythm were used in the analysis. Stability was determined by visual inspection of individual animals' actograms. To determine the phase relationships of the locomotor and body temperature rhythms (ψ_{AT}), and the phase relationships of the rhythms to an entraining light cycle (ψ_{RL}), we used the waveform feature of the Tau program. For determining ψ_{AT}, 5 consecutive days of LAR and BTR data were used to create an average waveform of daily activity or temperature. The average waveforms were aligned, and the difference between the onsets of the two was measured. The onset of the LAR was considered to be the beginning of a bout of locomotor activity longer than 2 hours in duration. The onset of the BTR was defined as the time at which the body temperature reached its mean daily value, as calculated by the software program Tau (see Decoursey et al., 1998). In LD, the difference between the onset of activity and the onset of light was measured to determine the phase angle of entrainment (ψ_{RL}). The phase relationship of the BTR and the light cycle was not considered since the onset of light often masks the rising body temperature (see Fig. 6). Masking was observed as a steady increase in body temperature immediately after lights-on and a steady decline after lights-off. The differences between conditions were analyzed using repeated measures ANOVA with a paired Student's *t* test as a post hoc analysis. Variance between the control and experimental group was tested using an *F* test.

RESULTS

The effects of pinealectomy and enucleation were measured on four aspects of circadian rhythmicity.

Locomotor Activity Rhythms

Pinealectomy significantly lengthened τ of the LAR in DD (τ of control = 24.22 ± 0.17 h; τ of pinealectomized = 25.36 ± 0.27 h; $p < 0.05$, *t* test; see actograms in Fig. 1a, 2a, and 4a). Enucleation produced no change in τ (τ of enucleated animals = 24.06 ± 0.25 h; see Fig. 2a, 4a). Pinealectomy combined with enucleation produced arrhythmicity in both locomotor activity and body temperature in 4 of 16 animals tested (Fig. 3a). For those animals whose LAR remained after both pinealectomy and enucleation, τ was not statistically different from that of pinealectomized animals (τ = 25.52 ± 0.45 h; Fig. 4a).

BTRs

Pinealectomy abolished the BTR in approximately two-thirds of the animals tested. A typical body temperature record from an animal that lost its BTR can be seen in Figure 1b. In lizards that still had a BTR after pinealectomy, τ was lengthened (τ of control = 24.20 ± 0.24 h; τ of pinealectomized = 25.96 ± 0.74 h; $p < 0.05$). The average τ of the BTR was not significantly different from that of the LAR in these animals (Fig. 4b). Enucleation did not change τ of the BTR (τ = 24.07 ± 0.25 h; Fig. 4b). The combination of pinealectomy and enucleation lengthened the τ of BTR but no more than pinealectomy alone.

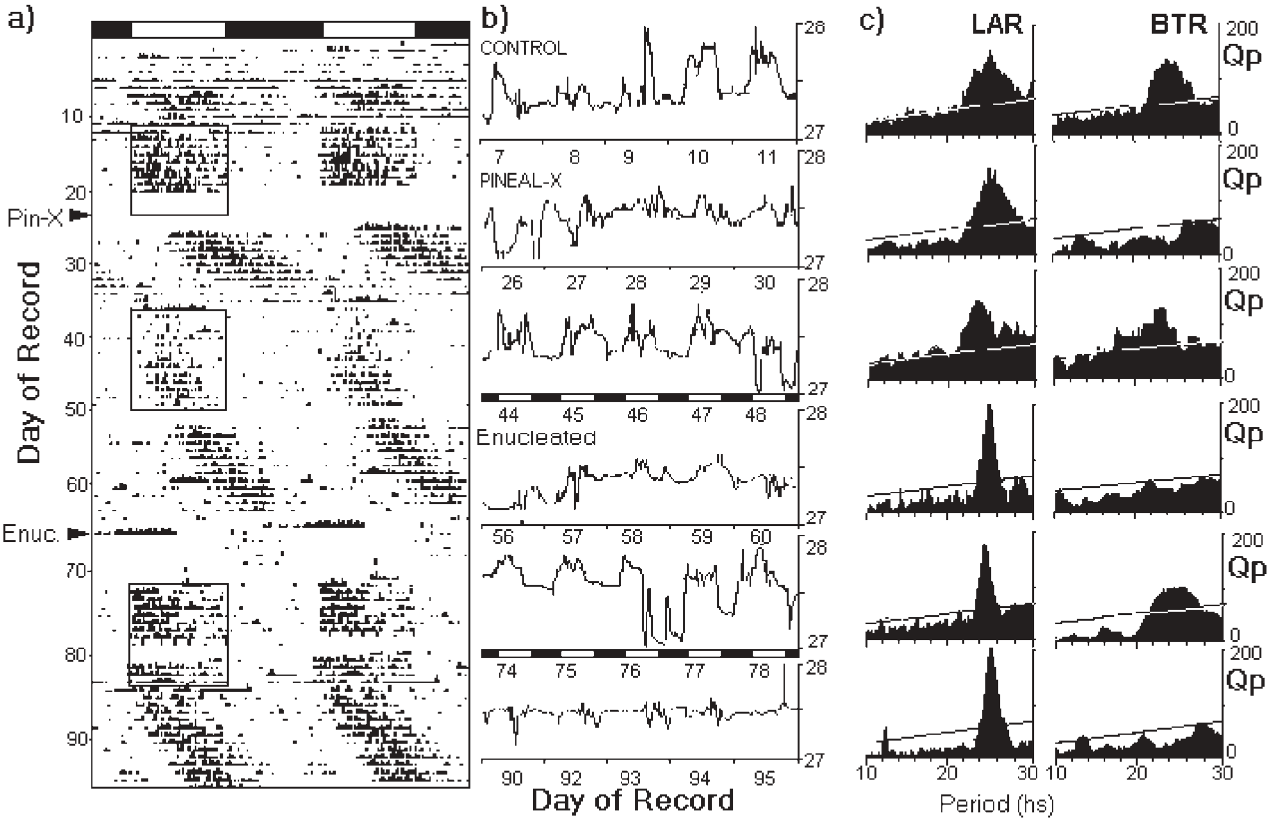

Figure 1. **A double-plotted actogram of locomotor activity (a), the waveform of body temperature (b), and the corresponding periodograms (c) from an iguana while intact, pinealectomized, and enucleated. The day of each surgery is denoted by an arrow in each actogram. The body temperature recordings have the corresponding days listed below each individual record. The periodograms shown were constructed using 5-day segments under each condition and correspond to the days shown in the temperature record. This animal lost its body temperature rhythm (BTR) after pinealectomy while locomotor activity rhythm (LAR) persisted. The box in the actogram shows when an LD cycle was given, and the open and filled bars at the top of the actogram correspond to light and dark periods. The gap in the actogram between days 21 and 25 is the result of lost data due to a computer program malfunction.**

Phase Relationships in Constant Darkness

In DD, the mean daily rise in body temperature occurred shortly after the onset of locomotor activity ($\psi_{AT} = +0.87 \pm 0.39$ h). In pinealectomized animals that still expressed both rhythms, the onset of LAR was delayed ($\psi_{AT} = -3.03 \pm 1.7$ h) relative to that of BTR; therefore, the daily rise in body temperature began well before the onset of activity (Fig. 5a, 6c; $p < 0.05$, *t* test). The most profound effect on ψ_{AT}, however, was seen in enucleated animals (Fig. 5a, 6b), in which ψ_{AT} is shifted approximately 180 ° so that BTR and LAR are completely out of phase with each other ($\psi_{AT} = -11.08 \pm 0.55$ h). Surprisingly, the BTR and LAR of animals that were both pinealectomized and enucleated assumed a phase relationship similar to that of intact animals, although with considerably more variability ($+2.475 \pm 2.26$ h; Fig. 5a).

Phase Relationships in LD Cycles

In intact animals, the onset of locomotor activity occurred within an hour of lights-on, and the daily rise in body temperature began about an hour later (ψ_{RL} of controls = $+1.38 \pm 0.21$ h; Fig. 5b, 6a). Neither pinealectomy nor enucleation affected the average phase angle of entrainment of the LAR to the LD cycle (ψ_{RL} of pinealectomized = $+1.75 \pm 0.46$ h; ψ_{RL} of enucleated = $+2.2 \pm 0.64$ h; Fig. 5b). However, enucleation increased variability in ψ_{RL} among animals when compared with controls (*F* test, $p < 0.05$;

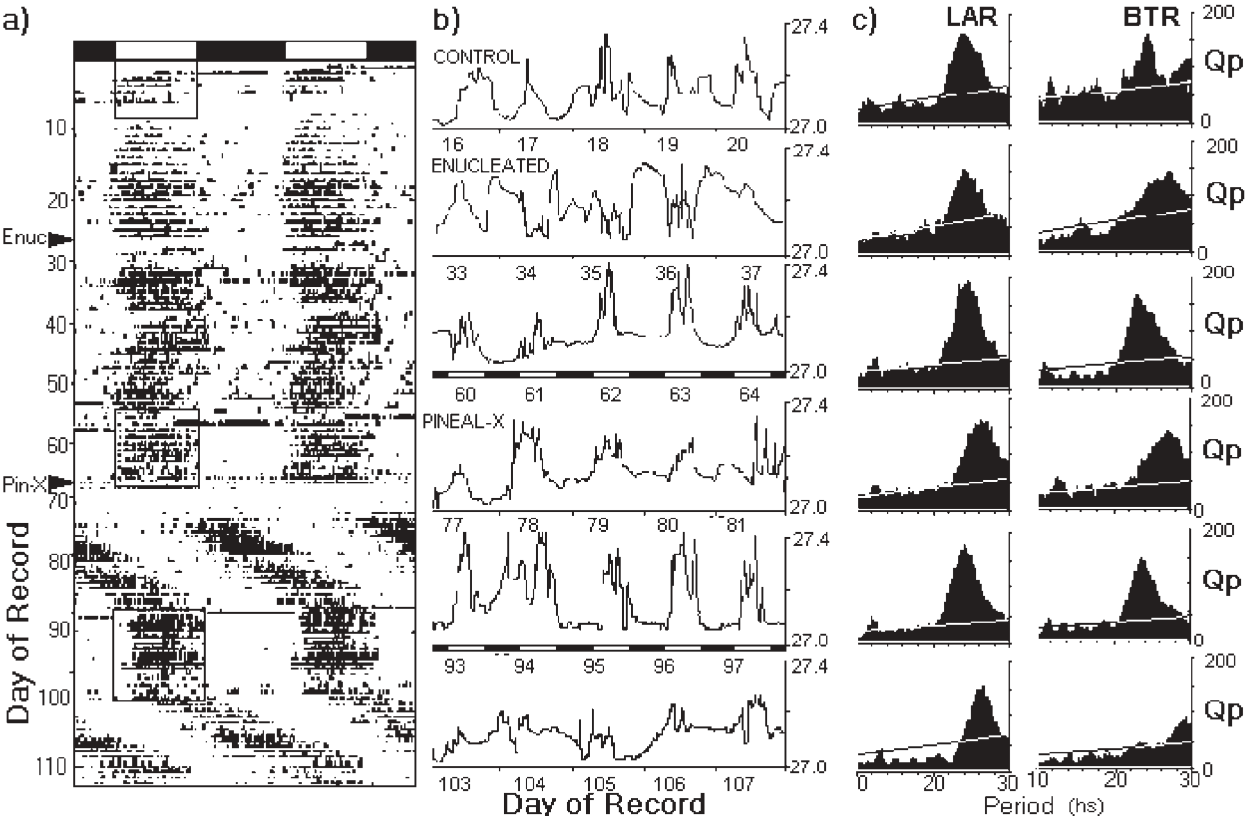

Figure 2. A double-plotted actogram of locomotor activity (a), the waveform of body temperature (b), and the corresponding periodograms (c) from an iguana while intact, after enucleation (arrow), and after pinealectomy (arrow). In this case, pinealectomy did not abolish the body temperature rhythm (BTR). Details as in Fig. 1. LAR = locomotor activity rhythm.

Fig. 5b). The ψ_{RL} of animals that were both pinealectomized and enucleated was also more variable compared to control animals ($\psi_{RL} = +2.05 \pm 0.72$ h; F test, $p < 0.05$; Fig. 5b). On close examination, ψ_{RL} of animals that had been enucleated or pinealectomized and enucleated did not show a unimodal distribution, as in control animals, but rather a bimodal distribution (enucleated $\psi_{RL} = +0.58 \pm 0.16$ h; $n = 4$ and $+3.82 \pm 0.29$ h; $n = 4$; enucleated and pinealectomized $\psi_{RL} = +0.22 \pm 0.16$ h; $n = 7$ and $+3.88 \pm 0.3$ h; $n = 5$).

In LD, the BTR of the animals appeared to be masked by both locomotor activity and light. The body temperature immediately began to rise at light onset (Fig. 6). Often during the light phase, there were fluctuations in body temperature that appeared to coincide with changes in locomotor activity. Masking thus made the determination of independent BTR phase unreliable. Phase and τ of BTR and LAR were not altered in sham pinealectomized animals kept under DD or LD cycles. Furthermore, there were no observed differences in phase or τ of BTR or LAR as a result of the order in which the lesions were given.

DISCUSSION

Our data demonstrate that both the eyes and the pineal gland are major components of the circadian system in *Iguana iguana*. This is most explicitly demonstrated by the lack of circadian rhythms of both body temperature and locomotor activity in approximately one-fourth of the animals tested after removing both the pineal gland and the eyes. Further support comes from the observed changes in period and phase of all animals tested after removal of either the pineal gland or the eyes. However, because circadian rhythms of locomotor activity and body temperature do exist in some animals after removing the eyes and the pineal, an additional circadian pacemaker responsible for maintaining this rhythmicity must exist. The most

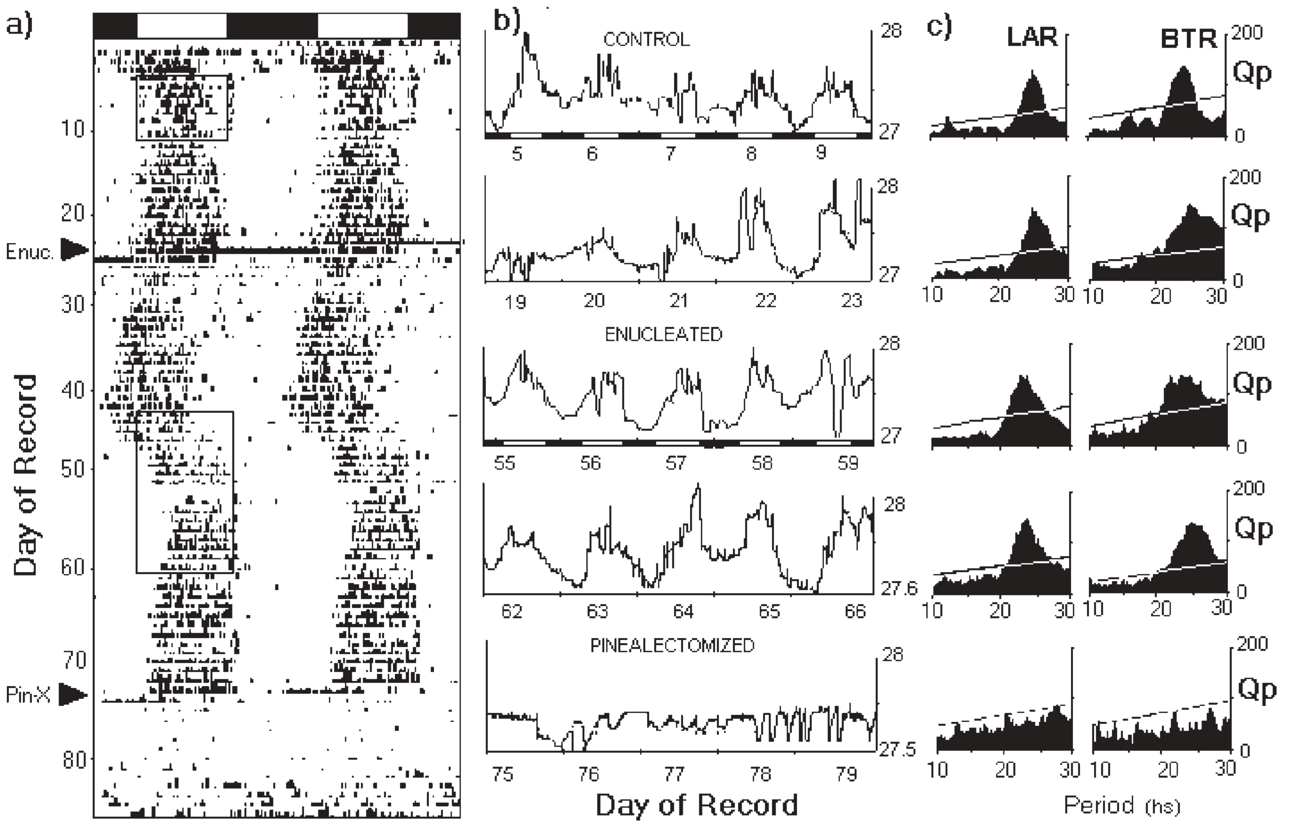

Figure 3. A double-plotted actogram of locomotor activity (a), the waveform of body temperature (b), and the corresponding periodograms (c) recorded from an iguana in which the combination of pinealectomy and enucleation abolished both locomotor activity rhythm (LAR) and body temperature rhythm (BTR). Details as in Fig. 1. After enucleation in DD, a phase shift and changes in the amplitude of locomotor activity are seen in (a). These are attributed to trauma of surgery; after being placed in DD subsequent to being housed under an LD cycle, there is no change in Tau, while the typical changes in phase are observed.

probable location of this additional pacemaker is in the SCN of the ventral hypothalamus. A hypothalamic pacemaker has been previously assumed to exist in *I. iguana* (Tosini and Menaker, 1998) and *S. occidentalis* (Underwood, 1981) and has been clearly demonstrated to exist in *D. dorsalis* (Janik and Menaker, 1990b) and *P. sicula* (Minutini et al., 1995; Bertolucci et al., 2000). Indeed, the SCNs of lizards have many characteristics similar to the SCNs of mammals; they show similar cytoarchitecture and neurochemical markers (Magnone et al., 2003; unpublished data from Grace et al.), cause behavioral arrhythmicity when lesioned (Janik and Menaker, 1990b; Minutini et al., 1995; Bertolucci et al., 2000; unpublished data from Bartell et al.), and are required by both mammals and lizards to entrain locomotor activity to daily melatonin injections (Cassone et al., 1986; Bertolucci and Foà, 1998). Under normal circumstances, the eyes and pineal work in concert with the presumed pacemaker in the SCN to regulate the circadian rhythms of locomotor activity and endogenously regulated body temperature. These structures coordinate the phases of these rhythms with the external environment using photic cues perceived by one or more of several photoreceptive structures.

The importance of the pineal gland as a component of the iguana's pacemaking system is emphasized by the effects of its removal. Pinealectomy abolished the BTR in 11 of 16 lizards and lengthened the free-running period of LAR in all animals tested. In those animals in which BTR remained, a similar lengthening in period was observed, and body temperature began to rise before the onset of the daily bout of locomotor activity; the opposite was true of controls. These differences in phase, and the fact that in a majority of animals pinealectomy abolishes BTR but not LAR, suggest that separate oscillators may be controlling the two rhythms. Melatonin may directly regulate body temperature in iguanas (Tosini and Menaker, 1996b, 1998), and it is possible that structures other than the pineal

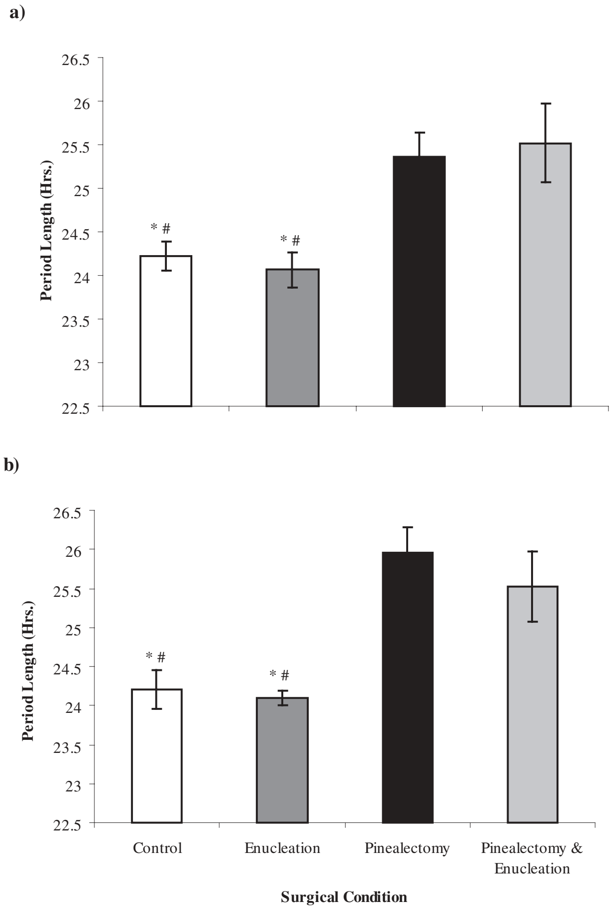

Figure 4. The average lengths of the free-running period (τ, ±SE) of the locomotor activity rhythm (LAR) (a) and body temperature rhythm (BTR) (b) in DD during control, enucleated, pinealectomized, and pinealectomized/enucleated conditions. A significant change ($p < 0.05$, paired Student's *t* test) in τ of LAR is seen after pinealectomy or pinealectomy with enucleation but not after enucleation alone. τ of BTR was lengthened after pinealectomy or pinealectomy with enucleation in those animals still displaying a BTR. * = different than pinealectomy, # = different than pinealectomy and enucleation ($p < 0.05$, paired Student's *t* test).

may produce enough melatonin to maintain the BTR in young animals. Indeed, the concentrations of blood-borne melatonin are higher in younger animals than in adults, and nonrhythmic melatonin can still be measured in blood after pinealectomy (Miranda-Anaya et al., 2000, and unpublished data).

The eyes, like the pineal gland, are also an integral component of the iguana's circadian system. However, the greatest effects of enucleation that we observed were on phase not period. Enucleation alone had no measurable effects on period of either LAR or BTR, and the combination of enucleation with pinealectomy produced no greater effects on period of either LAR or BTR than pinealectomy alone. The effects of enucleation on circadian period in the iguana are quite different from most other lizards; significant changes in the period length of locomotor activity have been described in *P. sicula* (Foà, 1991), *S. olivaceus*, and *S. occidentalis* (Underwood and Menaker, 1976; Underwood, 1981).

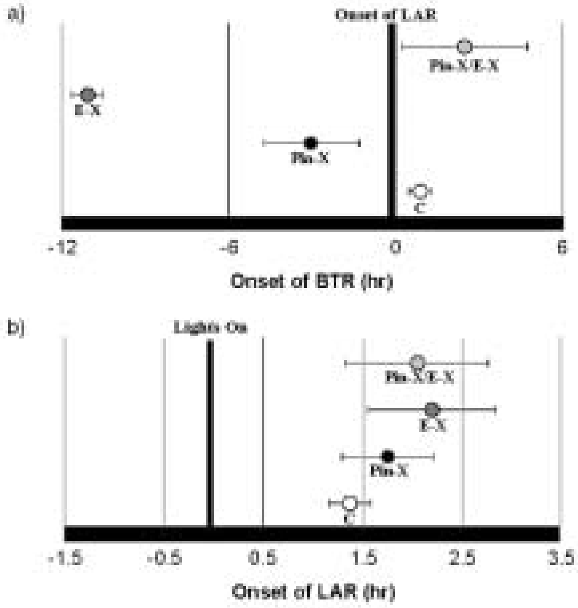

Figure 5. The phase relationship of locomotor activity rhythm (LAR) and body temperature rhythm (BTR) in DD (a) and the phase relationship of LAR and a light cycle (b) of control (c), pinealectomized (Pin-X), enucleated (E-X), and pinealectomized/enucleated (Pin-X/E-X) iguanas. After pinealectomy, the daily rise in temperature is advanced with respect to LAR onset (a). Enucleation caused a large change in the phase relationship between BTR and LAR, while the combination of enucleation and pinealectomy was not different from controls. While enucleation and enucleation with pinealectomy did not affect the mean phase relationship with the light cycle, these treatments did increase its variability (*F* test, $p < 0.05$).

Both Underwood (1981) and Foà (1991) have suggested that the eyes act as coupling devices in the circadian systems of lizards. Our data demonstrate directly that the eyes are involved in coupling the oscillators that control BTR and LAR, since enucleation caused a dramatic change (180 °) in the phase relationship between these two rhythms in constant darkness. However, it is likely that other mechanisms coupling these two rhythms exist, since the postenucleation phase relationships between them appear to be stable. Disruption of these coupling relationships is likely to be the reason for the increased variance and the bimodal distribution of the values of

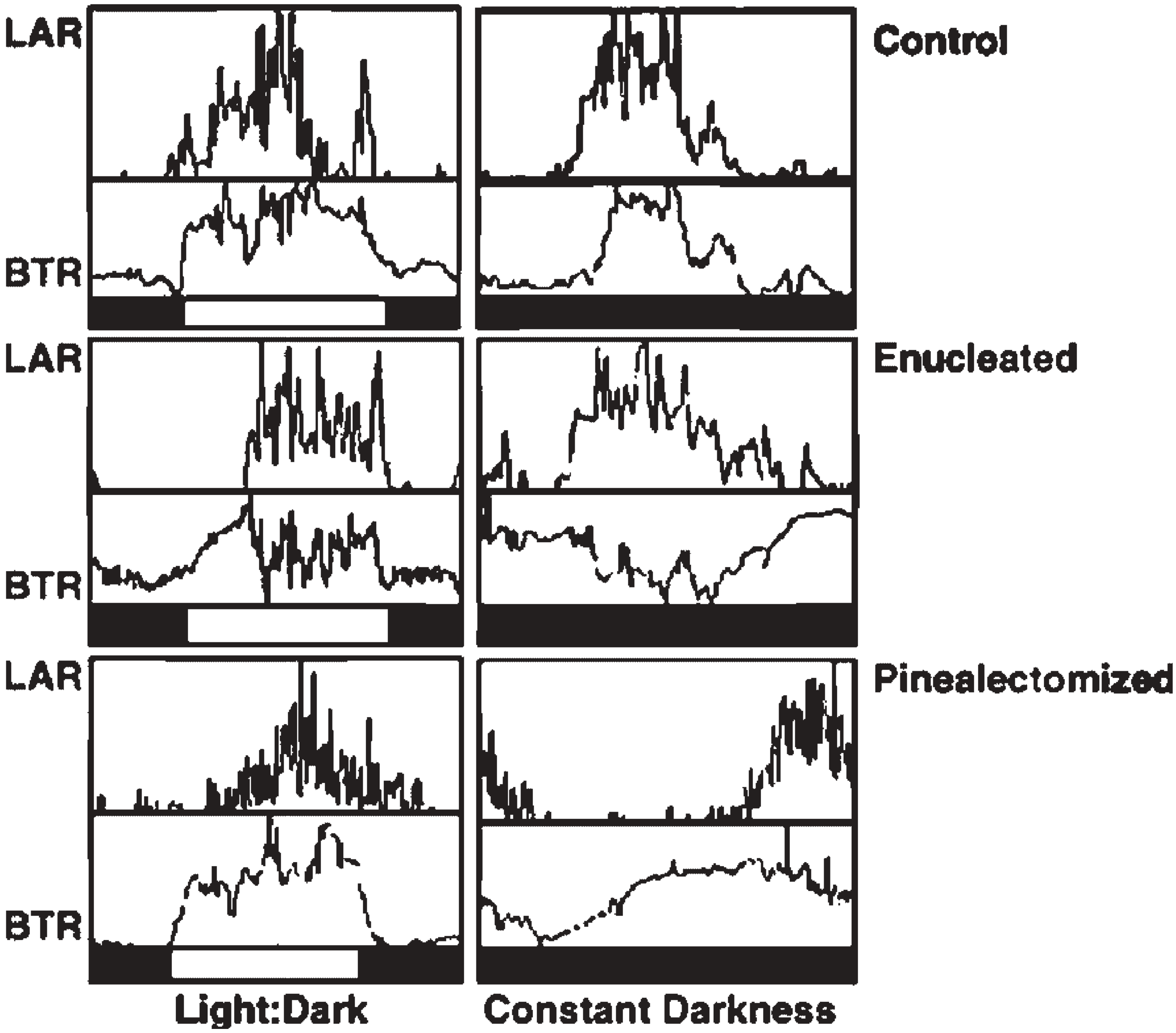

Figure 6. The average waveforms of the locomotor activity rhythm (LAR) (top trace of each panel) and body temperature rhythm (BTR) (bottom trace) from 5 consecutive days under LD (left column) and DD (right column) in intact, enucleated, and pinealectomized iguanas. The BTR is clearly masked by onset of light. During the light phase of LD, bouts of lowered body temperature are often seen in enucleated animals.

ψ_{RL} that we observed following enucleation. In a somewhat similar vein, Underwood (1983) reported that the onset of activity of both intact and pinealectomized *Anolis* assumed one of two preferred phase relationships with an entraining light cycle. He attributed this to the presence of two oscillators governing locomotor activity; depending on which oscillator led, ψ_{RL} assumed a value of either ≈ +0.6 h or ≈ +3.8 h.

Pinealectomy and enucleation in combination did not have a greater effect on the free-running periods of the LAR or BTR than that produced by pinealectomy alone, but the ψ_{AT} of animals that were both pinealectomized and enucleated was different from that of either enucleated or pinealectomized animals. In pinealectomized-enucleated animals, ψ_{AT} is similar to but more variable than ψ_{AT} of intact animals (with the BTR rising shortly after the onset of the LAR). Removal of both the pineal and the eyes produced a ψ_{RL} similar to that observed in enucleated animals. One of two preferred ψ_{RL} was observed, either ≈ +0.2 h or ≈ +3.9 h. The occurrence of two alternative phase relationships indicates that two different oscillators may be driving activity, with one oscillator being dominant. Since these animals have neither pineal nor retinae, both oscillators ultimately controlling LAR are likely to reside within the SCN, as is perhaps the case in mammals (Pittendrigh and Daan, 1976). It will be important to assess the effect of SCN lesions in iguanas.

The effects of pinealectomy and/or enucleation on circadian rhythms in iguanas are quite variable. This may be due to differences among animals in coupling strength among the several oscillators composing the circadian system. The weaker the coupling within an animal's hypothalamic pacemaker, the more susceptible that animal is likely to be to the effects of pinealectomy and/or enucleation. Variability in the effects of pinealectomy has been observed previously. In the spiny lizard (*S. olivaceus*) and the European starling (*Sturnus vulgaris*), pinealectomy can induce either arrhythmicity or changes in the period of locomotor activity (Underwood, 1977; Gwinner, 1978).

The circadian system of the green iguana may undergo changes as the animals age. The degree to which particular circadian behaviors rely on different pacemakers appears to change with age. This may explain the differences between the results reported here and those in a previous study from our laboratory. In the earlier study, using older iguanas (estimated to be older than 1 year of age), pinealectomy abolished the BTR in all 10 animals tested (Tosini and Menaker, 1995), whereas in the current experiments pinealectomy abolished the BTR in only 11 of 16 animals. More important, in the previous study when intact animals were placed in DD, the onset of activity occurred after the daily rise in body temperature; in the experiments reported here, the onset of activity occurred before the daily rise in body temperature. Age-related differences in melatonin levels are known to exist in green iguanas (Miranda-Anaya et al., 2000, and unpublished data) as well as in the pineal of mammals (Miguez et al., 1998). There are also age-related changes in SCN neuronal firing frequency in mice (Aujard et al., 2001) and SCN gene expression in rats (Yamazaki et al., 2002). Thus, the differences in rhythmicity and phase between our two studies can be plausibly accounted for by the differences in age of the animals used.

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