Arrhythmically Singing Crickets: Thermoperiodic Reentrainment After Bilobectomy

Abstract. *The circadian control of the calling song of crickets is abolished by severance of the optic lobes. The arrhythmic singing activity of operated animals is unaffected by all possible light conditions, yet the singing can be reentrained by a daily temperature cycle. The characteristics of this reentrainment indicate that temperature is acting as an actual Zeitgeber. In light of these results the current hypothesis which ascribes a driving oscillator function to the optic lobes must be reevaluated.*

One of the major problems in the investigation of the physiological and biochemical properties of circadian oscillations in animals has been the elusive nature of the oscillators' anatomical location. Only two driving oscillators have been characterized conclusively, both in *Aplysia* (*1, 2*). The existence of two other clocks, in the protocerebrum of silkmoths (*3*) and in the optic lobes of cockroaches (*4, 5*) and crickets (*6, 7*), have been suggested, although they have not been shown to oscillate independently. This report demonstrates that in the cricket, *Teleogryllus commodus* Walker, bilateral removal of the optic lobes renders the calling song (*8*) of the animals arrhythmic in all light conditions. The rhythm can, however, be reentrained by a daily temperature cycle. The hypothesis that the optic lobes are the site of the exclusive driving oscillator for stridulation must therefore be reexamined.

We have explored the effect of temperature on stridulation under constant light conditions in normal and bilobectomized crickets by exposing them to a daily temperature cycle (*9*). Figure 1 illustrates the daily singing activity of an unoperated cricket in response to 12 hours light : 12 hours dark (LD), constant light (LL), and a 12-hour high temperature (35°C) : 12 hour low temperature (25°C) cycle (HTLT 12 : 12). In LD the animal sings primarily during the dark phase, while when exposed to constant light and a temperature cycle singing activity is concentrated in the cold phase. This is to be expected, because in nature the cold part of the day corresponds to the dark phase. The total amount of singing in LD, constant temperature, and in LL, HTLT, is approximately the same over a 24-hour period. However, the phase angle of singing relative to the two different environmental transitions varies considerably. In LD the animal anticipates the light transitions by beginning to sing 1 to 2 hours before the onset of darkness and terminating 2 to 3 hours before the onset of light; synchronization of singing to the cold phase shows no anticipation of the temperature steps but begins approximately 30 minutes after the cold phase has started. The nearly simultaneous onset of singing and the cold phase might lead to speculation that temperature is not acting as a Zeitgeber. The synchronization of the calling song to the cold phase could be due to thermokinetic stimulation (*10*) or suppression (*11*), release of a sublimated underlying rhythmicity (*12*), or rhythmic release of stress factors (*13*) which might affect behavioral output (*14*).

Evidence that temperature is acting as a true Zeitgeber (*15*) comes from three sources. Transients provoked by the initiation of entrainment and by a single cold pulse provide the first piece of evidence. Examination of Fig. 1 (days 18 to 20) and Fig. 2 (days 16 to 19) shows that upon initiation of the first temperature step the acoustic behavior does not become immediately synchronized with the temperature step down. If the effect were an exogenous one, we would expect immediate synchronization, barring the unlikely possibility of adaptation. And when crickets held in constant illumination and temperature were exposed to a single 12-hour cold pulse (*16*) they showed a marked transient synchronization of singing correlated with the onset of the cold pulse for the next 2 days. If the cold pulse acted as an exogenous trigger we should find no subsequent transients under constant temperature conditions. The second argument for the true Zeitgeber function of the temperature cycle comes from the lack of any masking effect (*17*). Figure 1 shows that singing after termination of the temperature cycle (day 34) occurs at a phase angle related not to the previous free-running rhythm but to the onset of stridulation on the last day of the thermoperiod (*18*). This indicates that the temperature cycle does actually reset the clock each day, rather than simply releasing the behavior. The final and most compelling piece of evidence comes from the observation that crickets will not entrain to a 15 hours 35°C : 15 hours 25°C temperature cycle (*19*), but instead continue to free run. If the effect were exogenous and therefore not restricted to a circadian clock, synchronization to a 30-hour period should occur. The successful synchronization of the calling song with temperature cycles under constant light conditions, the presence of transients, the lack of any masking effect, and the inability to entrain to a 30-hour thermoperiod prove the role of temperature cycles as true Zeitgebers.

Previous work (*6*) has demonstrated that removal of the optic lobes causes crickets to sing arrhythmically (*20*) under any light conditions. The second portion of our in-

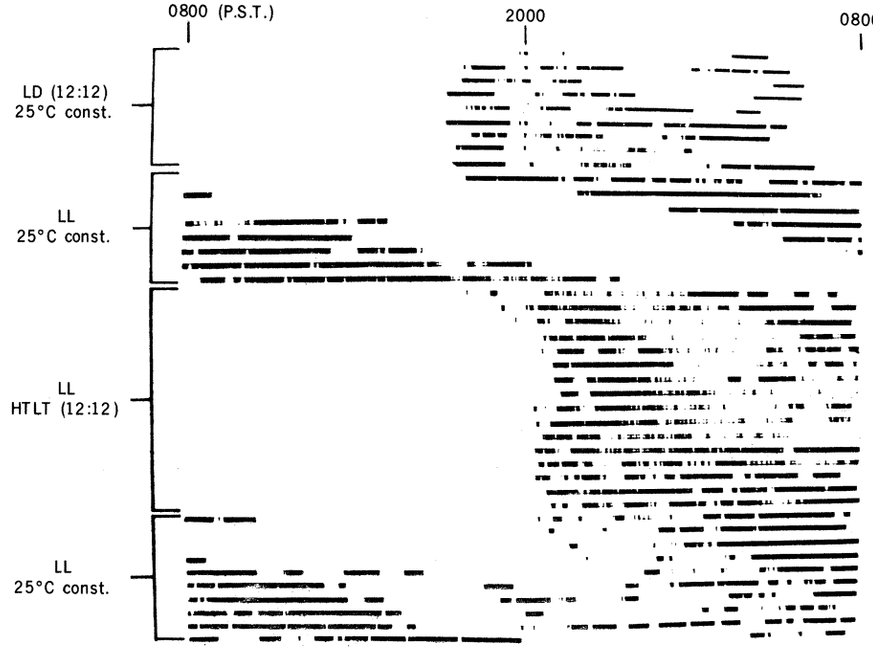

Fig. 1. Daily singing activity record of an unoperated normal *T. commodus*. Days 1 to 10: LD 12 : 12 and 25°C constant; days 11 to 18: LL and 25°C constant; days 19 to 34: LL and HTLT 12 : 12; days 35 to 44: LL and 25°C constant. The L (30 lux) and HT (35°C) phases occur from 0800 to 2000 P.S.T. and the D and LT (25°C) phases from 2000 to 0800 P.S.T.

vestigation consisted of an attempt to use temperature cycles for reentrainment of arrhythmic singing activity. Animals were exposed to constant light and temperature conditions, bilobectomized, and then subjected to a daily HTLT 12 : 12 cycle. Figure 2 shows that the free-running rhythm of singing in LL and 25°C constant temperature is rendered arrhythmic by the optic lobe removal, and that entrainment occurs upon initiation of a ± 10°C temperature cycle. The phase angle of entrainment to the temperature cycle in the bilobectomized crickets is identical to that of normal animals. Figure 3 presents the averaged results from all operated crickets which were exposed to the temperature cycle. The fact that a statistically significant (Student's t, $P < .001$) amount of the activity occurred in the 12-hour cold phase, while singing during the 4 days prior to and after the temperature cycle is randomly distributed over the 24-hour period, indicates that entrainment of bilobectomized crickets has taken place. The presence of transients at the beginning of this entrainment (Fig. 2, days 16 to 19), coupled with the induction of transients in crickets after a single cold pulse and their inability to entrain to HTLT 15 : 15, demonstrates that for bilobectomized crickets the temperature cycle is acting as a true Zeitgeber.

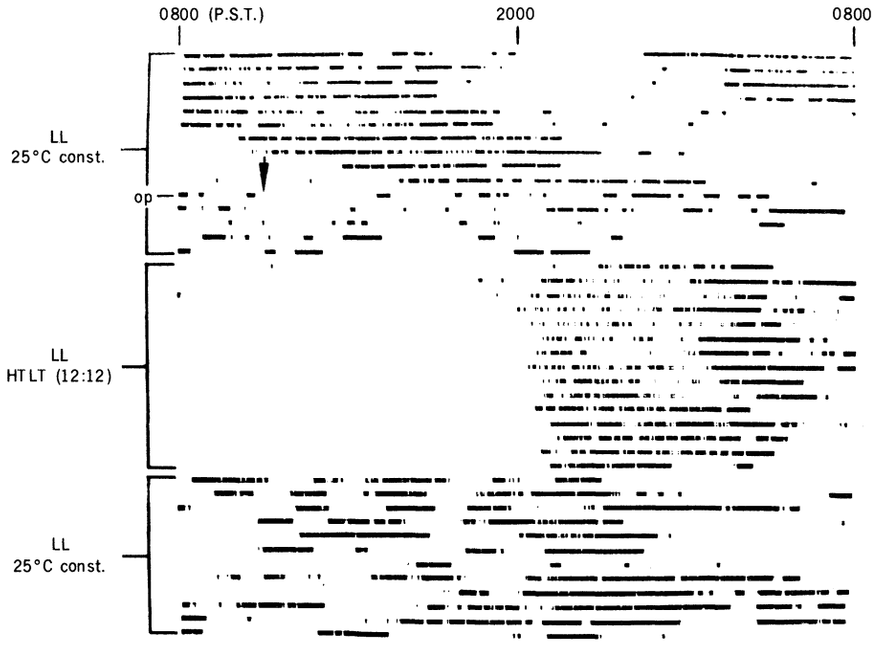

Fig. 2. Daily singing activity record of an operated (bilobectomy on day 11 indicated by arrow) *T. commodus*. Days 1 to 15: LL and 25°C constant; days 16 to 30: LL and HTLT 12 : 12; days 30 to 42: LL and 25°C constant. The HT (35°C) phase occurs from 0800 to 2000 P.S.T. and the LT (25°C) phase from 2000 to 0800 P.S.T.

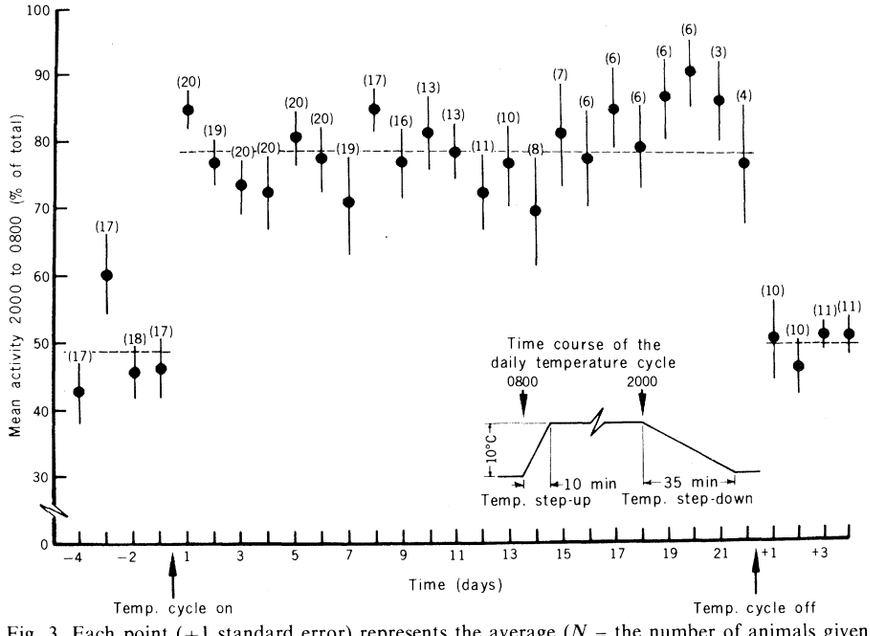

Fig. 3. Each point (±1 standard error) represents the average (N = the number of animals given above each point) percentage of the total singing activity which occurs from 2000 to 0800 P.S.T., which is the cold phase when the temperature cycle is on. The dashed lines represent the mean percentage of singing in the 2000 to 0800 time period for all the animals before (49.0 percent), during (78.1 percent), and after (49.9 percent) the temperature cycle. The inset is a schematic of the time course of the daily temperature cycle.

The current (*21*) hypothesis concerning the cockroach locomotor activity rhythm (*4, 5*) and, by implication, the locomotor and stridulatory rhythms of the crickets (*6, 7*) is that a circadian pacemaker resides in each optic lobe and that these oscillators control entrainment and free running of the various behavioral outputs. The results presented here suggest that this hypothesis requires modification. The synchronization of stridulation with temperature cycles is similar in normal and bilobectomized crickets, indicating that surgery has not destroyed the entrainability of the calling song. Therefore, at least one timing device for that behavior pattern must still be intact. However, the oscillator or oscillators responsible for the timing of the calling song is not unaffected by the removal of the optic lobes, as is indicated by the loss of free-running rhythmicity. This result argues for an important role of the optic lobes in the expression of free-running periodicity. Their effect on the free-run must therefore transcend the circuits of the visual pathway, for free running continues to occur in the absence of light or after severance of the ommatidial nerves (*6*). Although the classic defining feature of a clock is its ability to free run (*22*), a condition that cannot obtain after optic lobe removal, we can still attribute the temperature entrainment of bilobectomized crickets to an endogenous circadian oscillator because of the presence of transients and the inability to entrain to a 30-hour period.

There are a number of alternative explanations as to how and where the circadian control of singing behavior takes place. Our results show that light and temperature can each act independently as a Zeitgeber for singing and that the cricket can entrain to temperature cycles after bilobectomy although it cannot respond to light or free run. One hypothesis would call for the existence of two mutually coupled clocks—a light-sensitive clock in the optic lobes and a temperature-sensitive clock in the central brain mass. These two clocks could also both be located in the central brain mass, but then the light-sensitive oscillator would have to maintain a con-

nection with the optic lobes. This pathway might or might not be part of the visual system. As suggested for the pineal of sparrows (*23*), the optic lobes of the cricket could house a self-sustaining oscillator, driving a damped oscillator in another part of the brain which directly controls singing activity. Entrainment, then, of bilobectomized crickets would be due to direct driving of the damped oscillator by the temperature cycle. Previous studies have argued against multiple oscillators for different behaviors in this cricket species (*24*). It seems even more likely that one behavior is not controlled by as many oscillators as there are Zeitgebers. Therefore, a more parsimonious explanation demands a single timing device which receives afferent input of hierarchically ordered environmental stimuli which act as Zeitgebers.

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8. Hereafter any use of the words singing, calling, or stridulation refers to the calling song of male *Teleogryllus commodus*.
9. Housing, recording, rearing, and surgical techniques are described in detail elsewhere (*6*). Forty unoperated and 20 bilobectomized crickets were recorded. The light intensity in all LL and LD experiments was 30 to 35 lux. All LD cycles had the lights on from 0800 to 2000 and the lights off from 2000 to 0800 P.S.T., and all HTLT cycles were at 35°C from 0800 to 2000 and 25°C from 2000 to 0800 P.S.T. Parallel experiments were run in DD (constant dark) with results similar to those obtained in LL conditions. Results of exposure of bilobectomized crickets to LD have also been described (*6*). The daily temperature cycle (inset, Fig. 3) was effected by combining a high-wattage heater with a forced air uptake and removal system. The temperature regime was recorded in each individual cricket chamber by copper-constantan thermocouples attached to a Honeywell recording potentiometer at 6-second intervals for the period around the transitions and 5-minute intervals for the rest of the 24-hour period. The total time for an experiment in *T. commodus* was restricted to between 40 and 60 days, which is the average lifespan (from imaginal molt to death) of a normal male.
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15. "Zeitgeber" is used here in its strictest sense as that forcing oscillation which entrains a biological rhythm [J. Aschoff *et al.*, in *Circadian Clocks*, J. Aschoff, Ed. (North-Holland, Amsterdam, 1965), p. xiv]. We mean to make a sharp distinction between those driving oscillations that simply trigger or release the behavior and those that actually reset the biological clock with each cycle.
16. *T. commodus* housed in constant light (30 lux) and constant temperature (25° ± 1°C) were placed for 12 hours (0800 to 2000 P.S.T.) at 5°C, LL (30 lux), and then returned to the constant light and temperature conditions. Statistical analysis of the period beginning 24 hours after the initiation of the cold pulse in normal ($N = 2$) and bilobectomized ($N = 5$) animals shows a significantly (Student's t, $P < .01$) greater singing activity during the 12-hour period corresponding to the cold pulse (0800 to 2000) as compared to the subsequent 12-hour period (2000 to 0800). This difference lasts for approximately 2 days after the cold pulse and then begins to damp out.
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18. Best fit projection of the free-running rhythm in Fig. 1 reveals that if the thermoperiod was merely masking the expression of the free run we would find the singing on day 34 beginning at 0800 rather than at 2000. The 3.5-hour bout of singing that does begin at 0800 on day 34 is a transient extension of the previous day's singing activity due to the novel absence of the step up in temperature.
19. Normal ($N = 5$) and bilobectomized ($N = 3$) animals were allowed to entrain to HTLT 12 : 12, LL (30 lux) and then subjected to HTLT 15 : 15, LL (30 lux). Normal animals returned to a free-running singing rhythm and lobectomized ones to an arrhythmic pattern during exposure to the 30-hour cycle. Fourier analysis of the lobectomized animals showed no significant (± 2 standard errors) periodicities over the range from 1 hour to infinity. Both groups entrained again to HTLT 12 : 12 after termination of the 30-hour cycle.
20. Arrhythmia here means a total desynchronization with no apparent periodicities. For instance, Fourier analysis of the activity depicted in Fig. 2 for days 30 to 42 (LL, 25°C constant) shows no significant periodicities within 95 percent confidence limits. The similarity in the total amount of singing in lobectomized and normal crickets (*6*) indicates that the operation is not quantitatively releasing or suppressing the singing.
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Heteropolypeptides from Poly-α-Cyanoglycine and Hydrogen Cyanide: A Model for the Origin of Proteins

Abstract. *Poly-α-cyanoglycine, a homopolymer synthesized from the* N-*carboxyanhydride of α-cyanoglycine, is converted by cumulative reactions of hydrogen cyanide to heteropolypeptides that can be hydrolyzed to protein amino acids, including glycine, alanine, valine, aspartic acid, and glutamic acid. These results are consistent with the hypothesis that the original heteropolypeptides on the earth arose spontaneously from hydrogen cyanide and water without the intervening formation of α-amino acids.*

During the past two decades, extensive research (*1*) on chemical evolution has led to the widespread belief that the formation of primitive proteins occurred in two stages: α-amino acid synthesis initiated by the action of natural sources of high energy on the components of a reducing atmosphere followed by polycondensation of the accumulated monomers in the oceans or on land. A more critical examination of the evidence for the second step, however, suggests that the inherent thermodynamic barrier to spontaneous polymerization of α-amino acids has only been overcome by invoking specific environments—anhydrous locales, high-temperature milieus, or acidic bodies of water, for example—that may not be characteristic of a young, developing planet. We now present experimental results consistent with an alternative route proposed for the origin of proteins—the direct synthesis of heteropolypeptides from hydrogen cyanide and water without the intervening formation of α-amino acids.

According to this hypothesis (*2*), a low-energy pathway exists (Fig. 1) that allows hydrogen cyanide (**1**) to polymerize readily to polyaminomalononitrile (**4**) via dimeric hydrogen cyanide (**2**) and its polymer (**3**). Semiempirical quantum-mechanical calculations (INDO) suggest that **2** is probably azacyclopropenylidenimine (*3*) rather than iminoacetonitrile or aminocyanocarbene (*4, 5*), other possible structures that could also lead to **3**. Successive reactions of hydrogen cyanide with the reactive nitrile groups of **4** then yield heteropolyamidines (**5**) which on contact with water are converted to heteropolypeptides (**6** and **7**) after a series of hydrolysis and decarboxylation steps (*6, 7*).

To demonstrate the feasibility of this postulated conversion of a homopolymer to a heteropolymer it would be desirable to synthesize **4** and transform it to **7** by treat-

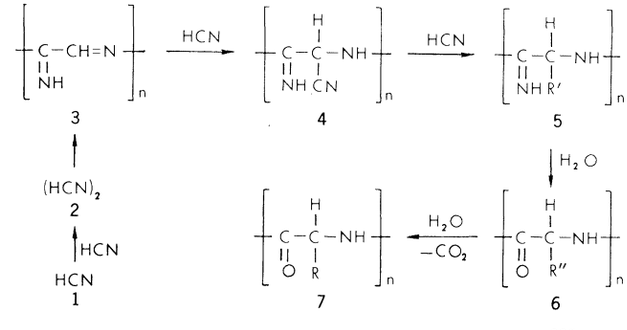

Fig. 1. Proposed route for heteropolypeptide synthesis from hydrogen cyanide and water (**1** → **7**). R″ and R′ are precursors of protein side chains R.