Periodic Analysis of the Viking Lander Labeled Release Experiment

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ABSTRACT

Did Viking Lander biology experiments detect life on Mars? The strongest evidence for biology resulted from the Labeled Release (LR) experiment[1]. A radiolabeled (^{14}C) nutrient solution was added to a Martian soil sample and the subsequent evolution of radioactive gas was observed. Flight data showed an initial release of labeled gas followed by strong periodic fluctuations in the amount of gas in the headspace above the soil, superimposed on a slow rise in release. Current analyses show, at steady state, these fluctuations exhibit a periodicity of 24.66+/-0.27 hr, statistically indistinguishable from the Martian solar period. The gas fluctuation appears synchronized to a mean 2º C periodic fluctuation in internal temperature in the experimental chamber, which in turn is synchronous with almost 50º C daily fluctuations in ambient Mars surface temperature. Calculations based on LR data indicate that the daily gas fluctuation amplitude could be in part accounted for by change in temperature-dependent soil solubility of CO_2, but total amount of gas accumulated cannot be accounted for in this way. Recent observations of circadian rhythmicity in microorganisms and entrainment of terrestrial circadian rhythms by low amplitude temperature cycles argue that a Martian circadian rhythm in the LR experiment may constitute a biosignature.

Keywords: circadian rhythm, Labeled Release experiment, life on Mars, phase shift, Viking mission

1. INTRODUCTION

The possibility of life on Mars has excited human imagination since well before the time of Percival Lowell and has been depicted in countless works of imagination by such authors as H. G. Wells, Edgar Rice Burroughs, and Ray Bradbury. But an empirical test of this possibility had to await the Viking mission to Mars in 1976. In this mission three independent experiments were designed to detect putative Martian microbes. One, the Labeled Release (LR) experiment[1], gave results that satisfied pre-mission criteria agreed upon for demonstrating the existence of life. However, other interpretations have mired the results in controversy for the last 25 years.

2. METHODS

The LR experiment involved collection of a sample of Martian soil by a robotic arm. A small portion of the sample was placed in a sealed test cell and injected with a drop of a ^{14}C-labeled nutrient medium. The sample was maintained at approximately 10º C. Evolution of radiolabeled gas (most likely CO_2, but possibly CO, or even CH_4) was monitored by a beta detector in a chamber connected to the test cell by a 13" stainless steel tube (i.d.= 0.105"), through which the evolved gas traveled. A total of nine soil samples was tested, four in the first lander (VL1) and five in the second lander (VL2), the landers being approximately 4000 miles apart. Positive responses (n=4) were followed by control tests (n=5) on duplicate soil samples, after heating to "sterilization" temperature or storing for long periods, to distinguish whether the responses were biological or chemical.

3. RESULTS

Nutrient incubation with a fresh Martian soil sample generated a large evolution of radiolabeled gas, compared to background. Sterilization of the sample via heating to 160º C prevented this effect. Prior heating to 46º C significantly attenuated the nutrient-induced rise in detected radiolabel. Storage of the sample in the dark at 10-15º C for either three or five months resulted in a 90% loss of signal upon subsequent nutrient injection. Arrhenius analysis indicated that the activation energy of the release process was 35-43 kcal/mole, well within the range of a biological process[2]. Ground-based simulations[3] with Mars soil analogs suggested that the evolved radiolabel may have been CO_2, but experiments with peroxides and superoxides[4] provided no support for such chemical agents being the cause of the observed response on Mars.

The focus here will be on one of the nine experiments, the "active" phase (no sterilization) Viking Lander 2 cycle 3 (VL2c3) experiment. One aspect of the data that has not been previously subjected to detailed statistical analysis is the periodic fluctuation in radiolabel superimposed on a longer term logarithmic increase in signal (VL2c3; Figure 1 upper; expanded in Figure 1 middle, lower). About 40 sols following the second nutrient injection, the amplitude of the periodic fluctuations diminishes, eventually dampening out. These data were subjected to classical circadian analysis.

Cosine spectrum (Figure 2 upper) and F periodogram analysis (Figure 2 lower) indicated that the period of the fluctuations in radioactive gas (labeled LR in the figures) present in the detector chamber is precisely 24.66 hr, one Martian sol.

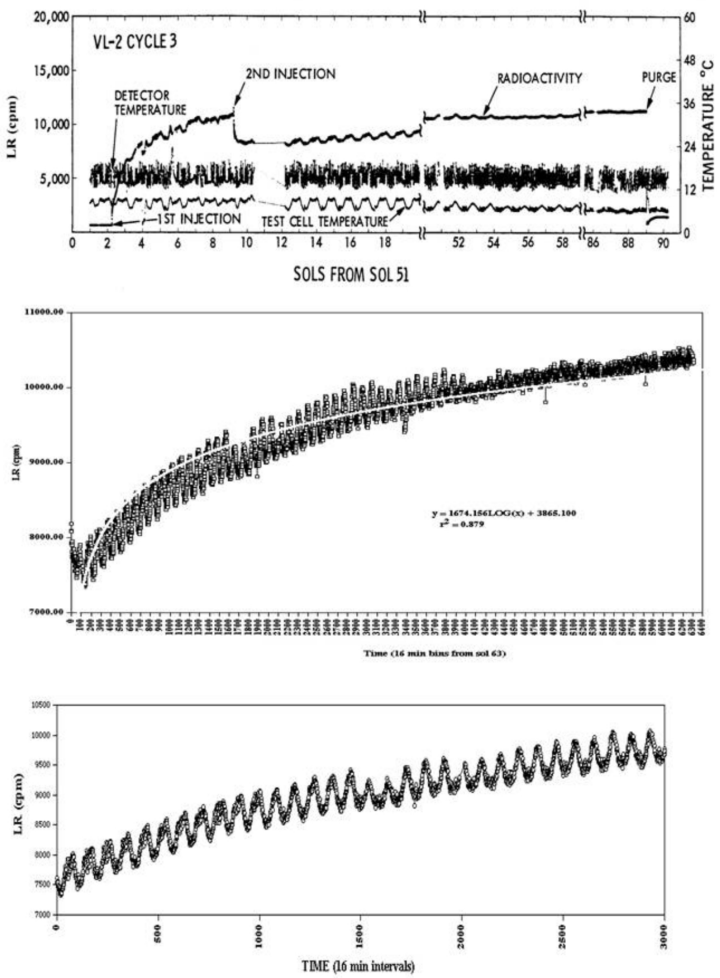
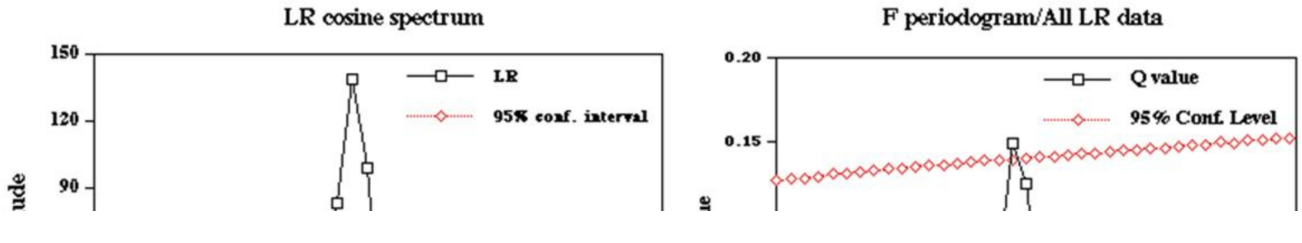


Figure 1 (upper, middle, lower) The upper graph is taken from Levin and Straat, 1977 and shows labeled release (radioactivity), test cell temperature (HT), detector temperature, and nutrient injections for VL2c3. The LR data from sol 63 on is expanded in the middle graph and fit to a log function (white line). The lower graph expands 30 highly periodic oscillations from sol 63 up to the signal reduction late in the experiment.



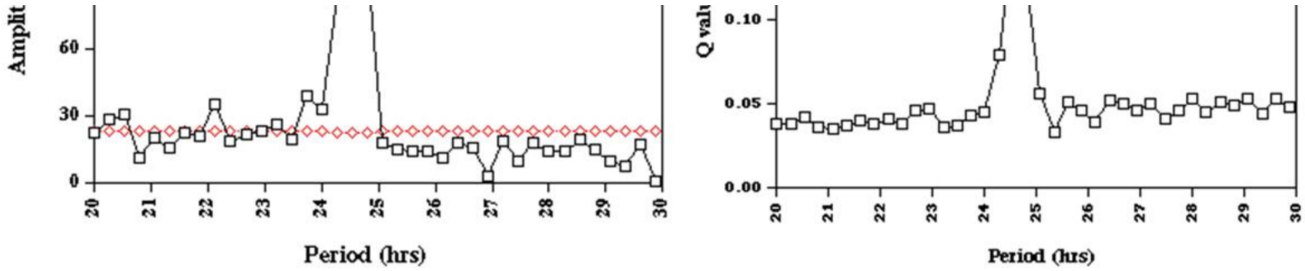


Figure 2 (upper, lower). The upper graph shows that the best fit (highest amplitude of the fit cosine) to the LR data is a cosine function with a period of 24.66 hr. The lower graph similarly shows that the best fit (highest Q value) is a periodic function with period equal to 24.66 hr. 95% confidence intervals for the best fits are indicated in both graphs.

The average waveform of the LR signal for the first two months following the second nutrient infusion is plotted in Figure 3 upper. It is apparent that the average amplitude of the signal in the first month (Figure 3 middle) is substantially higher than in the second month (Figure 3 lower), although the period remains 24.66 hr.

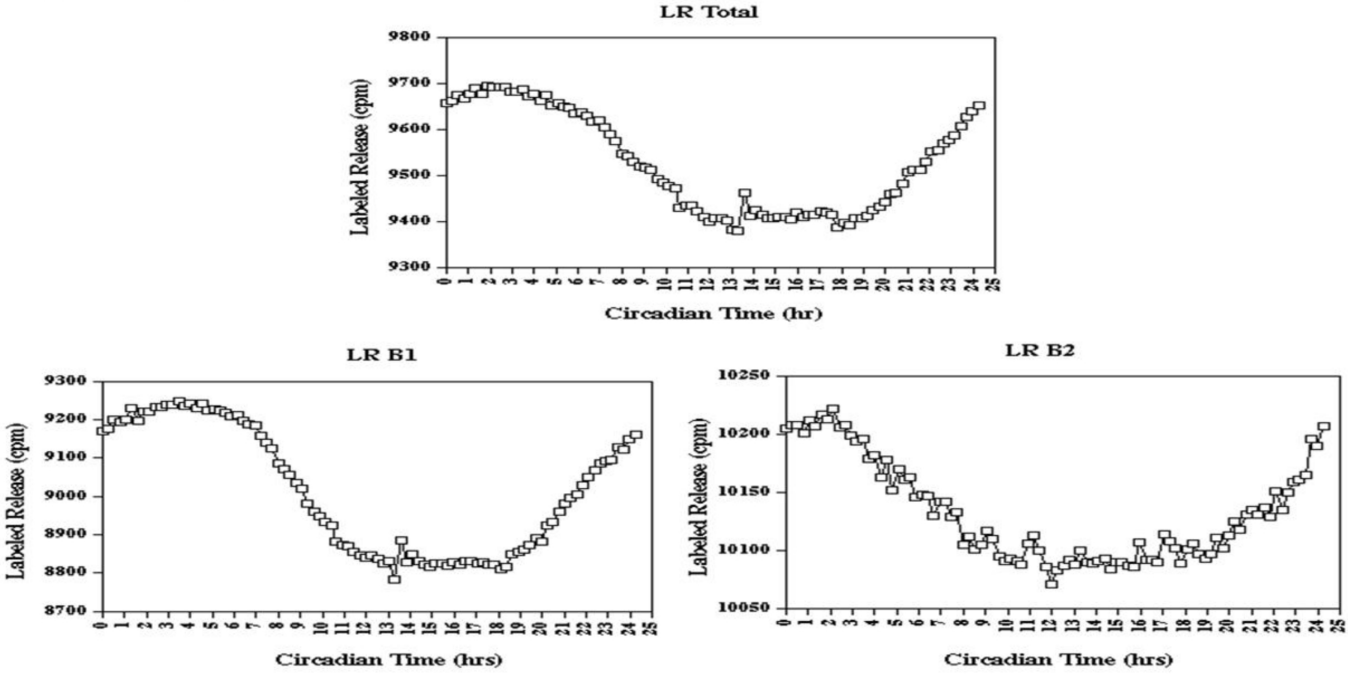


Figure 3 (upper, middle, lower) These graphs are mean plots of the circadian waveform plotted at a period of 24.66hrs for 60 sols from sol63 (LR TOTAL, upper graph) and for the first 30 sols (LR B1, middle graph) vs. the final 30 sols (LR B2, lower graph). Note the amplitude decrease in LR between B1 and B2.

A common method of plotting circadian data is the actogram. This graphical presentation plots a periodic variable across circadian time on the x-axis for some number of days on the y-axis. The data are typically plotted at the period of the variable (either 24 hr in the usual light/dark cycle or the intrinsic period under constant conditions, determined from F periodogram analysis). Figure 4 shows the LR data from VL2c3, subsequent to the second nutrient infusion, plotted in this fashion. The data are double-plotted on the x-axis to visualize any shift in phase, which may not be apparent in a single plot. The black bars represent the time periods during which the LR values exceed the daily mean of LR. The circles represent the daily acrophase, the timepoint at which LR reaches its maximal value. Best-fit regression lines are drawn through the acrophases. When free-running, non-entrained rhythms are plotted in this way, the acrophase occurs at the same time each day and the regression lines are perpendicular to the x-axis. The data between first nutrient infusion and second nutrient infusion (diamond symbol) are indicated at the top of the actogram.

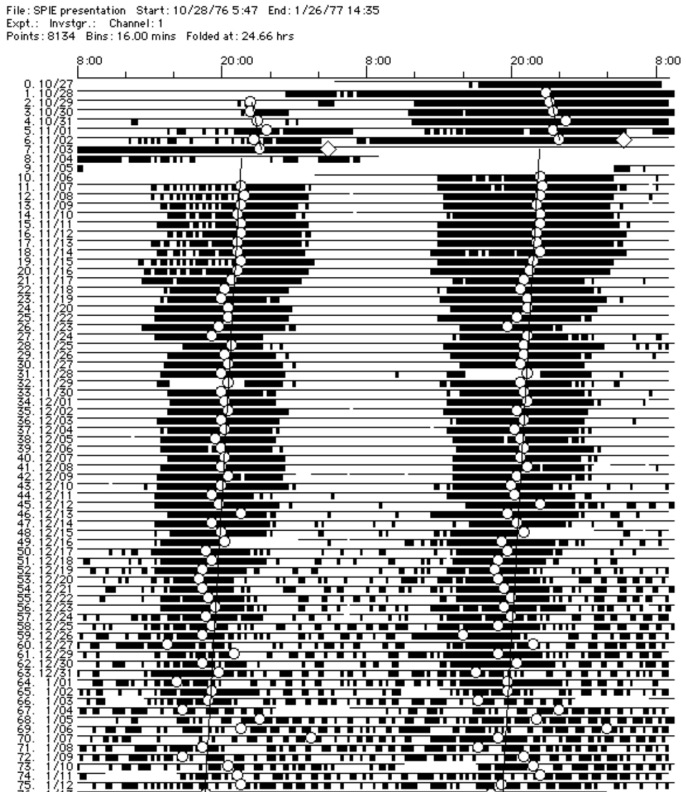


Figure 4 Actogram analysis of the VL2c3 data. The y-axis is sols of LR data and the x-axis is double-plotted at the best-fit period (one Martian sol or 24.66 hrs) in hrs (see axis across top of figure). Black bars represent those time intervals when the LR values exceeded the daily mean. The diamond symbol represents the time of nutrient injection. The circle symbols are the daily acrophases (times of daily maxima in LR). Regression lines are drawn through the acrophases following the nutrient injection and for available data before that injection. When actogram data is plotted at the intrinsic circadian period, acrophase regression lines are perpendicular to the x-axis. The small daily advance in the acrophase after the nutrient injection probably reflects the advancing time of sunset in Martian winter (see text). Note the large difference in acrophase slope before and after nutrient. This may indicate a phase-shifting effect of nutrient infusion, similar to food availability resetting of terrestrial circadian rhythms. It is also apparent that the rhythmicity breaks down late in the experiment, consistent with the reduction in LR amplitude noted in Figure 3, LR B2.

This plot has several interesting features. First, it is apparent that the LR data are highly periodic, particularly for the first five weeks of data following the second nutrient injection. Secondly, it is apparent that the rhythmicity begins to fragment in the last four weeks of data, consistent with the reduction in mean amplitude seen in the average waveform analysis (Figure 3 upper, middle, lower). Thirdly, the acrophase (maximum) and bathyphase (minimum) values (not shown) precess or phase advance every sol by about 2.5 min. This suggests either that the period of the LR oscillation is not precisely 24.66 hr (i.e., it is free-running at a slightly different period) or that the LR rhythm is entrained to some variable that is advancing every sol. Finally in the six day period between the first and second nutrient injections (top of actogram) the period (25.46 hr) is markedly different from the 24.66 hr period seen in the remainder of the data set. These acrophases are delaying every day instead of advancing, as in the data following second nutrient. The acrophase then advances about two hrs between the last pre-infusion value on November 3 to a new stable value on November 7 (estimated acrophases on November 4 and November 5 are not trustworthy because of a loss of signal throughout much of the time interval November 4 to November 6).

Similar analysis of the head end assembly (of the test cell) temperature (HT, as measured directly over the soil sample) indicates that it is also highly periodic with a periodicity of 24.66 hr, exhibiting acrophases and bathyphases that similarly advance by about 2.5 min every day. Temperature measured at the beta detector itself (DT) is significantly periodic, but much noisier (compare average waveforms in Figure 5 upper, lower and period estimates derived from F periodogram analysis in Figure 6 upper, lower). The daily means of LR were highly predictable from the daily HT means, with HT accounting for 88% of the variance in LR (e.g., Figure 7 upper-Lissajous ellipses occur when two periodic functions are plotted against each other). Note that the HT means phase-lead the LR means by as much as two hours (Figure 7 lower). Virtually every LR acrophase could be predicted from the HT acrophase, but LR acrophases could not be predicted from DT acrophases.

It should also be noted that LR does not reflect every high frequency deviation in HT, as may be seen from one cycle of data (Figure 8 upper) and from five cycles of data (Figure 8 lower). Clearly, there are uncorrelated sources of noise in both waveforms.

Mars ambient surface atmospheric temperatures are highly periodic (Figure 9). Daily fluctuations in ambient temperature are as large as 50º C, compared to the maximum fluctuation of 4º C observed in HT. The ambient temperatures[5] showed no evidence of the 2.5 min/sol phase advance that was observed in the LR and HT data. However, the averaging procedure employed to obtain these values may have obscured this small phase shift. Indeed an analysis of sunset times through the course of VL2c3[6] indicated that all but about 18-sec of the LR phase advance could be accounted for by the daily advance in sunset during the Martian winter. The residual 18-sec is well within the error variance of the linear regression through the daily acrophases (Figure 4).

Previous ground-based experiments[3] have strongly suggested that the gas released in the Mars LR experiment was CO_2. Simulations with CO_2 and sterile Mars analog soils were able to demonstrate a minor daily fluctuation when temperature was cycled from 7-16ºC. However, these simulation experiments were unable to replicate the magnitude of the daily LR oscillation correlated with the 2ºC mean daily temperature fluctuation observed in the flight data.

We reconsidered the hypothesis that temperature-regulated CO_2 solubility in Martian soil samples could explain the daily LR oscillations. The pH of the Mars soil-water mixture was calculated from the 30-35% drop in LR following the second injection using the carbon dioxide/water/bicarbonate ($CO_2/HCO_3^-/CO_3^{2-}$) equilibrium. This calculation yielded a pH of 8.06, the first experimentally determined value for moistened Martian soil (details will be presented elsewhere). Using this pH, equilibrium calculations indicated that at best the daily temperature fluctuation could account for 55-78% of the LR oscillation. Thus, the amplitude of the daily rhythm (maximum minus minimum) as well as the precipitous drop in signal after 2nd injection[3] could be substantially explainable in terms of physical chemistry. However, not as easily explained are: 1) the 2 hour phase delay relative to temperature oscillation 2) the failure of the LR signal to follow slavishly the HT waveform (Figure 8) 3) substantial decrease in the daily LR fluctuations in the 160º C "sterilization" 4) absence of the continual slow rise in LR signal (as inVL2c3) in the "sterilization" cycle. All of these four phenomena (as well as the large increase in LR after 1st injection and the continuing slow rise in VL2c3) can be accommodated by a biological explanation.

4. CONCLUSIONS

The existence of a circadian rhythm (or, more properly a circasolar rhythm) is a presumptive biosignature. All terrestrial lifeforms examined to date exhibit circadian rhythmicity, from primates to blue-green algae. In fact, circadian rhythmicity in blue-green algae[7] is superimposed on a growth curve analogous to the apparent growth function observed by Levin and Straat[1]. Levin and Straat concluded, on the basis of the "active" LR response following first nutrient injection, compared to the large attenuation in LR response to nutrient injection caused by pre-nutrient "sterilization" at 160º C, as well as the smaller attenuation in response associated with pre-nutrient soil sample heating to a lower temperature ("partial sterilization"), that the LR response following first nutrient injection was consistent with biological origin. Further study of these data and other relevant findings led Levin[8] to conclude that the LR experiment had detected living microorganisms in the soil of Mars. In contrast, the focus of this report is on the LR data following second nutrient injection. The logarithmic

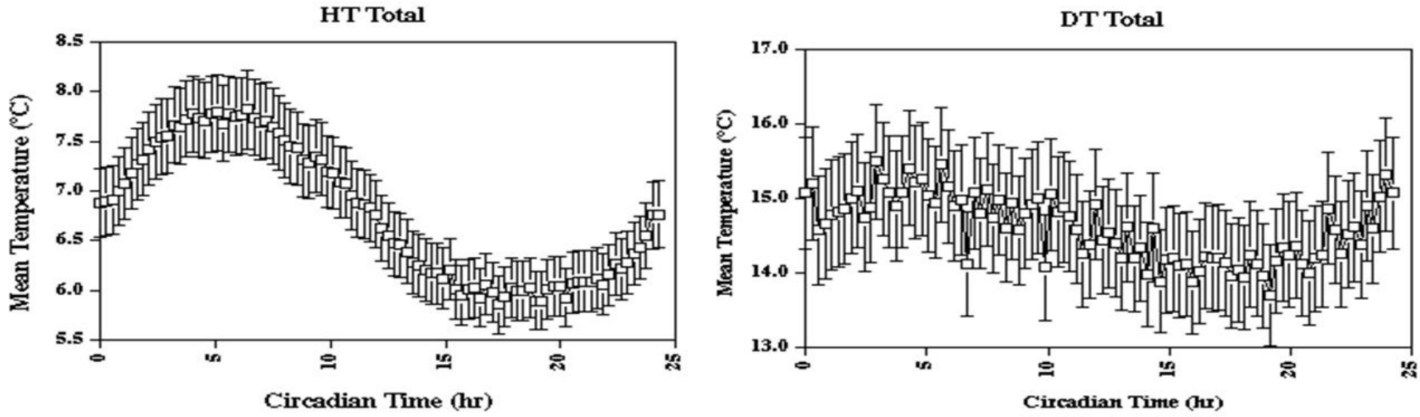


Figure 5 (upper, lower). The upper graph is the mean plot, averaged at 24.66 hr, of the total HT data; the lower graph is the corresponding plot for DT. Vertical bars are standard deviations. Note the small amplitude and large variance of the DT data relative to the HT data.

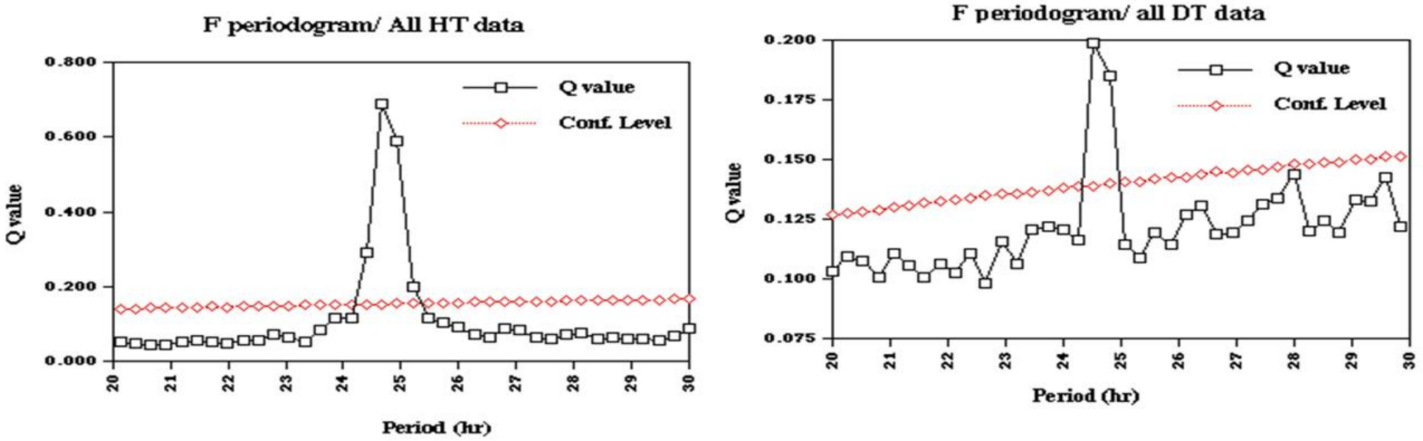
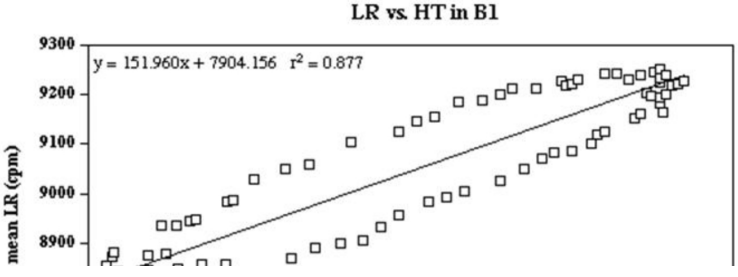


Figure 6 (upper, lower). These graphs are plots of the dominant periodicity in the HT measure (upper) and DT measure (lower). 95% confidence intervals are indicated in each plot. In spite of the great variance in DT, there is considerable power (Q value) at 24.66 hr.



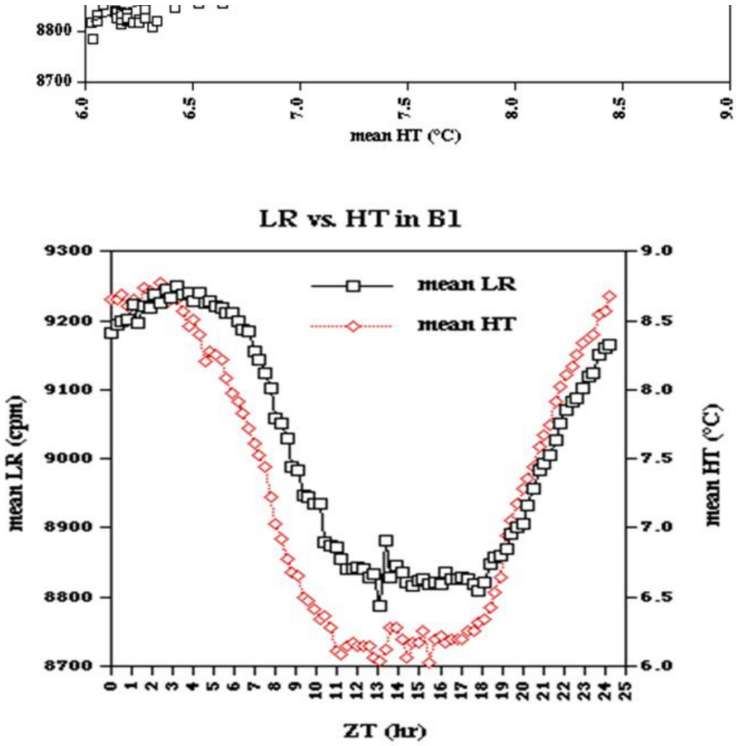


Figure 7 (upper, lower). The upper graph plots the periodic HT function vs. the periodic LR function, yielding a Lissajous plot. The width of the data ellipse is a measure of the phase difference in the variables. Almost 90% of the variance in LR may be accounted for by variation in HT. The lower plot graphs both variables against the Martian sol. Since both rhythms appeaentrained to external ambient temperature, which in turn reflects the Martian light/dark cycle, this axis is most appropriately labeled zeitgeber time (ZT) where ZT0 is approximately sunrise. Note that HT is advanced by about two hrs relative to LR at most ZT.

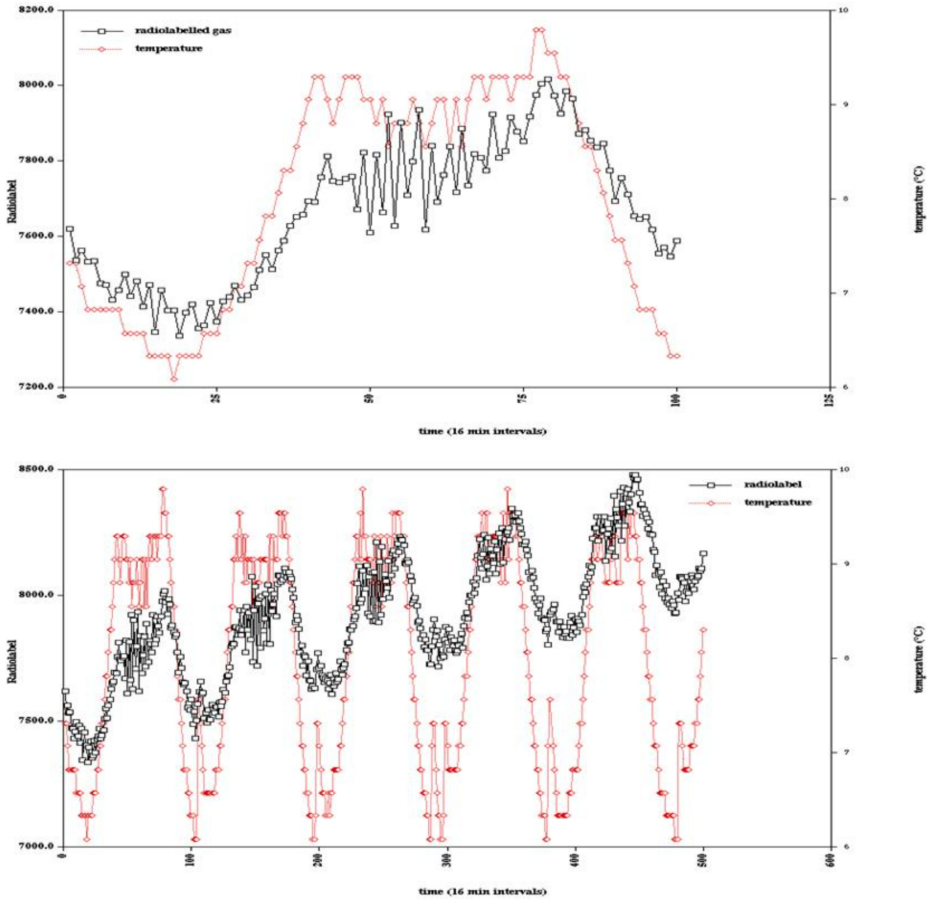
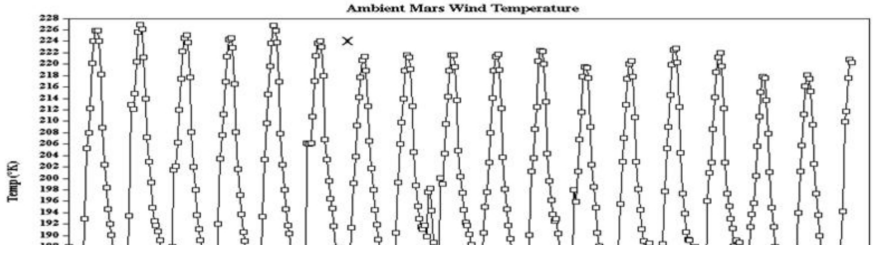


Figure 8 (upper, lower). The upper plot graphs one sol of LR data vs. the HT temperature. The lower plot graphs five sols of the same data. Note the uncorrelated high frequency variance in both LR and HT.



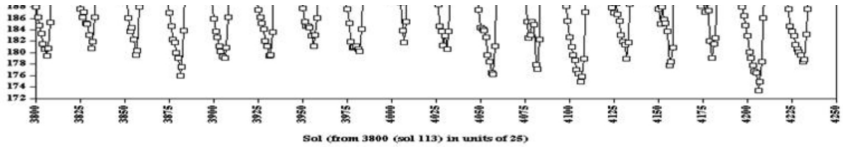


Figure 9 Plot of ambient Mars wind temperatures (°K). The Mars ambient temperatures are highly periodic, with high temperatures in day and low temperatures at night. The X symbol marks the mission time at which atmospheric opacity greatly increased (possible dust storm) and the amplitude of the Martian daily temperature cycle decreased correspondingly. This corresponded to the decrease in LR amplitude noted in the last weeks of the VL2cycle 3 data set.

function fit to these data is a mathematical construct; it does not imply any particular biological measure of growth, cell division or metabolism. One speculation is that the function represents metabolism during a period of slow growth or cell division to an asymptotic level of cellular confluence, perhaps similar to terrestrial biofilms in the steady state. Furthermore, the independent periodic component in the LR data may indicate a circasolar rhythm in a putative Martian microorganism, similar to the circadian rhythm previously observed in terrestrial cyanobacteria[7]. This rhythmicity has been observed in all the "active" LR cycles analyzed so far by the techniques of this paper. In the soil sample that was sterilized at 160º C, there was a residual (and significant) fluctuation (~50 cpm) approximately twice the radioactivity remaining after the purge from the preceding cycle. This fluctuation is marginally periodic but is attenuated by 91.5% compared to the active cycle. In the active cycle of VL2c3, a robust rhythmicity persists for as long as nine weeks. Such persistence is difficult to explain in terms of, for instance, a superoxide chemical reaction[9] since such superoxides are immediately destroyed in aqueous media such as the nutrient solution employed in the LR experiments.

The periodic oscillation in LR observed here is highly correlated with a periodic oscillation in HT. A temperature-regulated change in CO_2 solubility could at least partially account for the amplitude of the LR oscillation. However, the HT oscillation phase leads the LR oscillation by as much as two hours, an unusual circumstance if this were simply a chemical oscillation driven by thermal fluctuation. (Admittedly there is uncertainty concerning the delay between change in temperature at the head end assembly, perhaps one inch over the 0.5 cc soil sample, and soil sample temperature per se. However, a two-hour lag seems quite long for what is presumably a convective and radiative process. Similarly, thermal-induced movement of gas between the soil sample and the beta detector requires only about 20 minutes.) Furthermore, the LR oscillation does not slavishly follow the thermal variation; rather, it seems that the LR rhythm is extracted from the HT oscillation, while high frequency noise is not. This is very common in terrestrial organisms in which a low frequency periodic stimulus (i.e., a zeitgeber) such as a 12:12 light/dark cycle can entrain a circadian rhythm, while high frequency transients in the same stimulus are ignored (e.g., turning on the light in the bathroom at night for a minute or two does not alter normal entrainment to the light/dark cycle). Furthermore, there is abundant evidence that as little as a 2º C temperature cycle can entrain circadian rhythms in terrestrial organisms such as lizards, fruit flies, and bread molds[10-13] and entrainment can be preferential to the diminution phase of the temperature cycle[12], in analogy to the temperature fall that occurs at sunset on Mars).

We believe that the Martian light/dark cycle and its associated ambient temperature cycle drives the rhythm in HT observed here, probably because of less than total shielding of the LR experiment from ambient temperature fluctuations on the surface. Internal heaters in the head end assembly prevented the temperature from falling to anything like Mars ambient at night, but internal temperatures were not quite constant, probably because of thermostat hysteresis. Thus a periodic oscillation in HT (but not DT) could have synchronized or entrained the LR rhythm. This interpretation gains considerable support from the analysis showing that the daily phase advance in LR and HT is entirely accountable for in terms of the daily advance in sunset on Mars.

What would be the "smoking gun" from a circadian biology perspective for the existence of life on Mars? Probably the strongest evidence would come from the observation of a free-running circadian rhythm with a period significantly different from a Martian sol. Endogenous rhythms of this kind are present in all terrestrial organisms observed under constant conditions. However, the temperature oscillations in HT may have precluded such observations in the current data. Nonetheless, for about a week between the first and second nutrient infusion, there is some indication of a free-running rhythm with a period of 25.46 hr. The second nutrient infusion appears to phase shift and entrain this rhythm to the HT rhythm of 24.66 hr. Such an interpretation suggests that nutrient infusion itself is a potent zeitgeber and such zeitgebers are known to alter the period and phase of terrestrial rhythms. However, the amount of data between first and second nutrient injections is rather small. Continuing analysis of the Viking Lander data will target possible occurrence of non-sol periodicity and large phase shifts that might be expected resulting from the action of a potent phase-shifting stimulus on a biological rhythm. A NASA-sponsored forthcoming publication[14] of the complete Viking LR Mars data will support such inquiries by other interested scientists and us.

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