Circadian Rhythms and Evidence for Life on Mars.

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ABSTRACT

In a previous reanalysis of the Viking Labeled Release (LR) experiments of Levin and Straat[1] we demonstrated what appeared to be a temperature-entrained circadian rhythm in radiolabeled gas release following incubation of a Mars soil sample with a ^{14}C labeled nutrient solution. Here we have extended these analyses to all nine LR experiments performed on Viking Landers I and II. All nine experiments exhibited a temperature-entrained circadian rhythm. Furthermore, we show here the gradual development of the rhythm over the first week of the LR experiments and the nearly complete abolition of the rhythm by sterilization or storage of the soil sample in the dark for four months before nutrient administration. These phenomena are typical for terrestrial organisms. In addition, we present evidence that a major component of the labeled gas was probably carbon dioxide, rather than methane or carbon monoxide.

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

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. 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[2,3], 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 28 years.

In previous work[1] we demonstrated the existence of a robust periodic oscillation in LR (headspace gas) in the third LR experiment performed on board Viking Lander II (VL2c3). The oscillation had a period of 24.66 hr, the period of a Martian day or sol, under essentially steady state conditions. Furthermore, the oscillation was entrained to an approximate 2°C temperature cycle on board the lander which represented the hysteresis of the heaters in compensating for a nearly 50°C daily cycle in Mars ambient temperature. Thus the heaters were able to maintain the interior of the lander at 10-12°C, depending on time of day. Late in this experiment an apparent dust storm, which attenuated the 2ºC temperature cycle by about 50% (probably due to thermal buffering of ambient temperature by the dust particles) nearly eliminated the circadian oscillation in LR.

In this report we extend our analyses to the remaining eight LR experiments and show both the development of the rhythm in non-steady state conditions, and its near-abolition by sterilization of the test cell or storage of the soil sample in the dark for four months before nutrient administration. Furthermore, we present evidence that the LR gas was probably not composed primarily of methane or carbon monoxide, but more likely was primarily composed of carbon dioxide, in agreement with past laboratory experiments of Levin and Straat[6].

2. METHODS

The LR experiment[2,3] 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 10ºC. Evolution of radiolabeled gas (i.e., Labeled Release or LR) was monitored by a beta

detector in a chamber connected to the test cell by a 13" stainless steel tube (i.d.=.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 various temperatures or storing for long periods, to distinguish whether the responses were biological or chemical.

We have applied a variety of analytical techniques to these data common to the circadian field (cosinor analysis, periodogram analysis, and harmonic regression for period determination, actogram-based linear regression of acrophase time to estimate phase over sols, waveform averaging to determine rhythm amplitude). We have also used phase angle analysis and cycle by cycle and mean double plots to examine the relationship between internal lander temperature (Head end temperature or HT) and LR.

In addition we have examined methane solubility in brines at Martian temperatures and atmospheric pressures to determine if this gas could have been a substantial component of the LR gas. Assuming that the 30% reduction in LR seen in the sterilization control (see below) indicated a non-biological absorption of some component of the LR gas by the soil/nutrient slurry, we calculated the solubility of that component. Next we calculated methane solubility under Mars ambient conditions. In an equilibrium between a liquid and vapor phase, the solubility of methane in the liquid phase can be calculated from its concentration in the vapor phase using Henry's Law:

$$k^\circ = (Ø*P)/(\gamma*x)$$

where k° is the Henry's Law Constant for methane at a certain temperature, P is the partial pressure of methane in the vapor phase, and x is the mole fraction of methane in the liquid phase. The variable Ø is the fugacity coefficient of methane, so that Ø*P gives the fugacity of methane, or its tendency to escape from one phase to another. Under the conditions relevant here, Ø was very close to unity so the term was dropped from the calculation. The mole fraction, x, is modified by the activity coefficient γ of methane in the liquid phase. Using methane solubility data[4], given in the form of the modified Henry's Law Constant, $\gamma*k^\circ$, the solubility of methane in NaCl brines of varying molality (NaCl 1-4, distilled water and Salton Sea for comparison) at equilibrium with a Martian atmosphere (8 mb) was determined for a range of temperatures. (The brine densities used for these calculations were based on interpolated density data[5]). Similar calculations for CO and CO_2 solubilities under Martian conditions were also performed.

3. RESULTS

Figure 1 shows representative actograms (panels A, C) for two of the five active Viking LR experiments. In Figure 1A the acrophases became more dispersed late in the experiment during an apparent Martian dust storm. Note that dispersion of the temperature acrophases (panel B) was much less. It is also apparent that small changes in period (i.e., slope of the acrophase) occurred at the nutrient injections, as is especially evident in Figure1C. These changes in slope are not evident in the temperature data (panels B, D).

Figure 2 shows that the test cell chamber or head stage temperature (HT) covaried with the LR signal on a cycle to cycle basis (panels A, C) as well as in the overall mean daily waveforms for these experiments (panels B, D). These data also show that the phase angle between LR and HT varied, with LR leading HT at some circadian times. This is not consistent with a thermally driven physico-chemical process.

Figure 3A shows the development of LR entrainment to HT over the first sols following first nutrient injection in VL2c3. Figure 3B plots the mean and standard error of the average LR rhythm amplitude across all nine experiments in which each "sol" following nutrient injection represents the mean of consecutive quintiles of the data points in each experiment in the non-steady state "growth" phase of the LR rhythm. It is apparent that rhythm amplitude increases monotonically over time. Figure 3C likewise shows that the standard deviation of the HT/LR phase angle, averaged across all experiments as in 3B, is very large in the "growth" phase (approximately the first week following nutrient injection), but then diminishes to a stable, although substantial level for the remainder of the experiments. This suggests that entrainment of the LR rhythm by the HT cycle takes about a week to develop, and that HT is not a perfect predictor of variation in LR.

Figure 4A shows the data from the full sterilization control in which the soil sample was raised to a temperature of 160°C for three hours (full sterilization) before nutrient was added. Small LR fluctuations were still seen, about 10% of the amplitude seen in the active LR experiments. A second nutrient injection at the end of sol 5 caused a 30% drop in the residual LR signal. Figure 4B shows the mean and standard errors of the LR rhythm amplitude for the active experiments, the intermediate heat controls (test cell temperature raised to either 46°C or 51°C), the full sterilization control and a sample kept in the dark for four months at 7-10°C and Mars ambient atmospheric pressure before nutrient was added. It is apparent that intermediate heating was not as effective as full sterilization, while storage in the dark for four months before nutrient was added had an effect similar to full sterilization.

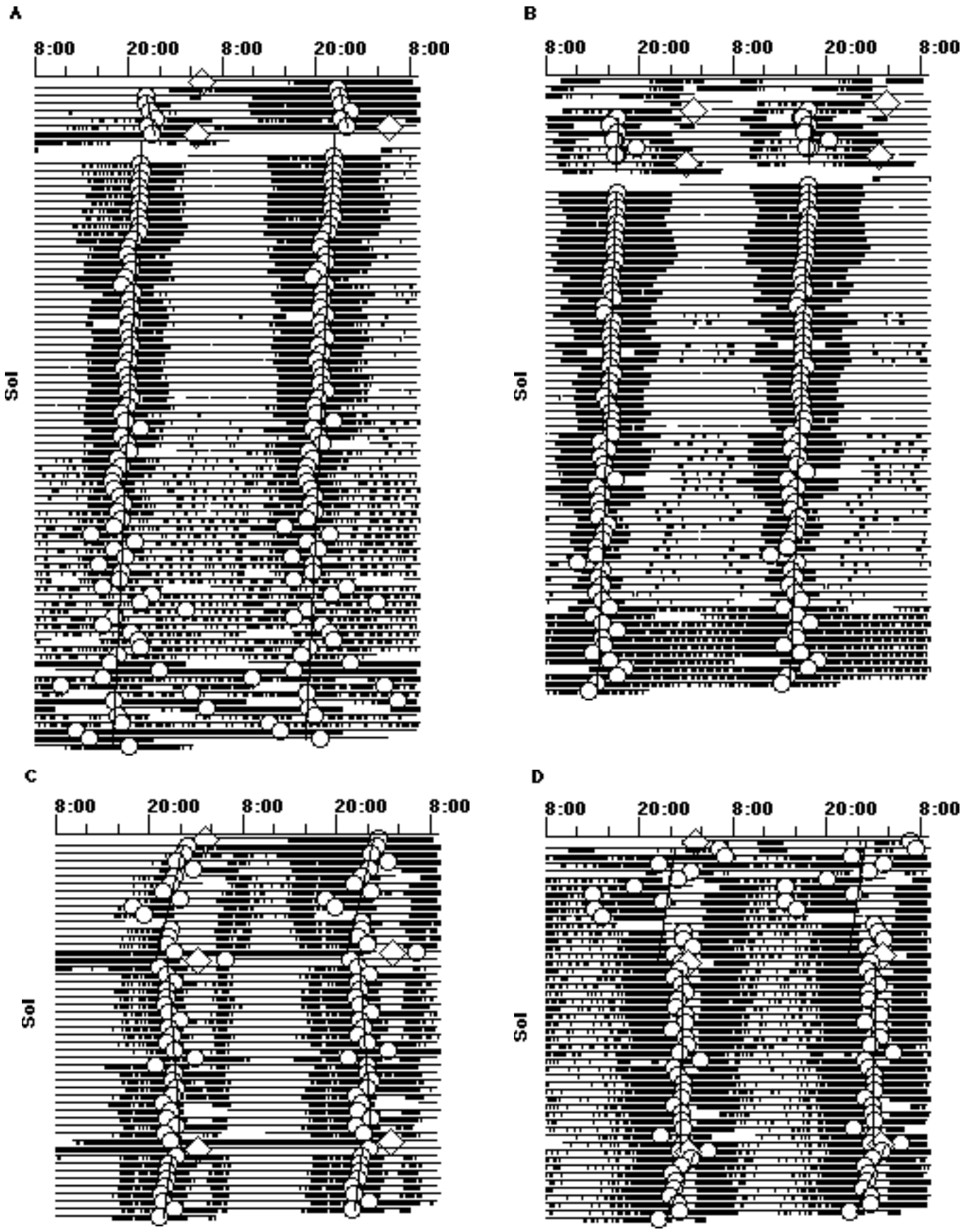

Figure 1 (A, B, C, D). Double-plotted actograms for LR (A, C) and HT (B, D) in VL2c3 (top panels) and VL1c3 (bottom panels). Y axis plotted in sols, X axis in normalized zeitgeber hours (24.66hr/24 hr or 1.03 hr units). Circles indicate acrophases (daily maxima) and rectangles indicate nutrient injection. Linear regression lines are drawn through the acrophases. Note the changes in slope (i.e period) following nutrient injection.

A 30% drop in LR was seen in all the experiments when nutrient was administered the second time. However, in the sterilization experiment (160°C for three hr before first nutrient injection) the 30% drop in signal after second nutrient administration (Figure 4A) is not likely explainable by a biological mechanism. Extrapolating from previous work[6], this could represent reabsorption by the soil/nutrient mixture of about 8.5 nanomoles of CO_2 per 0.5 cc slurry or 0.1 nanomole of some other carbon-containing gas per 0.5 cc mixture. However, Figure 5 shows that methane solubility under Mars ambient conditions is approximately seven orders of magnitude too low to allow the degree of reabsorption observed in the sterilization experiment, let alone in the active experiments.

CO is 60 times as soluble as methane in aqueous media, and the partial pressure of CO in the Martian atmosphere is five orders of magnitude greater than methane (compared to three orders of magnitude less in the terrestrial atmosphere). By Henry's law these data indicate CO could only constitute about 2 femtomoles of the resorbed gas. Indeed, Earth-based simulation experiments failed to show reabsorption of CO in aqueous medium under similar conditions[6].

In contrast, CO_2 is 24 times more soluble than methane in terrestrial conditions and its Martian partial pressure is some eight orders of magnitude greater than that of methane (compared to about two orders of magnitude greater in the terrestrial atmosphere). These data yield a CO_2 solubility in the test cell at 69 torr of about 3 umol of CO_2 per cc H_2O. Assuming partitioning into an aqueous fraction of about 0.1 ml in the test cell mixture, this would equate to about 300 nanomoles of absorbed CO_2, within a factor of two of the predicted 170 nanomoles in the active experiments. The molarity of this fraction should have been relatively high compared to pure H_2O (see Figure 5) which would explain the difference between these predictions. Since CO2 solubility is temperature-sensitive we were able to calculate that approximately 3% of basal LR gas should be reabsorbed each night and released from the soil the following day. This corresponds to about half the amplitude of the circadian rhythm in LR.

4. CONCLUSIONS

Sec. 4.1The biological interpretation

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[2] (e.g., Figure 2A). 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 ("intermediate heating"), 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.

Our previous work[1] focused on the LR data of VL2c3 following second nutrient injection, in which a slow increase in LR continued to be present, on which circadian oscillations were superimposed. We speculated that this increase represented 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 periodic component in the LR data may indicate a circasolar rhythm in a putative Martian microorganism, similar to the circadian rhythm observed in terrestrial cyanobacteria[7]. An important caveat is that a substantial fraction (~50%) of the LR rhythm amplitude probably reflected a contribution from daily absorption and release of CO_2 (see Sec. 4.2).

We report here that this rhythmicity is present in all the LR cycles of both Viking landers. In the soil sample that was sterilized at 160º C, there was a residual (and significant) fluctuation (~50 cpm) which was approximately twice the level of radioactivity remaining after the purge from the preceding cycle. This fluctuation was marginally periodic but attenuated by more than 90% compared to the active cycle. Intemediate heating resulted in a smaller attenuation of the circadian amplitude, while sample storage in the dark before nutrient injection attenuated the subsequent circadian rhythm in LR to almost the same degree as full sterilization.

The periodic oscillations in LR observed were highly correlated with the periodic oscillations 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 in VL2c3 (Figure 2D), an unusual circumstance if this were simply a chemical oscillation driven by thermal fluctuation. On the other hand, in some instances the LR oscillation actually leads the HT oscillation (Figure 2B). The phase angle between LR and HT is variable, particularly in the first week when the LR rhythm is beginning to appear and beginning to entrain to the HT temperature (Figure 3).. 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[9-12] and entrainment can be preferential to the diminution phase of the temperature cycle[11], in analogy to the temperature fall that occurs at sunset on Mars.

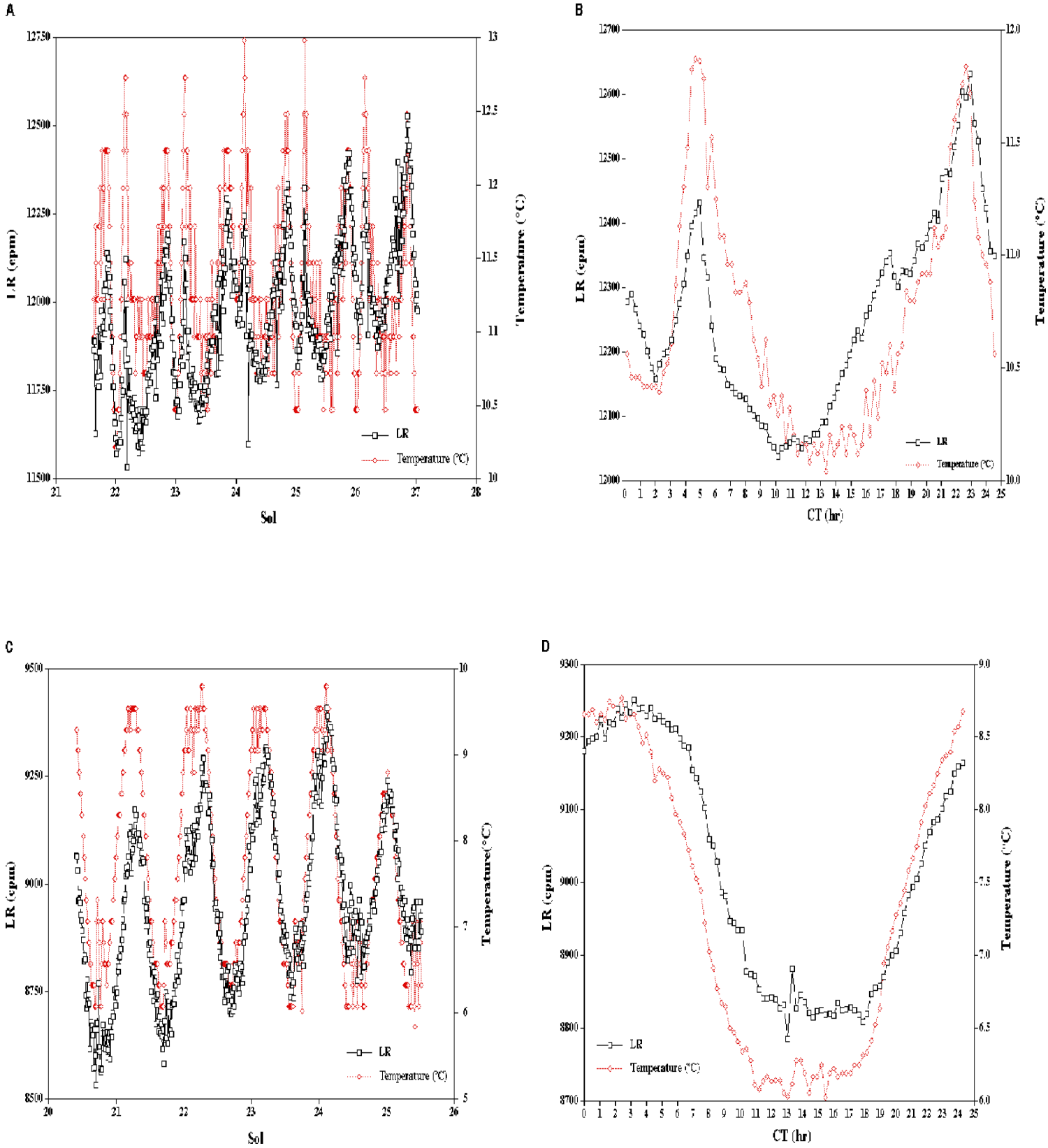

Figure 2 (A, B, C, D). Double plots of LR and HT in VL1c3 (top panels), and VL2c3 (bottom panels). Left panels show 5 cycles, right panels are 1 cycle means across the entire experiment duration. X axis plotted in sols or normalized zeitgeber hours.Y axis plotted in counts per minute (cpm) of radioactivity.

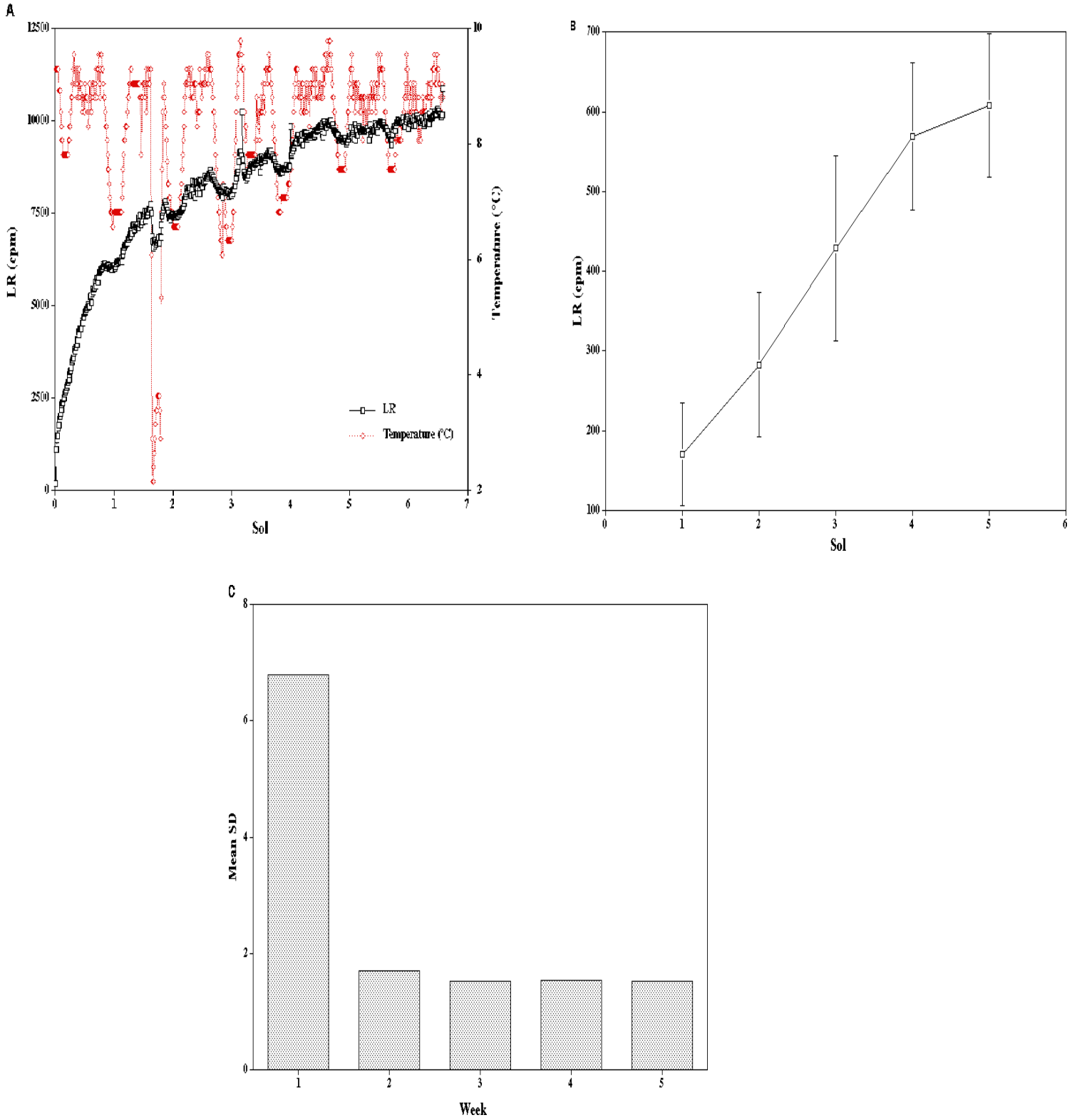

Figure 3 (A, B, C). Top left panel (A) is the first six sols of double-plotted LR and HTdata for VL2c3 following first nutrient injection. Top right panel (B) is the mean and standard deviation for the non-steady state data following first nutrient injection (sols 1-5) averaged across all nine VL experiments. Bottom panel (C) plots the mean standard deviation of the phase angle between HT and LR for every week following first nutrient injection averaged across all nine VL experiments.

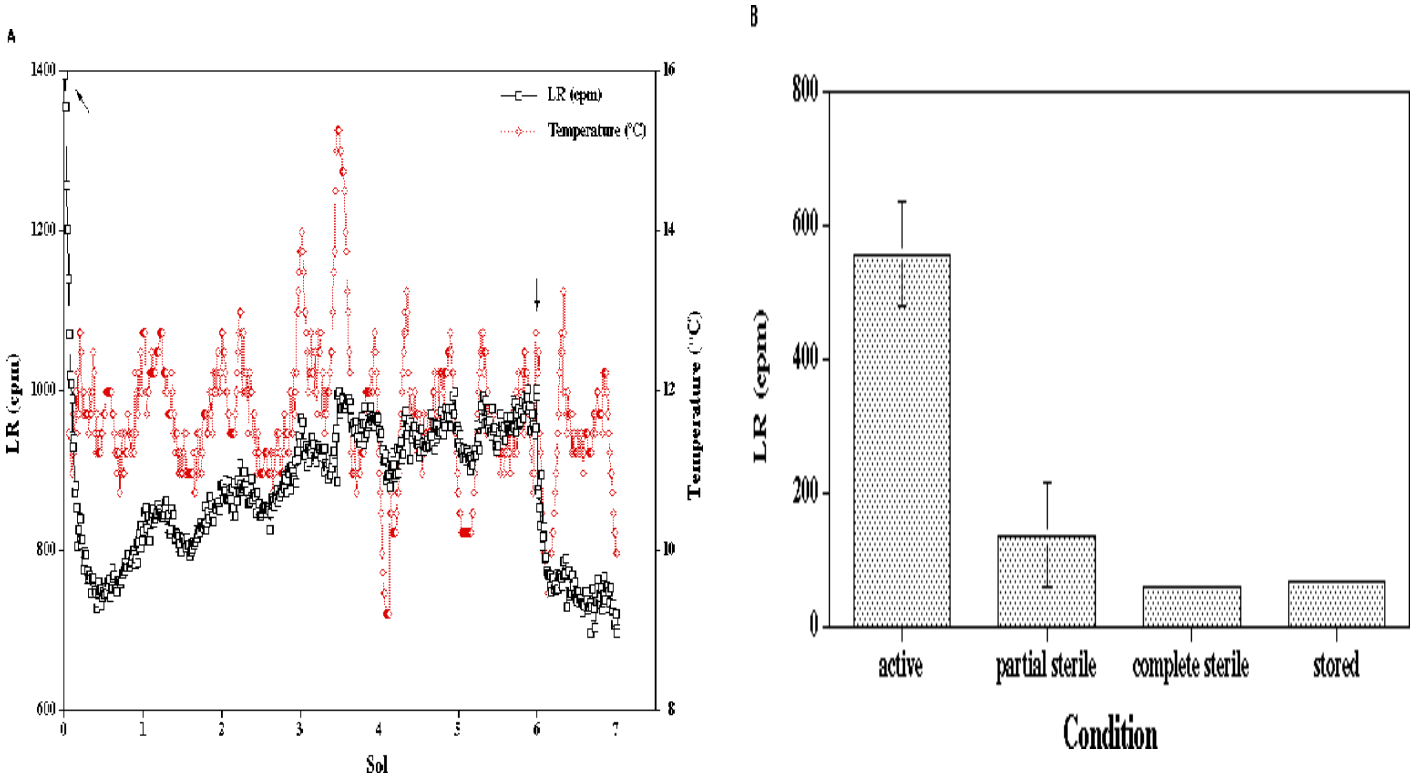

Figure 4 (A, B). Panel A shows double-plotted LR and HT data from VL1c2, the 160° C sterilization control. Note the precipitous drop in LR late in sol 5 following second nutrient administration. Panel B shows the average LR rhythm amplitude (mean and standard error over experiments) as a function of condition (four active experiments, two intermediate temperature controls at ~ 50°C, one full sterilization control at 160°C, or one storage in the dark for 4 months before nutrient injection control).

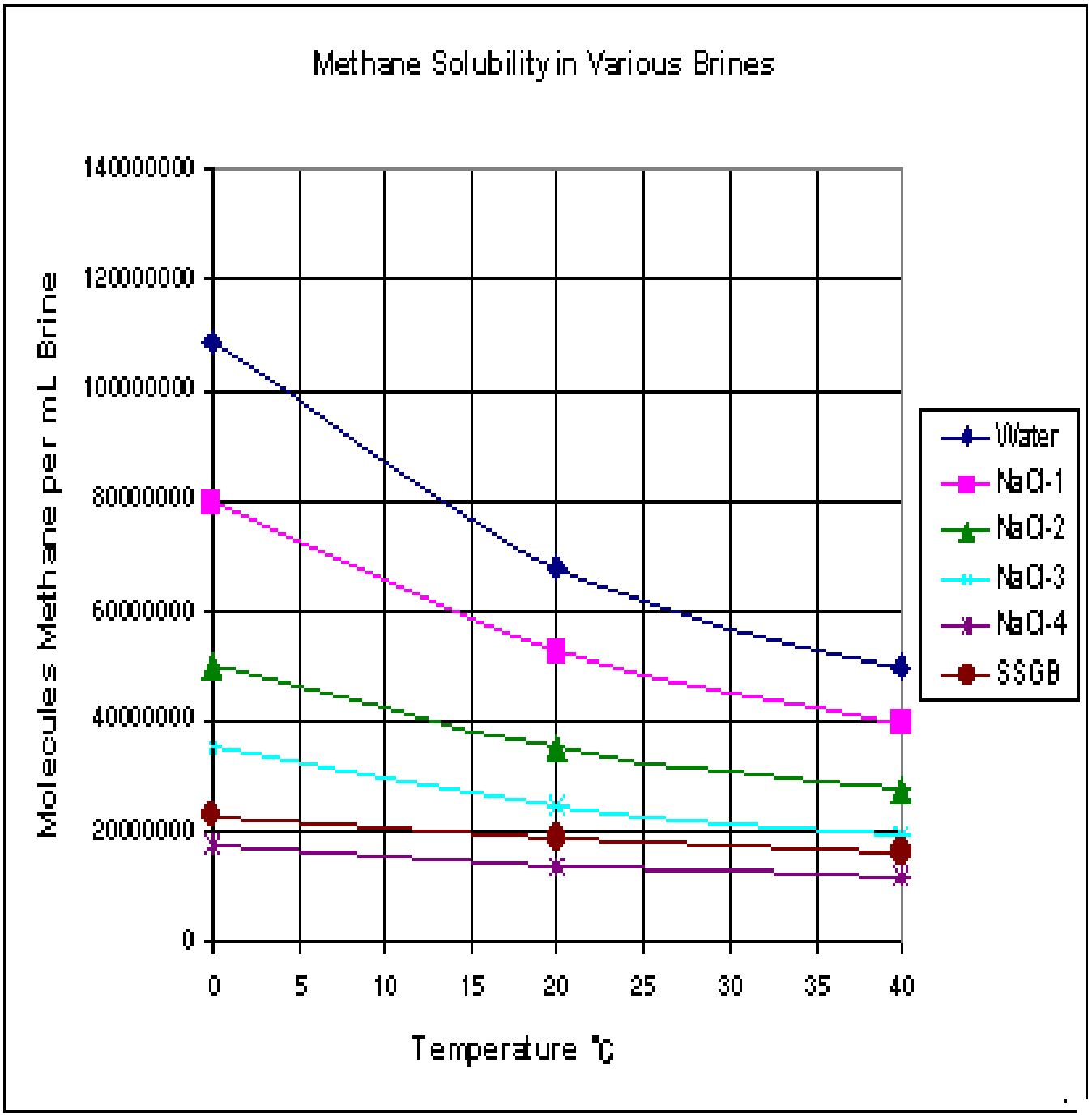

Figure 5 Methane solubility in various brines at Mars atmospheric pressure. SSGB=Salton Sea Geothermal Brine

Brine:	Molality	Molarity	Ionic Strength
NaCl-1	0.81	0.79	0.81
NaCl-2	1.95	1.86	1.95
NaCl-3	3.18	2.97	3.18
NaCl-4	4.70	4.26	4.60
SSGB	4.05	3.55	6.18

We conclude that the Martian light/dark cycle and its associated ambient temperature cycle drives the oscillation 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, varying from a typical day average of 12° C to a night average of 10° C. Thus a periodic oscillation in HT probably synchronized or entrained the LR rhythm with a degree of stochastic variability characteristic of a somewhat weakly entrained biological rhythm.

In addition, the small changes in period associated with nutrient injection (Figure 1) are reminiscent of nutrient-entrained circadian rhythms in terrestrial mammals. In conjunction with the observations that the LR rhythm develops over time, entrains to the temperature cycle over time, does not follow apparent high frequency alterations in temperature, has a varying phase angle with temperature, particularly prior to entrainment (Figure 3C), and is attenuated by stimuli that either reduce the strength of the entraining stimulus (e.g., dust storm in Figure 1A) or would be expected to reduce the number of viable microbes (sterilization, "starvation" in the dark). These data, taken together, strongly support a biological interpretation of the LR Viking data, although it remains difficult to prove that they could not have reflected a thermally driven physico-chemical phenomenon.

Sec. 4.2 Nature of the LR gas

One observation that is difficult to explain from a biological perspective is the approximately 35% drop in LR signal that occurs in all experiments following second administration of the nutrient. This is even seen in the full sterilization control (Figure 4A). The biological expectation would have been another fast rise in LR, similar to what is seen after first nutrient administration. Since the test cell/scintillation counter was a closed system, the strong implication is that the aqueous soil/nutrient solution must have resorbed the LR gas.

The Mars Express Planetary Fourier Spectrometer group and two other independent groups[13,14] have recently made observations of methane in the Martian atmosphere. The apparent absence of volcanic activity suggests that the methane is biogenic in origin. We investigated the possibility that the LR gas could have been methane. We conclude on the basis of past analyses[6] and the results depicted in Figure 5 that the reabsorbed gas was not methane. CO could also be excluded on the basis of solubility considerations. In contrast the solubility and partial pressure of CO_2 in the Viking test cell make it the most likely candidate for the reabsorbed gas, as has been previously suggested[6]. Similarly, about half of the circadian rhythm in LR can be accounted for by temperature cycle-driven daily changes in CO_2 solubility[1]. However, these constraints do not apply to the gas fraction which is not reabsorbed or the remaining 50% of the LR rhythm. We cannot exclude the possibility that some fraction of this non-reabsorbed gas was indeed biologically-produced methane. In fact, it can be shown that biogenic production of less than one picomole of methane per cc of Martian soil per sol, planet-wide, could completely replenish atmospheric methane.

Sec. 4.3 The smoking gun

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 these data. Statistical analyses are currently underway to clarify this point.

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