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Characterization of *Neurospora* Circadian Rhythms in Space

Final Technical Report

Principle Investigator: Dr. James S. Ferraro
Department of Physiology
Southern Illinois University
School of Medicine
Carbondale, IL 62901-6512

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Technical Officer: Dr. Charles Winget
NASA - Ames Research Center
Mail Stop 240A-3
Moffett Field, CA 94035

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Abstract

To determine whether the circadian rhythm of conidiation in *Neurospora crassa* is endogenously derived or is driven by some geophysical time cue, an experiment was conducted on space shuttle flight STS-9, where inoculated race tubes were exposed to the microgravity environment of space. The results demonstrated that the rhythm can persist in space. However, there were several minor alterations noted; an increase in the period of the oscillation and the variability of the growth rate and a diminished rhythm amplitude, which eventually damped out in 25% of the flight tubes. On day seven of the flight, the tubes were exposed to light while their growth fronts were marked. It appears that some aspect of this marking process reinstated a robust rhythm in all the tubes which continued throughout the remainder of the flight.

We have hypothesized that the damping found prior to the marking procedure on STS-9 may have been a result of the hypergravity pulse of launch and not due to the microgravity of the orbital lab; furthermore, that the marking procedure, by exposing the samples to light, had reinstated rhythmicity. To test this hypothesis, we conducted an investigations into the effects of acute and chronic exposure to hypergravity. The acute studies consisted of a 10 minute 3G exposure (similar to launch). A chronic (7 day) 3G exposure was used in order to determine if there were any long term effects of hypergravity. Both BND and CSP strains of *Neurospora* were used. The onset of both the acute and chronic exposures to the 3G force was evenly distributed through-out the circadian cycle; i.e. packages filled with inoculated race tubes were placed on the centrifuge at circadian time (CT) 3, 9, 15, and 21. Two groups of the acute exposure group were exposed to light shortly after being removed from the centrifuge to determine whether the effects of the 3G force could be reversed through the use of a light pulse as had been suggested.

While chronic exposure of *Neurospora* to a 3G force had no damping effect, an acute exposure to hypergravity causes significant damping of the circadian rhythm of conidiation. Furthermore, a brief light pulse given 36 hours after the acute exposure eliminates any effect of the acute 3G exposure on damping. BND was more susceptible to the hypergravity perturbation than CSP. The average free-running period increases with chronic hypergravity similar to what was seen during spaceflight. Moreover, the period of each individual cycle continues to increase throughout the exposure period. It is not known whether this increase in cycle length possesses a threshold, since the period continued to increase for the entire length of the experiment. These results suggest that most, if not all, of the aberrant effects observed on STS-9 spaceflight can be explained by the hypergravity encountered during launch and not due to the lack of geophysical zeitgebers in space.

Description of Research

The ultimate long range goal of our research is to determine whether circadian rhythms, thought to be endogenously derived, can persist normally in the abscence of all known geophysical and environmental cues (See Photograph 1). A rhythm is thought to be circadian if it can persist in constant conditions, free-run with an endogenously derived period of about 24 hours in these constant conditions and entrain to 24 hour environmental time cues. It has been proposed by some, that the free-running rhythms we observe, in conditions which we consider constant (i.e constant light, temperature and humidity), are not free-running rhythms in constant conditions at all; rather rhythmic phenomenon in known and unknown geophysical cycles induce rhythmicity in the organisms. It is their hypothesis that there are no circadian rhythms driven by an "internal clock", only passive oscillations responding to rhythmic environmental influences. By observing the rhythmicity of an organism in an environment removed from the geophysical rhythms of Earth, we can determine whether these rhythms are endogenous by their ability to persist without these purported exogenous time cues.

The filamentous fungus, *Neurospora crassa,* displays rhythmic growth patterns in certain specific conditions. This rhythmic growth consists of an alternating low growing surface mycelium and a surface mycelium with aerial hyphae which pinch off to form conidia (asexual spore formations). The mycelium that contains the aerial hyphae is clearly seen as a band when grown on media in petri dishes or long cylindrical tubes, known as race tubes. In constant conditons the cycle of banding is repeated approximately once every 22 hours (i.e. the rhythm is in the circadian range).

To determine if this circadian rhythm of conidiation is endogenously derived or is driven by some geophysical time cue, an experiment was conducted on STS-9 where race tubes innoculated with growing *Neurospora* were exposed to the microgravity environment of space. The results demonstrated that the rhythm can persist in space (Sulzman, 1984; Sulzman *et al.*, 1984). However, there were several minor alterations noted. There was an increase in the period of the oscillation and the variability of the growth rate. Furthermore, the rhythm of the conidiation possessed a diminished amplitude and eventually damped out in 25% of the flight tubes. However, on day seven of the flight, the tubes were exposed to light while their growth fronts were marked. It appears that some aspect of this marking process reinstated a robust rhythm in all the tubes which continued throughout the remainder of the flight. These results lead us to question why the rhythm, before the marking procedure, had been so variable and even damped out in some cases.

Much of the past year has been devoted to the materials, hardware and procedures preparation for our mid-deck experiment BGE NS402 which was scheduled to fly on EOM1/2 in late summer/early fall 1986. Due to the tragic accident of January 28^{th} it became apparent that we could expect substantial delays in our flight. Therefore, we shifted our major work emphasis from flight preparation to expanding our ongoing investigation of components of the question that can be examined utilizing ground based research. These areas of research have included experiments on the effects of: 1) a clinostat {rotating the tubes in a 1G vector in order to evenly distribute the force and obtain a simulation of zero G}, 2) different orientations of the 1G gravity vector, 3) chronic and acute exposure to hypergravity (3G), 4) genetic strain, and 5) media composition on the rhythmic conidiation and growth of *Neurospora*. *Neurospora crassa* band strain (#1859) and band CSP strain (#4548) (obtained from the Fungal Genetics Stock Center, Humbolt State College, Arcata, California) were used. Stock cultures are grown on slant tubes containing Vogel's salts, 1.2% sodium acetate and 0.5% Difco casamino acids. Experimental cultures are grown by inoculating one end of a "race" tube containing medium. Cultures are grown in constant bright light for 24 to 48 hours then the growth front is marked on the glass tube and the tubes are placed in constant darkness. In the laboratory, the growth fronts are marked at regular intervals during the course of an experiment. Marking is done under a red "safe" light which has been shown not to affect the free-running conidiation rhythm. At the end of the experiment the growth front is again marked. Since growth is linear at constant ambient temperatures, the time of occurrence of each conidiation band can be determined from the growth front marks. These marks are recorded by a digitizer and analyzed by an Apple II+ microcomputer.

Since these ground based results are so interesting and promising, we intend to continue, and in fact, step up our efforts in this area. The reasoning behind why we conduct ground based research becomes very evident from the results of these experiments in particular. It is very possible that these results suggest that most, if not all, of the aberrant effects observed on SL-1 can be explained by the hypergravity encountered during launch. These studies may then suggest possible procedural changes, including changes in how we ask the questions. These changes can then be integrated into our flight experiment and not only alter how we interpret the results of flight experiments, but affect our experimental design before the experiment is flown. This attitude toward space research of planning ahead through ground based scientific investigation allows for a greater return on the flight dollar and milks every available flight opportunity in order to reap the greatest scientific return. Obviously, we must await the results of our BGE NS402 experiment, now scheduled to fly on the Atlas mission, to confirm the suspicions raised by these experiments, but we have a heightened anticipation for the amount and quality of scientific information that will be brought back due to these ground based investigations. Thus, we may be one step closer to strongly supporting the theory of endogenous rhythmicity in living organism.

Accomplishments

1. Design Changes of NS402
   - increased sample size from 24 to 45-50 (without an increase in the size of the total package and without a substantial increase in overall package weight)
   - subdividing the package into three sections
   - the addition of a second strain of *Neurospora* (CSP)
   - the addition of gas sampling syringes
   - an earlier marking of the tubes in flight.

2. Combined EVT and biocompatability test was conducted at Ames, February '86

3. Meetings
   - Principal Investigators' meeting, June '86,
   - FASEB Summer Conference on Gravitational Biology, July '86
   - Annual ASGSB Meeting, October '86.
   - 14th Annual Fungal Meeting, April '87
   - NASA Space Life Science Symposium, June '87
   - Gordon Conference on Biological Rhythms, July '87
   - NASA Lighting Conference, July '87

4. Clinostat Studies
   - Definition: a clinostat rotates the race tubes about their axis, thus distributing the gravity vector equally in all directions, therefore simulating a 0 gravity vector (see Figure 1)
   - the rhythm damping and the decreased amplitude seen in orbit on SL-1 was not simulated by the clinostat at any of the circadian times
   - the growth rate was significantly slower on the clinostat ($P<0.01$)
   - a clinostat providing a rotating 1G vector about the *Neurospora* race tube axis appears not to be capable of simulating growth patterns, rates or circadian periods seen during spaceflight

5. Orientation Studies

- Definition: different orientations of the normal 1G gravity vector involved 5 sets of tubes:
  (1) tubes horizontal and upright (1G control)
  (2) tubes horizontal and upsidedown
  (3) tubes vertical
  (4) tubes at a 45 degree angle with the media upsidedown
  (5) tubes at a 45 degree angle with the media upright (Figure 2)
- *Neurospora* in group 2 displayed a decreased amplitude and rhythm damping (one tube appeared to become arrhythmic)
  furthermore, the period of the rhythm in this group was significantly longer than the upright controls ($P < 0.05$).
- *Neurospora* in group 4, grown upsidedown at a 45 degree angle, showed similar results to the tubes in group 2; however, the response was somewhat more attenuated with regard to the damping and decreased amplitude
- *Neurospora* in groups 3 and 5 displayed no damping or changes in amplitude
- *Neurospora* growing vertically showed a decreased growth rate ($P<0.001$)

6. Hypergravity Studies (See Figure 3)

- Definition: preliminary investigations into the effects of chronic and acute exposure to hypergravity; a 3G load was chosen as an adequate simulator of the gravitational forces encountered during liftoff
  - chronic (7 day) 3G exposure was used to determine long term effects of hypergravity
  - acute studies consisted of a 10 minute 3G exposure (roughly similar to the launch time frame)
  - CSP and the BND strains of *Neurospora crassa* were used
  - the onset of both the chronic and acute exposures to the 3G force were evenly distributed through-out the circadian cycle
    i.e. packages filled with inoculated race tubes were placed on the centrifuge at CT 3, 9, 15, & 21
  - two groups of the acute exposure group were exposed to light shortly after being removed from the centrifuge to determine whether the effects of the 3G force (provided that there was an effect) could be reversed through the use of a light pulse as had been suggested following the STS-9 results
- chronic exposure of *Neurospora* to a 3G force has no damping effect
- acute 10 minute (liftoff like) exposure to this hypergravity force causes

significant damping of the circadian rhythm of conidiation
- a brief light pulse given 36 hrs. after the acute exposure eliminates any effect of the acute 3G exposure on damping
- BND is more susceptible to the hypergravity perturbation than CSP
- the average free-running period increases with chronic hypergravity
- the major effects observed on the STS-9 experiment were simulated by hypergravity, including the ability of a light pulse to correct the aberrations

Significance of Accomplishments

The increased number of experimental samples in the new package design will not only aid in our statistical analysis, but has also allowed us to divest our samples into a greater number of paradigms; thus resulting in the equivalent of several flight experiments in one. The new strain of *Neurospora* (CSP) grows at a somewhat faster rate and "bands" with an increased resolution and clarity. This second fact becomes very significant in light of the results of STS-9, where a significant number of samples appeared to become arrhythmic (i.e. no clear banding) prior to the marking procedure. The strain BND is retained in the package as a control in order to repeat the observations of the earlier flight. Throughout the year we have been conducting test using both strains in order to verify the proposed differences and substantiate the benefits of using the second strain. Division of the package into three sub-packages also was allowed through the increase in the sample size of the package. One package will be treated similarly to the original SL-1 experiment; i.e. the package and its' race tube complement will be exposed to the ambient (mid-deck) white light while a crew member marks the growth fronts of the race tubes. A second package will also be marked by a crew member in the ambient mid-deck light; however the tubes in this second package will be wrapped by a red filter which will allow the astronaut to see the growth front without the *Neurospora* being able to "see" the light. The third package will not be marked or moved and thus will not be exposed to light. Gas sampling syringes were added to the package to determine if, in the unique conditions of space, the gaseous enviroment within the race tube had a significantly higher concentration of CO_2 at the growth front, (CO_2 is known to inhibit rhythmicity and therefore banding). The final change from the STS-9 experiment was not a hardware change but a procedural one. We intend to mark the tubes early in the flight rather than late. It was hypothesized that the damping found prior to the marking procedure on SL-1 may have been a result of the hypergravity pulse of launch and not due to the microgravity of the orbital lab. We have also hypothesized that the marking procedure, by exposing the samples to light, had reinstated rhythmicity. By moving the marking procedure earlier and perhaps eliminating the arrhythmic growth, it can be determined if the microgravity environment of earth orbit can support normal cyclic conidiation in *Neurospora*. This procedure will allow sufficient time for the rhythm to damp after the marking procedure, if indeed the damping is due to being in space and not due to the hypergravity of launch.

The combined EVT and biocompatability test was conducted at Ames in Febuary '86 to train the crew in marking and gas sampling procedures, verify the procedural timeline and confirm experimental procedures and hardware capability. These efforts lead to minor design changes and an overall smoother operation of the experimental procedures.

Ground based experiments were conducted in order to develop a ground based paradigm that could simulate the effects of microgravity on the *Neurospora* rhythm of conidiation (i.e. increased period length, decreased rhythm amplitude, rhythm damping, and increased variability in growth rate). The methods used in these studies were clinostat, orientation and hypergravity. In each of the first two experiments only the BND strain was used. In the hypergravity study both CSP and BND were used. In this and other studies (not defined here) the CSP strain produced more clear and consistant results. Therefore, our opinion remains in favor of the addition of this strain to all future flight and ground based research.

While the clinostat studies failed to simulate the effects of microgravity on the conidiation rhythm, the orientation studies demonstrate that creating a gravity vector 180 degrees from normal is a relatively good model to simulate the spaceflight results (Ferraro *et al.*, 1986). These experiments are currently being repeated in order to determine the consistency of the results; but the method looks quite promising.

The results obtained in the hypergravity study are even more promising (Ferraro *et al.*, 1987). We proposed a hypothesis that the hypergravityy of launch caused the damping, arrhythmicity and the increase in period length of the *Neurospora* circadian rhythm of conidiation, rather than the abscence of geophysical cues found in space. The results of the hypergravity study follow this hypothesis to the letter and adds further credence to our proposal to mark the tubes (thus exposing the race tubes to light) as soon after launch as possible (i.e. L+36hrs). Furthermore, it is also quite evident that the BND strain is more susceptible to the hypergravity perturbation than the CSP strain. Thus, flight results obtained with CSP should be cleaner. Similar to what is seen during spaceflight, the average free-running period increases with chronic hypergravity. Furthermore, the period of each individual cycle continues to increase thoughout the exposure period. It is not known whether this increase in cycle length has a threshold or not, since the period continued to increase for the entire length of the experiment.

Publications

Abstracts Presented:

Ferraro, J.S., H.N. Krum, G.T. Wassmer, M.E. Friedman, R.C. Wollman and F.M. Sulzman: Clinostat and Orientation Studies of the Growth Rate, Period, Amplitude and Damping of the *Neurospora* Circadian Rhythm of Conidiation. *Am. Soc. Grav. Space Biol. Abst.* 2:18, Abst. #35, 1986.

Krum, H.N., F.M. Sulzman, C.A. Fuller and J.S. Ferraro: The Effects of Hypergravity on the Circadian Rhythm of Conidiation in *Neuropsora crassa*. *Soc. Neurosci. Abst.* (in press), 1987.

Non-Refereed Publications:

Ferraro, J.S.: Effects of Spaceflight on Circadian Rhythms. *NASA Tech. Memo.* 89809:46-48, 1987.

Ferraro, J.S.: Characterization of *Neurospora* Circadian Rhythms in Space. *NASA Tech. Memo.* 89951:54-58, 1987.

Refereed Publications:

Sulzman, F.M.: Preliminary Characterization of Presisting Circadian Rhythms during Space Flight. *Adv. Space Res.* 4:39-46, 1984.

Sulzman, F.M., D. Ellman, C.A. Fuller, M.C. Moore-Ede and G. Wassmer: *Neurospora* Circadian Rhythms in Space: A Reexamination of the Endogenous-Exogenous Question. *Science* 225:232-234, 1984.

Ferraro, J.S. and C.E. McCormack: Minimum Duration of Light Signals Capable of Producing the Aschoff Effect. *Physiol. Behav.* 38:139-144, 1986.

Ferraro, J.S. and F.M. Sulzman: The Effects of Feedback Lighting on the Circadian Drinking Rhythm in the Diurnal Primate (*Saimiri sciureus*). *Am. J. Primat.* (accepted), 1987.

Refereed Publications (Submitted):

Ferraro, J.S. and H.N. Krum: The Effects of Feedback Lighting on the Reproductive Maturation of the Male Djungarian Hamster (*Phodopus sungorus*). *J. Reprod. Fert.* (submitted), 1987.

Ferraro, J.S., H.N. Krum, G.T. Wassmer, A. Bartke, V. Chandrashekar, S.D. Michael and F.M. Sulzman: Reproductive and Circadian Function in the Syrian Hamster: The Effects of Short and Ultrashort Light-Dark Cycles on the Photoperiodic Response and the Circadian Rhythm of Locomotor Activity. *Endocrinology* (submitted), 1987.

Ferraro, J.S., H.N. Krum, A. Bartke, V. Chandrashekar, S.D. Michael and F.M. Sulzman: Reproductive and Circadian Function in the Syrian Hamster: The Effects of Normal, Reverse, and Neighbor Control Feedback Lighting on the Photoperiodic Response and the Circadian Rhythm of Locomotor Activity. *Endocrinology* (submitted), 1987.

Papers in Preparation:

Ferraro, J.S., H.N. Krum, C.A. Fuller and F.M. Sulzman: The Effects of Chronic and Acute Hypergravity Exposure on the Growth Rate, Period, Amplitude and Damping of the *Neurospora* Circadian Rhythm of Conidiation. (in prep.), 1987.

Sulzman, F.M., J.S. Ferraro, C.A. Fuller, M.C. Moore-Ede, V. Klimovitsky, V. Magedov and A.M. Alpatov: Thermoregulatory Responses of Primates During Spaceflight. (in prep.), 1987.

Photograph 1.

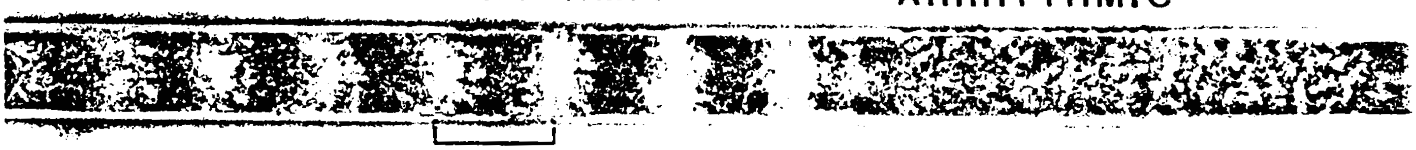
ORIGINAL PAGE IS
OF POOR QUALITY

QUESTION: CAN BIOLOGICAL CLOCKS CONTINUE TO MEASURE TIME IN SPACE ?

SPACELAB EXPERIMENT:

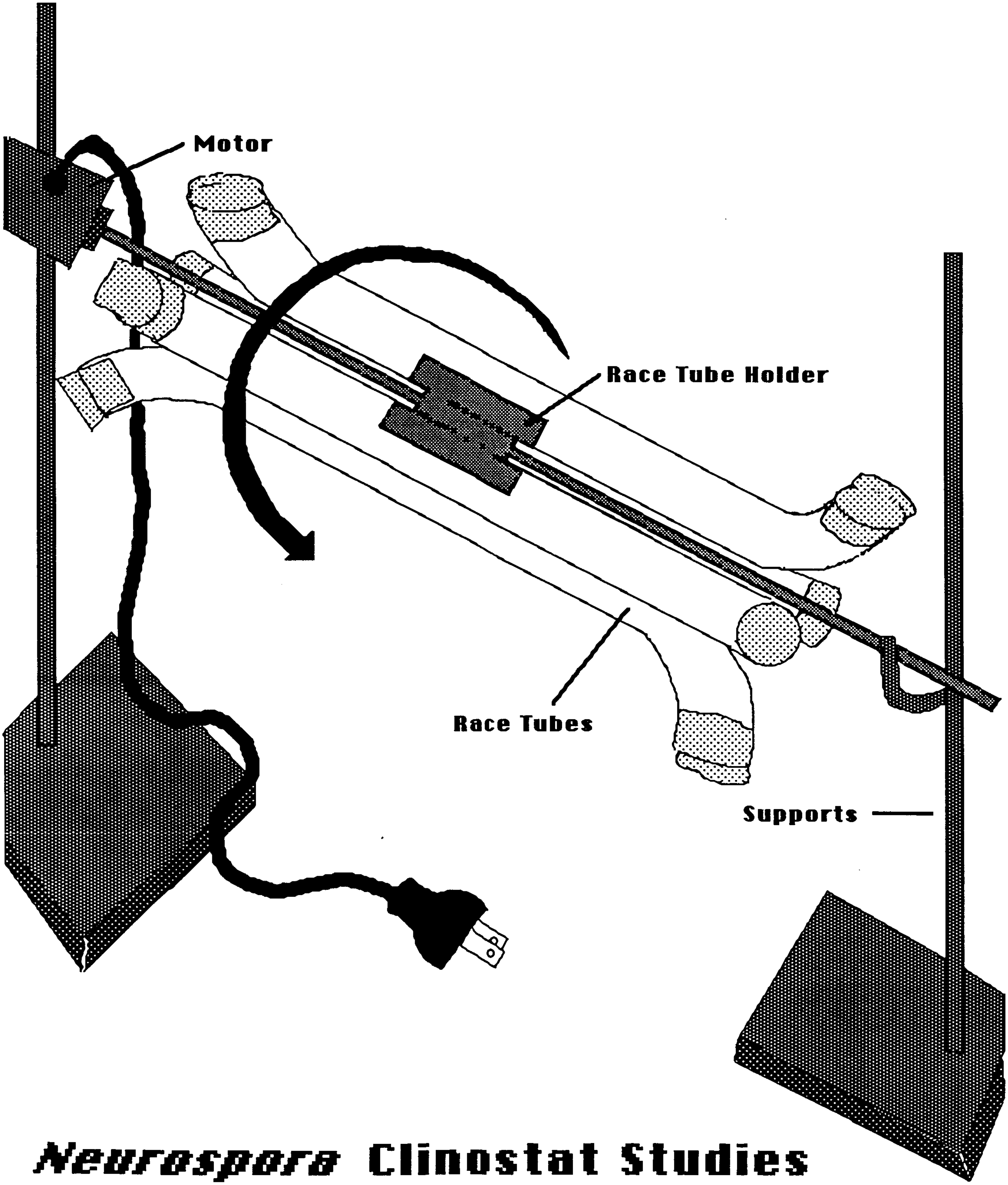
FUNGUS (NEUROSPORA) GROWTH RHYTHM

RHYTHMIC ARRHYTHMIC

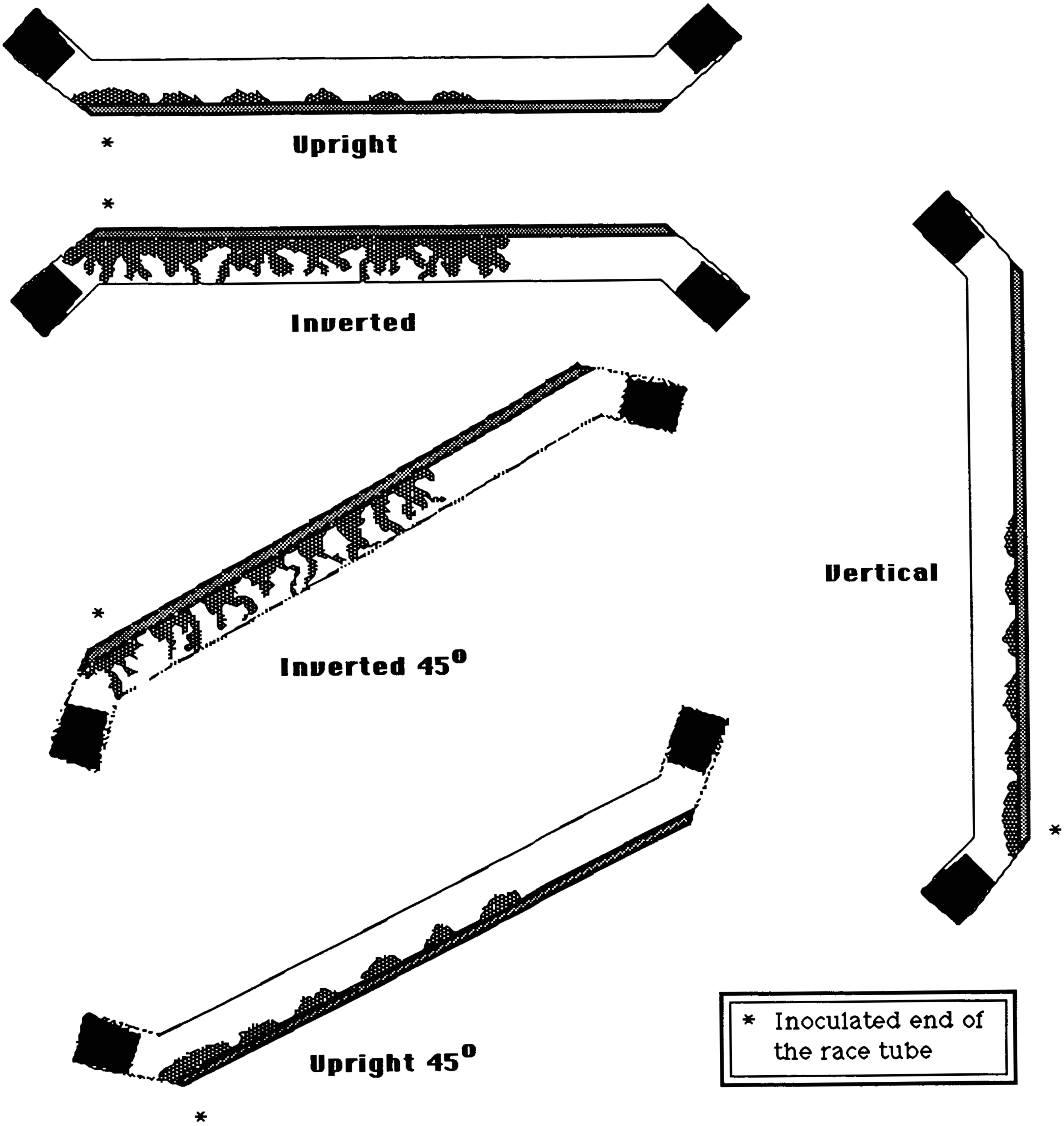


24 HOURS

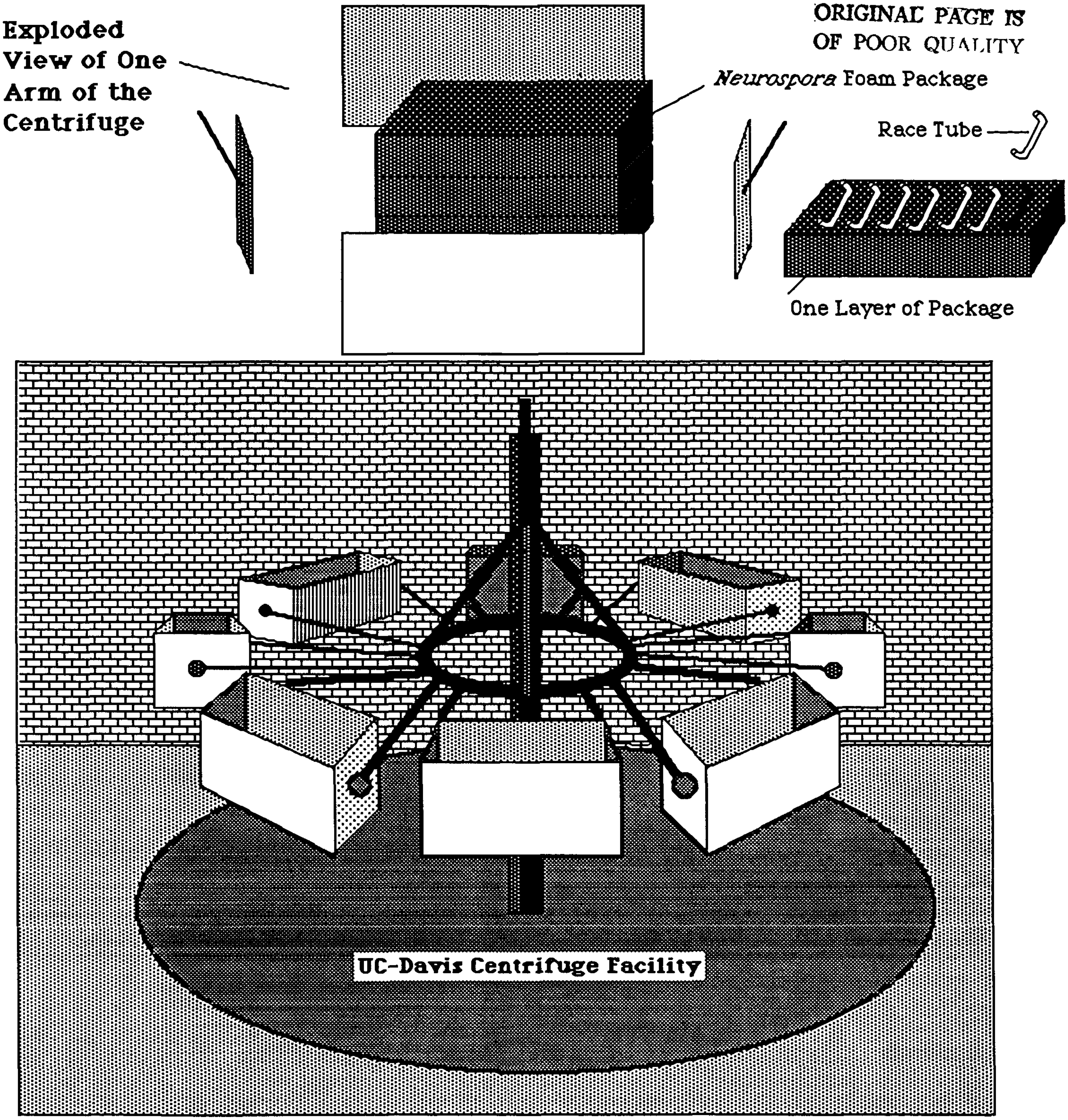
RESULTS: RHYTHMS CAN PERSIST IN SPACE
BUT THERE MAY BE SOME ALTERATIONS.



Neurospora Clinostat Studies



Neurospora **Orientation Studies**



Hypergravity Studies at UC-Davis