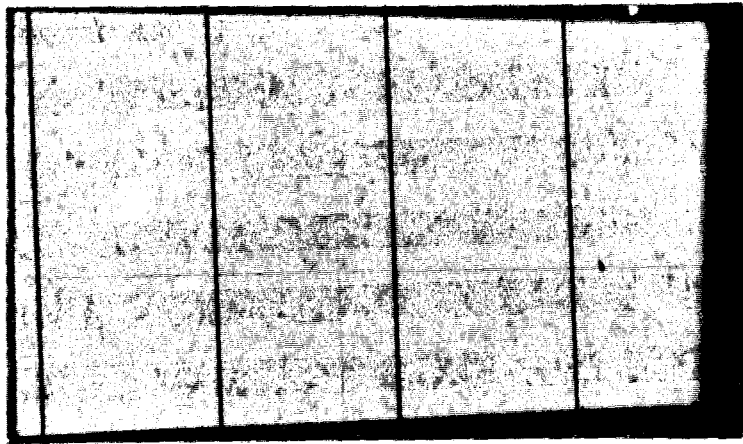# General Disclaimer

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UNIVERSITY of PENNSYLVANIA

Department of Biology

PHILADELPHIA, PENNSYLVANIA 19174

UNIVERSITY of PENNSYLVANIA

Department of Biology
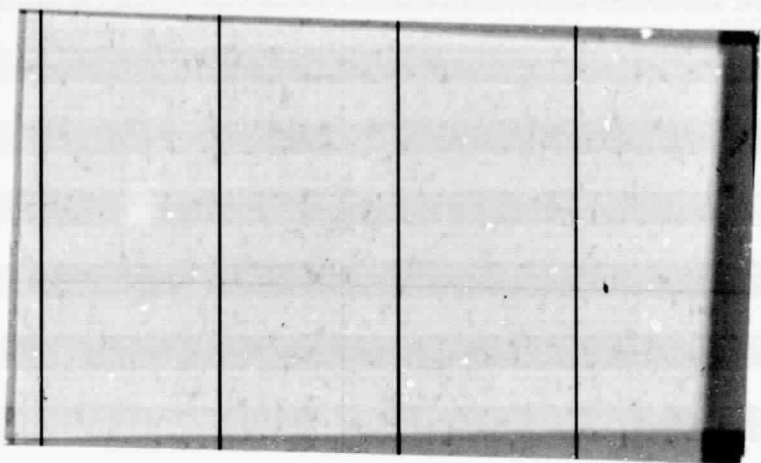
PHILADELPHIA, PENNSYLVANIA 19174

·Circadian Rhythm of
Leaf Movement in
Capsicum annuum Observed
During Centrifugation

CIRCAD-1

7 November 1975

by:

D.K. Chapman, A.H. Brown, and A.O. Dahl
Plant Centrifuge Laboratory, University City Science Center
and Biology Department, University of Pennsylvania

with the technical assistance of:

L. Goldstein, A.K. Venditti, and F.T. Vogel

ABSTRACT

Plant circadian rhythms of leaf movement in seedlings of the pepper plant (Capsicum annuum L., var. Yolo Wonder) were observed at different g-levels by means of a centrifuge. Except for the chronically imposed g-force all environmental conditions to which the plants were exposed were held constant. The circadian period, rate of change of amplitude of successive oscillations, symmetry of the cycles, and phase of the rhythm all were found not to be significantly correlated with the magnitude of the sustained g-force.

It is almost universally accepted that rhythmic oscillations in any given organism, as exhibited by at least some physiological characters, can be detected whenever the appropriate functions are adequately monitored. Rhythms, designated _circadian_, whose periods approximate 24 hours fall into an especially interesting class. Although circadian rhythms may not be ubiquitous, the organisms in which they have not been found after competent searching are limited to the prokaryotes; among metazoa, higher plants, and presumably all eukaryotes circadian fluctuations of divers functional attributes are overwhelmingly the rule, not the exception.

The nearly universal occurrence of circadian rhythms makes it difficult to consider them of only trivial importance to the organisms which display them -- a circumstance made all the more intriguing by our inadequate understanding of their biological roles and by our frustrating ignorance of their basic mechanism. We lack unifying concepts in both these areas and we are prone to justify the exploration of novel facets of circadian rhythm phenomena, if only because our teleological intuition warns that these rhythms which we understand so poorly relate to biological mechanisms of fundamental importance to the organism. Only in a few cases have we been able to deduce how some groups of organisms exploit their rhythmicities.

Early in the planning phase of the U.S. space program it was suggested that circadian rhythms would be a promising subject for investigation (1,6). Among the reasons given were first, to check on the possibility that, a test organism's rhythmic properties might be absolutely dependent on some (unidentified) periodic feature of the earth's environment from which it could be isolated only in space, and second, to determine whether the

organism's perception of earth's gravity is in some way a prerequisite for maintenance of a circadian rhythm. Neither of these possibilities should be dismissed out of hand even though each seems far fetched. Our skepticism is due chiefly to our ignorance of any mechanism which would account for the suggested effects of space flight; but ignorance is a poor base for rejecting a plausible even though tenuous scientific proposition.

Circadian oscillations of physiological parameters have been evident in data collected on astronauts. However, those rhythms could have been entrained by the mission schedules. No experiments have been performed in space which would constitute a rigorous test of whether free running circadian rhythms persist under weightlessness.

Because we know so little about the biological mechanisms which underlie circadian oscillations, the origin of the rhythmic pacemakers has attracted principal attention and there have developed exogenous and endogenous schools of opinion on this matter. We believe that the weight of experimental evidence, of reasoned argument, and of scientific intuition have persuaded most students of circadian phenomena that frequency control rests within the organism and, while a circadian rhythm is indeed subject to environmental influences which can entrain it, depress or enhance its amplitude, or shift it in phase, the continuance of the rhythm in an essentially constant environment is believed by only a minority of observers to be dependent on undetected (perhaps undetectable?) fluctuations in some unspecified environmental parameter. We frankly admit our preference for the majority view but admit also that the matter is still moot. Among all the problems of modern biology

there may be no more clearly drawn example of a scientific question
on which the cognoscenti defend viewpoints which appear to be forever
irreconcilable (2).

There is direct evidence that in some plants circadian rhythms are
not quite insensitive to alterations in the g-force vector. It has been
noted that the leaf movement rhythm of garden bean plants was suppressed
when the plant was rotated on a horizontal clinostat (7). However, other
reports indicate that the clinostat does not abolish the rhythms in most
cases. The circadian rhythm of broad bean root tip cell division was
maintained but was shifted in phase by 180° as a result of clinostat
rotation (3,4). This kind of effect of the clinostat was firmly
established also for Pinto bean which could be phase shifted reproducibly
by 180° depending on the phase of the circadian cycle at which the
clinostat rotation began (5). The authors of these latter reports were
unable to suggest a mechanism which could account for such phase shifts
but they speculated that the onset of weightlessness might have the
same effect as the initiation of rotation on a clinostat and they called
attention to important consequences of such an effect whenever the results
of space flight experiments are being interpreted.

It is unfortunate that so much has been said about possible studies
of circadian phenomena in the weightless environment (so-called zero-g)
but so little has been done to determine whether the rhythms are g-
dependent in the range from 1 g upward, a region of the g-variable
which can be attained by centrifugation here on earth. As far as

plants are concerned we are aware of only a single experiment in which

a well studied circadian rhythm (leaf movement) was observed during

centrifugation and the results of even that preliminary set of observations

were never published (7).

The principle objective of the research we report here was to

observe a plant circadian rhythm of leaf movement during experiments

which would span a range of g-levels in order to determine whether or

not the oscillatory behavior was g-dependent.

MATERIAL AND METHODS

Test Organism -- The pepper plant, Capsicum annuum L., var. Yolo

Wonder was used. All seeds were from the same seed lot. They were planted

in flats of sterilized soil and illuminated by a mixture of cool white

fluorescent and tungsten lamps at 650 f.c. on a daily regimen of 16L/8D.

At age 14 days seedlings were transplanted into "Jiffy" peat cubes. The

temperature during growth of the plants was $24 \pm 1^{\circ}$ C.

Plants showed pronounced circadian leaf movements when they were

between 24 and 30 days old. At age 28 days test plants were selected

for uniformity of height and evenness of leaf size. For a plant to be

used in an experiment leaf no. 1 had to be no more than 15% longer than

leaf no. 2 and the stem had to be nearly straight.

After selection the cotyledons were removed and the plants were

transferred to polyethylene beakers containing perlite. The peat cubes

and the perlite were saturated with glass distilled water which proved

to be a sufficient supply for the duration of the experiment. To reduce
evaporation the surface of each peat cube was covered with a sheet of 0.5
mil Saran Wrap fastened to the outside of the beaker with rubber bands.
The test plant's stem protruded through a small hole in the Saran Wrap.

For a plant undergoing centrifugation the stem was supported by
a small stake fashioned from a "Q tip". A section of the stem was wrapped
with teflon tape and this was secured to the stake with "Scotch Tape".

After an experimental plant had been prepared for the experiment it
was exposed to continuous illumination from above at either 450 f.c. or
200 f.c. by a bank of "Wide Spectrum GroLux" fluorescent lamps (Sylvania)
for the duration of the test. (Preliminary observations had shown that
circadian leaf movements were not significantly dependent on light
intensity between 200 and 450 f.c.) Thus a circadian rhythm was impressed
upon the plant by its illumination schedule for 4 weeks but at the
beginning of each centrifuge experiment the illumination was made constant.
Since the plant's leaf rhythm was then observed to damp out over a 3-5
day period our observations on effects of altered g-level were made during
this damping period while the plant's environment was constant.

The plants were placed in rectangular plexiglass "greenhouses"
(23 cm x 20 cm x 10 cm) mounted on plywood base boards to each of which
a television camera was mounted. The temperature within each "greenhouse"
was monitored with a thermistor and recorded on a strip chart recorder.
In all tests the temperature was $22.5 \pm 0.5^{\circ}C$.

Centrifugation -- Continuous exposure to elevated g for 3 to 6 days
was accomplished on the NASA-University City Science Center Plant Centrifuge.

The experimental assemblies each containing greenhouse, plant, lamps, and camera were mounted in centrifuge payload cradles which were free to swing so that during centrifugation the resultant acceleration vector was maintained parallel with the stem axis of the test plant.

Photographic Observations -- The video images which were continuously available for all test plants in any one experiment were transmitted through slip rings, carried outside the centrifuge rotunda, and displayed sequentially on a video monitor once every half hour. The monitor was located in view of an Acme camera which was focused on the tube face and programmed to expose one frame during the few seconds each camera image was displayed on the monitor. Kodak Plus-X Reversal film (emulsion no. 7276) was used and, upon termination of an experiment, the film was removed and developed by a Kodak Prostar processing unit.

Data reduction -- Leaf angle was defined as the angle formed by the stem axis and a line connecting the base of the petiole and the tip of the leaf. The angle notation was according to the convention that "6 o'clock" would be called zero. Thus, in anatomical terms, the angles were abaxial rather than adaxial. These angles were measured for each leaf on each film frame with a Vanguard Motion Analyzer linked to an IBM key punch machine. The resulting set of punched cards were processed on an IBM 360/75 computer. The computer print-out provided a tabulation of leaf angles at successive 30 min. intervals during the experiment. Preliminary data analysis showed no significant differences between the changes of angle for leaf no. 1 and leaf no. 2; therefore these data were pooled (except in the case of data shown in Fig. 1).

Several criteria were established which we thought would characterize a plant's circadian behavior so as to reveal even fairly subtle effects on altered g-levels. The plants' leaf rhythms were examined for g-dependence by each of the following criteria: (a) cycle duration, (b) rate of amplitude damping, (c) cycle shape (symmetry), and (d) cycle phase shift.

Table I shows a summary of all experiments performed in this CIRCAD test series. Useful data were obtained at g-levels up to 6. At 9 g the test plants were unable to raise their leaves for mechanical reasons.

RESULTS

Fig. 1 shows representative data for rhythmic movements of individual leaves of plants maintained at 1 g or at 6 g. The patterns were similar. At either g-level the rhythms were damping out under the condition of a constant environment. Insofar as these particular examples are concerned there was no striking effect of a six-fold difference in the g-force to which the plants were exposed.

Period -- Measurements on period length are shown in Table II. The mean values for g-levels above unity did not differ significantly from the 1 g mean in any of the g-levels tested.

The data of Table II are plotted in Fig. 2 which shows the regression line fitted by the method of least squares. A correlation coefficient for period on g-force was calculated for all the measurements which contributed to Fig. 1. The correlation was -0.19 which was not significantly different from zero $(p > 5\%)$.

Damping -- It is evident from Fig. 1 that amplitudes of circadian

cycles of leaf movement decreased progressively once the test plants

were introduced to a constant photic environement. Therefore, to detect

g-dependence of cycle amplitude, it was necessary to relate the rate of

damping to the g-parameter.

Damping was measured as a change in cycle amplitude. A "damping

coefficient" was defined in terms of successive amplitude differences

observed in the course of the first two full cycles measured after the

onset of constant light conditions (which coincided with the onset of

centrifugation). The coefficient was defined as:

$$\frac{C_I - C_{II}}{C_I}$$

Where C_I and C_{II} were mean amplitudes for the first cycle and second

cycle respectively. The mean amplitude of a cycle was operationally

defined for the first cycle (for example) as:

$$\frac{\Delta_1 + \Delta_2}{2}$$

where Δ_1 = amplitude difference from initial cycle maximum to subsequent

cycle minimum.

Δ_2 = amplitude difference from initial cycle minimum to subsequent

cycle maximum.

In this terminology an undamped rhythm would exhibit a coefficient of zero,

a rhythm which endures only for a single cycle would show a coefficient

of unity, and a rhythm which damps to half amplitude on each succeeding cycle

would have a coefficient of 0.5.

Table III gives the results of such damping coefficient calculations. These results were plotted against g-level as shown in Fig. 3. Although there appears to be an upward trend to the data (implying that increased g-force accelerated the damping), it is evident that the trend was not statistically valid.

Cycle symmetry -- Although oscillations were not shown to differ in period of in amplitude there could have been a form change which might be g-related. Sometimes it appeared that the rate at which a leaf angle increased was greater than the rate at which it subsequently decreased; i.e., not symmetrical about its maxima or minima. We measured the time for the leaf to move from its half-amplitude value durings its increasing phase and to achieve its maximal angle (defined as α) and we compared that with the time for the leaf to fall subsequently from its maximal angle and to reach its subsequent half-amplitude value (defined as β). The quotient of those values was called the "symmetry ratio". For an oscillation which was quite symmetrical, $\frac{\alpha}{\beta} = 1$. If the rate of leaf angle increase was greater than the rate of its decrease, the symmetry ratio would be less than unity, a result consistently observed with plants at 1 g.

Table IV summarizes the results obtained over the six-fold g-range which was explored. At 3 g and 4.5 g the mean symmetry ratios were not significantly different from the mean ratio for 1 g. At 6 g that difference was significant at the 5% but not at the 1% level.

In Fig. 4 the symmetry ratios are plotted against the sustained g-levels. The upward trend reflects a correlation coefficient of +.1893,

a number which is significantly different from zero (p=.0811).

Phase stability -- Reports from two other laboratories (4,5) that some leaf movement rhythms could be phase shifted by a g-perturbation (clinostating) led us to search for a similar effect associated with centrifugation. The earlier reports announced phase reversals (180° phase shifts) attributable to rotation on clinostats but with our material no such large shifts were observed as a result of centrifugation at up to 6 times the normal g-level. As Table V shows, all leaf angle maxima occurred within $\pm$ 2 hours of the same time of day at all g-levels tested.

DISCUSSION AND CONCLUSIONS

Compared with results obtained at 1 g the circadian leaf movement rhythm was not materially influenced by chronic centrifugation at least up to 6 g. The period, rate of amplitude damping, symmetry of the oscillations, and phase all were found not to be significantly correlated with the sustained g-level.

Our negative findings with respect to Capsicum differ from results reported by others for legumes for which the g-perturbation that affected the rhythms was clinostating rather than centrifugation.

It is possible that an influence on the plant rhythm is especially dependent on the phase of the cycle at which g-perturbation begins. Hoshizaki (5) showed this to be the case for Pinto bean. Possibly the pepper plant is even more finicky and the time of onset of centrifugation could be especially critical in determining whether or not its rhythm would be affected. In the experiments reported here we did not initiate centrifugation at all times of the plants'

subjective cycles. However, in three of our tests centrifugation was started at the time of subjective day which would have corresponded to the most sensitive part of the cycle had our test plant been the Pinto bean. The results in those three cases were substantially the same as for the rest of our tests. Therefore we concluded that the circadian rhythm of Capsicum was indeed insensitive to incrased g-level within the range we examined.

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TABLE I. SUMMARY OF EXPERIMENTS
SEARCHING FOR AN EFFECT OF G-FORCE
ON CIRCADIAN RHYTHM OF LEAF MOVEMENT

EXPT. IDENT. NUMBER	G-FORCE EMPLOYED	STARTING DATE	TIME OF DAY G-FORCE APPLIED	NUMBER OF LEAVES MEASURED
8	1.0	14 FEB '75	1030	4
9	1.0	17 FEB '75	1100	6
10	1.0	21 FEB '75	1300	6
11	1.0	24 FEB '75	1500	6
17	3.0	28 FEB '75	1600	4
20	3.0*	10 APR '75	1630	4
23	3.0*	29 APR '75	1200	4
24	4.5*	6 MAY '75	1200	4
18	6.0	3 MAR '75	1600	4
21	6.0*	15 APR '75	1030	4
22	6.0*	24 APR '75	1130	4
19	9.0	7 MAR '75	1330	4

*MEASUREMENTS ALSO MADE ON 2 LEAVES OF A PLANT AT 1G.

TABLE II. RELATION BETWEEN G-FORCE
AND LENGTH OF CIRCADIAN PERIOD

G-Force	Period* (Hours ± SE)	N	Probability that difference from value at 1 g was due to chance
1.0	24.07 ± .43	36	--
3.0	22.57 ± .38	24	5% > P > 1%
4.5	22.50 ± 1.67	8	P > 5%
6.0	23.13 ± .87	12	P > 5%

* Average of first two circadian cycles after application of altered G-force.

TABLE III. Relation Between Amplitude Damping and g-Force Exposure

G-Force	Damping Coefficient* (Coef. ± SE)	N	Probability that Difference From Value at 1g was Due To Chance
1.0	0.159 ± .091	23	--
3.0	0.184 ± .091	12	P > 5%
4.5	0.174 ± .102	4	P > 5%
6.0	0.317 ± .134	6	P > 5%

* Damping coefficient which is a measure of the rate of reduction of amplitude of rhythmic oscillation of leaf angle was calculated as described in the text.

TABLE IV. Symmetry Ratios* of Leaf Angle Oscillations in Relation to the G-Force

Cycle		1.0G		3.0G		4.5G		6.0G
	(N)	Mean ±SE	(N)	Mean ±SE	(N)	Mean ±SE	(N)	Mean ± SE
1ST	(10)	0.46±.15	(12)	0.38±.08	(4)	0.50±.14	(7)	0.73±.15
2ND	(10)	0.59±.08	(12)	0.98±.19	(3)	0.72±.20	(6)	0.50±.12
3RD	(7)	0.78±.06	(7)	1.65±.44	(1)	0.54	(6)	2.13±1.12
Average		0.59±.07		0.90±.15		0.50±.10		1.47±.34

* Defined and calculated as explained in text, the symmetry ratio is a measure of rate of decrease of leaf angle compared to rate of increase of the angle in respective phases of the circadian cycle.

TABLE V. TIME OF MAXIMAL
LEAF ANGLE (HOURS AFTER MIDNIGHT)

EXCURSION SEQUENCE	G-FORCE							
	1.0G		3.0G		4.5G		6.0G	
	(N)	MEAN±SE	(N)	MEAN±SE	(N)	MEAN±SE	(N)	MEAN±SE
1ST MAXIMUM	(12)	3.9±.4	(12)	3.5±.5	(4)	2.1±.1	(7)	2.9±1.5
2ND MAXIMUM	(20)	4.1±.5	(12)	1.9±.5	(4)	2.6±1.5	(7)	0.9±.5
3RD MAXIMUM	(16)	4.1±.5	(12)	0.6±.6	(4)	-0.9±.3	(6)	1.4±1.0
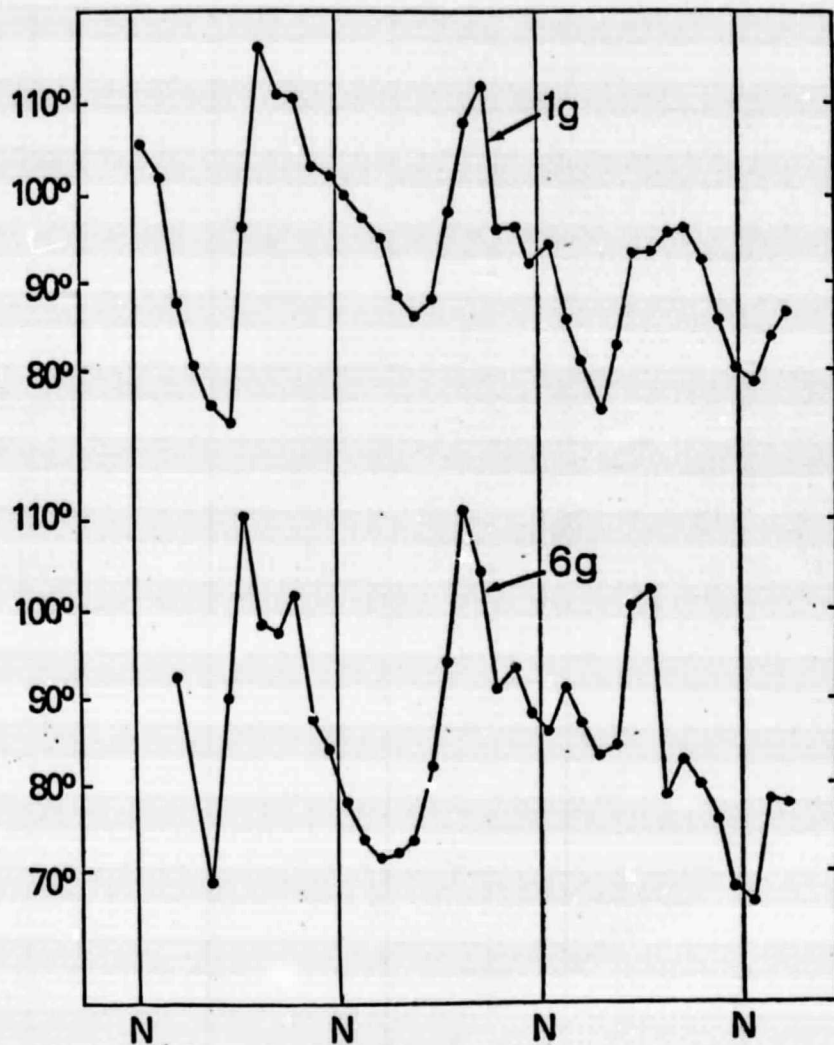
Fig. 1 Representative examples of the time course of mean leaf angle
for leaves #1 and #2 measured as described in text. Upper curve, data from
a plant maintained at 1 g. Lower curve, data from a plant maintained at 6 g.
Ordinate, average angle of departure from the plumb line (zero degress
corresponds to the 6 o'clock position.) Divisions along the abscissa
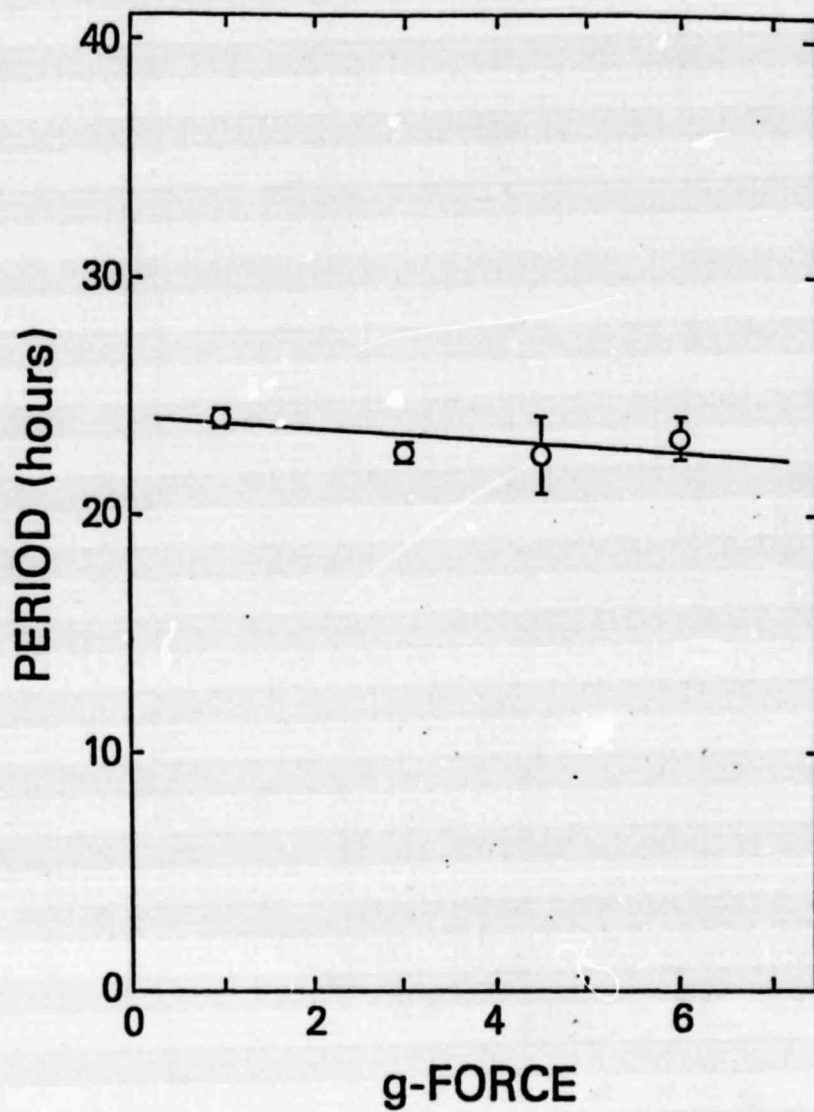represent 24 hour intervals (noon to noon).

Fig. 2. Relation between mean period length and chronically maintained g-force. Error bars on each plotted point indicate ± 1 SE unit from the mean. Data from Table II.
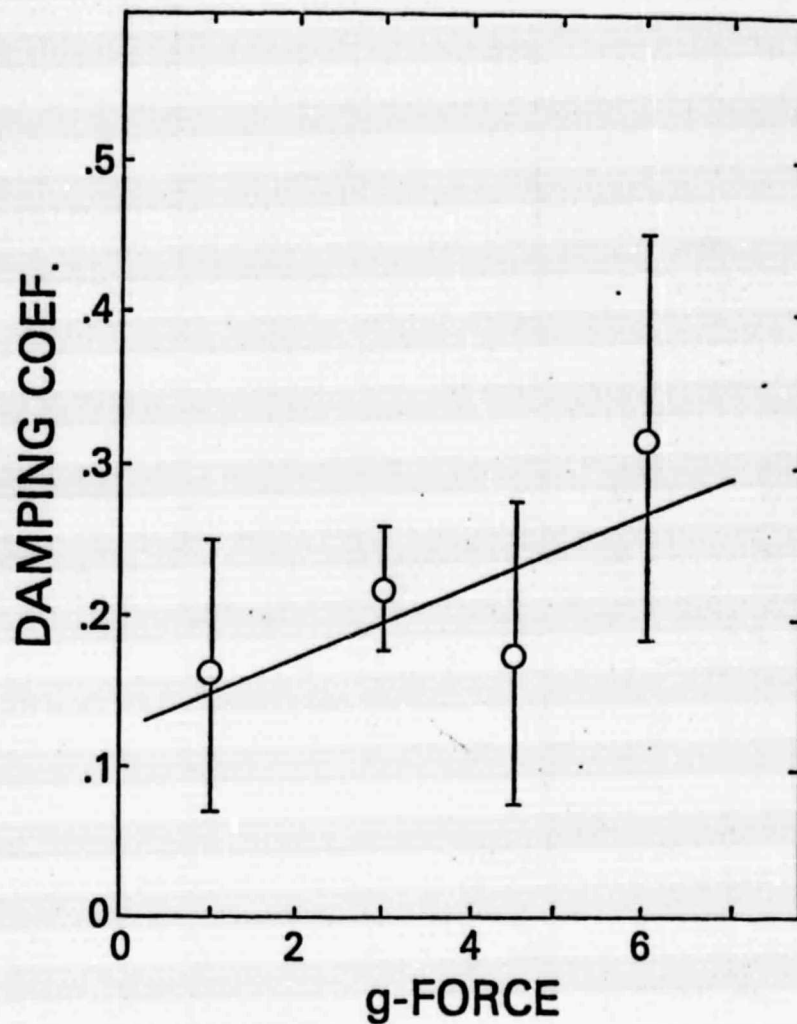
Fig. 3. Relation between amplitude damping and chronically maintained g-force. Ordinate, damping coefficient or relative change in cycle amplitude calculated as described in text. Abscissa, sustained g-force. Error bars on each plotted point indicate ± 1 SE unit from mean. Data from Table III.
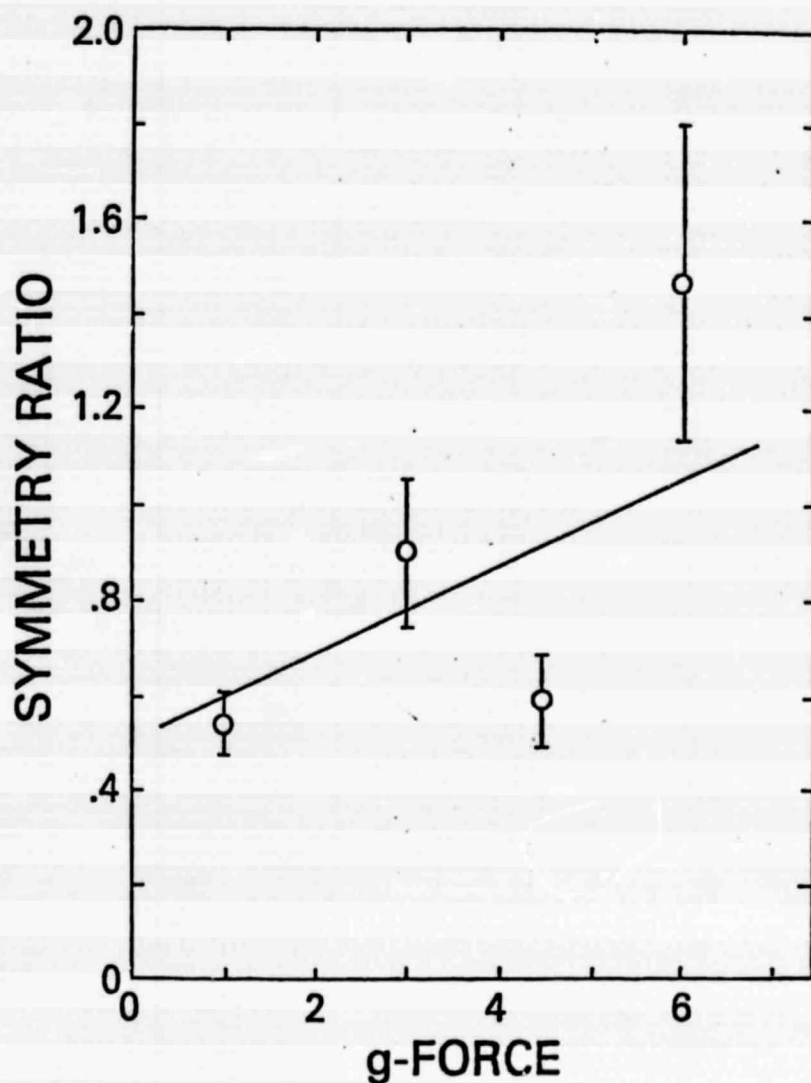
Fig. 4. Relation between the symmetrical character of circadian cycle of leaf movement and the g-force to which the test plants were exposed. Ordinate, symmetry ratio calculated as described in text. Abscissa, chronic g-force. Plotted points represent means of the first three circadian cycles after the beginning of centrifugation. Error bars indicate ± 1 SE unit from mean. Data from Table IV.