AN ABSTRACT OF THE THESIS OF

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Title: THE ACTIVITY PATTERN OF DIADORA ASPERA

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Diadora aspera is an intertidal keyhole limpet found in the mid-tidal zone. Its activity pattern was estimated under laboratory conditions using time-lapse-photography and manometric determinations of oxygen consumption.

Statistical and computer analyses of the photographic data suggest the presence of both a 24 hour and a 12-13 hour rhythm in activity. Both oxygen consumption and photographic data suggest that the diel rhythm has an activity peak at noon. The oxygen consumption data also suggest that the 12-13 hour rhythm in activity is peaked 3-4 hours before high tide. Protection from predation (possibly by Pycnopodia helianthoides) is suggested as a possible factor in selection for this phasing.

The Activity Pattern of *Diadora aspera*

by

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THE ACTIVITY PATTERN OF *DIADORA ASPERA*

INTRODUCTION

The activity pattern of *Diadora aspera* (Eschscholtz, 1833), a mid-level, rocky intertidal keyhole limpet found on the Oregon coast, has not previously been measured. Oxygen consumption and time-lapse-photography studies have been used to show that *D. aspera* may have both tidal and diel rhythms. The phasing of these rhythms suggests that protection from desiccation is not the primary factor in timing but that other factors may be involved, possibly predation by *Pycnopodia helianthoides* (Brandt, 1835) its only known enemy (Margolin, 1964).

Diel periodicity in animals has been observed since prehistoric times: stone-age hunters were probably aware of the times of day when their prey would be at the watering hole (Harker, 1964).

The first written account of periodicity appeared in Greece before the time of Aristotle, when a naturalist observed that certain legumes lowered their leaves at night, then raised them up in the morning (Brown, Hastings, and Palmer, 1970).

The first scientific measurements of a diel rhythm persisting under constant conditions was made by the astronomer DeMairan (1729), who kept plants in total darkness and found they maintained a 24-hour rhythm in leaf movements. Since that time, diel rhythms have been found in most phyla.

The literature of the general topic of biological rhythms or "biological clocks" is vast. Several excellent articles review the subject (Aschoff, 1963; Bruslé, 1969; Cloudsley-Thompson, 1961; Kleitman, 1949; Webb and Brown, 1959; Wolf, 1962; Harker, 1962, 1964; and Sweeney, 1969).

Definitions and Terminology

Under constant laboratory conditions of light and temperature the period of a naturally occurring 24 hour (diel) rhythm of activity tends to shorten or lengthen and gradually slip out of phase with control individuals maintained under natural conditions. This free running period of approximately 24 hours in duration has been termed circadian (L. circa about + di(em) day + an) (Halberg, 1959).

Other circa-rhythms may correspond to lunar rather than solar frequencies. Tidal rhythms, (12.4 and 24.8 hours) are common in many organisms, but are especially obvious in intertidal forms. Under constant laboratory conditions these also tend to slip out of phase in relation to natural conditions. The resulting free running rhythm has been termed circa-tidal (Palmer, 1963). Lunar rhythms of longer periods (14.8 and 29.5 days) have been observed in a variety of organisms and are often correlated with their reproductive cycles (Havenschild, 1960).

Seasonal rhythms are evident in reproductive cycles. Due to the length of the period involved, direct measurements of circa-seasonal rhythms are difficult. Indirect evidence of endogenous seasonal rhythms was given by Marshall (1952), when he noted that organisms

transported from one hemisphere to another often retained their reproductive timing in relation to the seasons of their former homes.

Correlations between biological rhythms and subtle environmental changes have been claimed. Brown and his co-workers have recorded similarities between activity measurements in numerous plants and animals (potatoes, carrots, fiddler crabs) and such environmental parameters as daily barometric pressure and magnetic and primary cosmic-ray fluxes (Brown, Webb, and Bennette, 1958; Brown, Webb, and Brette, 1960; Brown, Freeland, and Ralph, 1955).

Cloudsley-Thompson (1961) noted that short-period rhythms may reinforce each other to form rhythms of longer periods. If an organism possesses both a circadian and a circa-tidal rhythm, the effect would be to produce a complimentary rhythm of 12.4 days, when the two rhythms were synchronized with each other, as is the case with the fiddler crab (Brown et al., 1953).

Biological rhythms are essentially independent of temperature. The reaction rate of most biochemical reactions doubles with a 10°C rise in temperature, (i.e. $Q_{10} = 2$), but in surveying the literature Bünning (1972) found that the periods of biological clocks change only slightly ($Q_{10} = 1.0\text{-}1.2$) with temperature. Even if the temperatures are lowered to where normal life processes (e.g. respiration) are arrested, organisms returned to normal temperatures maintain their clock in phase with control organisms not subjected to the chilling (Bünning, 1972). If the animal is frozen, which in certain Arctic species may require temperatures several degrees below zero °C, the clock can be stopped (Bünning, 1972).

Circadian rhythms can be phased (reset) by several factors. Cycles in light and temperature seem to produce the most pronounced effects. Light flashes of milli-second duration can cause synchronization in plants (Bruce, Weight, and Pittendrigh, 1960). Weak, red (photographic) "safe" lights may cause phasing also (Bünning, 1972). In automated experiments (e.g. continuous recording respirometers) power supply voltage changes (which have a daily periodicity) may cause 24 hour rhythms (Heusner, 1965). An exact 24 hour period to a circadian rhythm probably indicates that some subtle phasing factor was neglected (Enright, 1965), and illustrates the need for meticulous controls to ensure unbiased results.

Adaptive Significance

Although adaptive significance of certain circa-rhythms is still the subject of debate, certain statements made by Cloudsley-Thompson (1961 p. 199) appear to be generally accepted:

1. "Biological clocks allow an organism to maintain rhythmic function in the temporary absence of environmental cues, such as occur on cloudy days, eclipses, or seasonally turbid waters."
2. "They may preadapt individuals to a forthcoming environmental change, (incoming tides, onset of night)."
3. "They may keep individuals in a population in phase with each other (reproduction or migration)."
4. "They may initiate seasonal phenomena (hibernation, diaphase)."

Persistent Rhythms in Intertidal Organisms

Rhythms in intertidal organisms were first studied about the turn of the century. The intertidal tubularian, Convoluta roscoffensis Graff, 1891, was observed to emerge on the sand surface at low tide and submerge when the tide rose. This tidal rhythm persisted for several days under non-tidal conditions in the laboratory (Gamble and Keeble, 1903; Bohn, 1903). The sea anemone, Actinia equina L., 1758, has a tidal rhythm of expansion and contraction which continues for 5 to 8 days in an aquarium (Bohn, 1906).

Gompel (1937) recorded the oxygen consumption of A. equina and a variety of intertidal animals, (a polychaete, Arenicola marina [L., 1758]; two gastropod species, Patella vulgata L., 1758, and Haliotis tuberculata L., 1776; a lamellibranch, Mytilus edulis L., 1758; and two species of intertidal fish, Rhombus maximus L., 1758, and Pleuronectes platessa L., 1766). He reported a general trend of maximal oxygen consumption values occurring just before high tide with minimal values occurring before the low.

After Gompel's work (1937), little research was done on the rhythms of intertidal organisms until the 1950's when F.A. Brown, Jr. and his co-workers reported a series of studies on the fiddler crabs, (Uca spp.). These animals were shown to have a diel rhythm in melanophore pigment dispersion with maximum darkening occurring around noon. In the laboratory under non-tidal conditions and constant illumination a maximum darkening occurred about the time of low tide. For the first time an organism with two persistent rhythms of different periods acting in

harmony was demonstrated, each reinforcing the other when low tide occurred near noon and interfering with each other when they were out of synchronization (Brown, et al., 1953). Since low tides occur approximately 50 minutes later each day, maximum melanophore dispersion due to the tidal and daily cycles would be superimposed every one half lunar month (approximately every 14.8 days), the resultant exaggeration of darkening forming yet another persistent rhythm. Fingerman (1955) found similar rhythms in melanophore dispersion in Callinectes sapidus Rathburn, 1895. This dual rhythmic system is not limited to melanophore dispersion; it has been observed, for example, in locomotor activity in several species of crabs (Palmer, 1967; Webb and Brown, 1965; Naylor, 1958; and Barnwell, 1966). Webb and Brown (1959) listed eight rhythms that have been observed in crustacea, (expecially Uca spp.).

1. Daily, tidal, and semi-lunar-monthly rhythms in integumentary color.
2. Daily, tidal, and semi-lunar-monthly rhythms in motor activity.
3. Daily, tidal, semi-lunar-monthly, lunar month, and possibly seasonal rhythms in oxygen consumption.
4. Daily and lunar-monthly rhythms in molting.
5. Daily rhythms in eye pigments.
6. Daily rhythms in response to illumination changes.
7. Daily rhythms in responses to temperature changes.
8. Possible daily rhythms in response to gravity (as a mechanism for vertical migration in zooplankton).

Circadian and circa-tidal rhythms are somehow interrelated. Barnwell (1966), using Uca minax (LeConte, 1855), was able to change

the phase of the circa-tidal rhythms of locomotor activity by altering the day-night cycle of illumination. Honnegger (1973) obtained the same frequency transformation using Uca crenulata (Lockington, 1876) but the display was not as obvious as that obtained by Barnwell.

The circa-rhythms shown by these animals reflect their particular habitat. Crabs of the same species collected from different zones in the intertidal show different phasings of their circa-tidal rhythms. Fiddler crabs that inhabit burrows nearer the high-tide level darken sooner than crabs that inhabit lower burrows suggesting that the time of exposure is important in setting the phase of the tidal rhythm (Fingerman, Lowe, and Mobberly, 1958; Barnwell, 1963).

The accuracy in timing of circa-rhythms to environmental cycles is well documented (Enright, 1963). The sand beach amphipod, Synchelidium sp., possesses such a persistent tidal rhythm that for the first few days under constant laboratory conditions, it not only matches the tidal period but matches the amplitude as well. Since this work was done where tides are asymetric in respect to amplitude and duration, this accuracy by Synchelidium is even more astounding.

The isopod Excirolana chiltoni (Richardson, 1905), was observed for two months under constant conditions (Enright, 1972). Swimming activity showed a rhythmic period of 24 hours and 55 minutes, only 5 minutes longer than the natural tidal cycle. Excirolana chiltoni also possessed a highly accurate bimodal circa-lunar month rhythm which was only one or two days longer than the natural cycle.

Persistent Rhythms in Molluscs

Lamellibranchs have been used by several authors to study circa-rhythms. Brown (1954) found that the oyster Crassostrea virginica (Gemelin, 1790), had a rhythm that was both circadian and circa-tidal and persisted for 46 days under constant conditions. Rao (1954) found circadian and circa-tidal rhythms in the rate of water propulsion in Mytilus californianus Conrad, 1837 and in M. edulis.

Circa-rhythms have been observed in several intertidal gastropods. Rhythms of activity were demonstrated by Stephens, Sandeen, and Webb (1953) in the mud snail, Nassarius obsoletus (Say, 1822). Maximum activity occurred at about the time of high tide at the site of collection, but no circadian component was observed. Sandeen, Stephens, and Brown (1954) recorded the circadian and circa-tidal rhythms of oxygen consumption in Littorina littorea (L., 1758) and Urosalpinx cinereus (Say, 1822). When the data were correlated with natural tidal cycles, minimal rates occurred one half-hour before high tides and maximal rates 2 to 3 hours before lows.

Ecological factors effecting zonation are also important in setting the phase of circa-tidal rhythms. Zann (1973) studied relationships between circa-tidal rhythms and zonation in ten species of Australian intertidal gastropods. Upper littoral forms showed no overt circa-tidal rhythms, possibly because they were not subjected to a regular tidal exposure. Mid-littoral species had an overt circa-tidal rhythm under non-tidal conditions, suggesting the influence of a regular tidal stimulus. They were active at ebb tide suggesting a

behavioral adaptation to avoid desiccation. Low littoral representatives were active at low tides. Since these organisms were less subject to desiccation, Zann suggested that other selective processes were at work, possibly predation or wave action at their perferred site. The only sublittoral species studies, Bembicium auratum (Quoy and Gaimard ? 1825) showed no overt circa-tidal rhythmicity, suggesting lack of a regular tidal stimulus.

In summary, research on intertidal organisms has shown that they possess rhythms that persist under laboratory conditions. These rhythms are temperature independent and in some way reflect the natural environmental cycles that occur in their habitats; they tend to "slip" under non-rhythmic conditions, but can be synchronized by a variety of stimuli (light having the most pronounced effect); and they help adapt the organism to its environment.

MATERIALS AND METHODS

An ideal method for determining the presence and phasing of circa-rhythms in D. aspera is one that measures some index of activity that is stable, is obvious for times exceeding the periods of interest, is indicative of the total physiological function of the organism, and is measurable by a means which minimizes stress. Two indices that conform to these criteria are oxygen consumption and locomotor activity. Both are subject to fluctuations due to the physiological state of the organism (presence or absence of food, temperature and salinity stress, handling shock, reproductive state, etc.). A certain amount of the fluctuation can be compensated for by combining and averaging many individual observations.

Measurement of Oxygen Consumption

Specimens of Diadora aspera were collected from their habitat at Yaquina Head, Oregon during minus tides. They were kept moist by covering them with wet sea weed during transportation to the laboratory. Elapsed time between collection and the start of experiments was usually three hours or less. In the laboratory each limpet was cleaned of encrusting organisms and gently brushed to remove algae since algae are known to produce measurable amounts of oxygen (Baldwin, 1968). The commensal polychaete, Arctenöe vittata (Grube, 1855), which often inhabits the mantle cavity was gently removed. Individual limpets were weighed and their volume determined before being placed in respiratory chambers.

Oxygen consumption experiments were done in a Warburg constant-temperature (12±0.1°C) water bath using standard Warburg manometers. Since the standard sizes of Warburg respiratory flasks were not adequate to accommodate individual limpets, 275 ml. water sample bottles were calibrated and adapted with a #8 rubber stopper to fit the manometer. Carbon dioxide was absorbed with a filter paper "fan" immersed in one ml. of KOH solution (20% wt/vol). This was contained in an ampule suspended from the stopper with a wire hook. The chamber (figure 1) was secured "champagne-cork" style by rubber bands stretched across the stopper to wire hooks on the chamber necks. To ensure sealing, Hi-Vac stop-cock grease (Dow Chemical) was liberally applied to the stopper.

Shaking of the chamber (to ensure oxygen saturation of the liquid phase) would have caused stress to the limpets. Therefore, the liquid phase was stirred from below with water driven submersible stir motors (Scientific Products) using magnetic stirring bars. The stirring motors were attached to a water supply in series so that ten experimentals and one thermo-barometer flask (to correct for barometric pressure and temperature fluctuation) could be used simultaneously. Individual limpets were placed in each respirometer in just enough filter-sterilized (millipore 0.45 micron) sea water obtained from the collection site, to cover them. The amount of this water was carefully measured and used along with variations in limpet volume in the calculation of oxygen consumption values. Each animal was suspended above the stirring bar in cages constructed of nylon screening (figure 2).

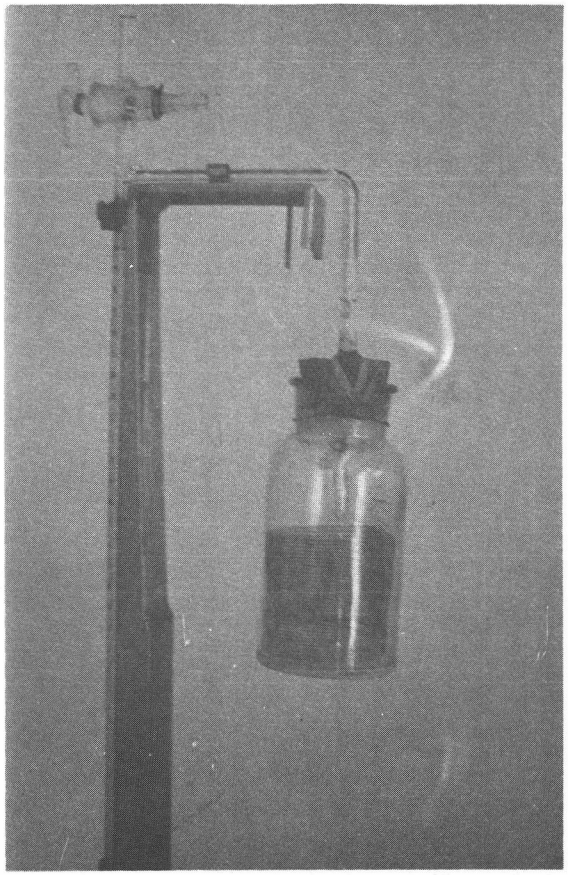

Figure 1. Manometer with attached respiratory chamber.

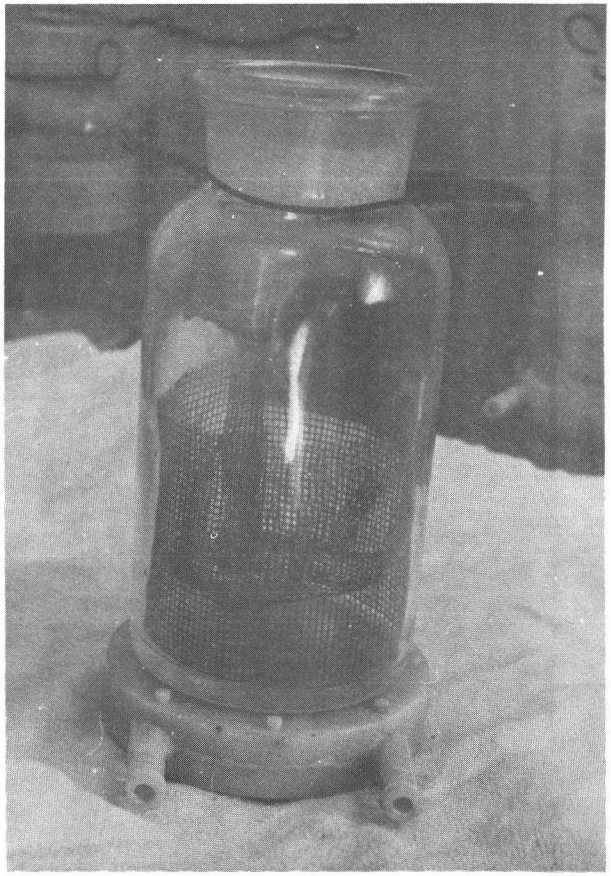

Figure 2. Respiratory chamber with cage and stirring motor.

Once sealed in the chamber, the manometer stop-cock was left open for a one-hour equilibration. After closing the stop-cock, readings of the Warburg manometers were taken every hour. The chambers were re-equilibrated for 15 minutes every three hours. Experiments lasted up to 35 hours.

Data from individual limpets were first changed from pressure to volume units and then expressed as microliters O_2/gram wet weight/hour. The average values for each hour of the experiment were plotted. Peaks of oxygen consumption were compared with tidal height at the same hour to check for any correlation.

Time-Lapse Photographic Experiments

Time-lapse photography was used for longer experimental periods (up to 35 days). Collection and laboratory handling were the same as that in the oxygen consumption experiments, except the limpets were not brushed or cleaned of encrusting organisms, nor were the commensal polychaetes removed.

Seven limpets were gently placed to the front of a 25 gallon aquarium filled with 15 gallons of sea water (30‰). The temperature was equilibrated to 13.5±0.5°C, by a Forma circulating refrigerated water bath. The sea water was placed in the aquarium one week before the experiment to allow the growth of algae on the glass. This would provide food for the limpets during the term of the experiment.

Plexiglass partitions restricted the animals to the same focal plane during the experiment. The water was recirculated from behind the partitions into each compartment by a peristaltic pump.

Photographs of the entire face of the aquarium were taken with a Nikon F 35 mm camera. The camera was equipped with an auto Micro-Nikkor lens and a Nikon F electric motor drive film advance which allowed bulk usage of film (250 exposures) and minimized film changes. A battery-powered timer exposed one frame every 16 minutes. This time was verified against a clock photographed with each frame. Lighting was provided by a Honeywell strobe (1/500 second at 480 B.C.P.S.).

An artificial day-night cycle was imposed using variable indirect flourescent light (22 or 10 foot candles at the aquarium front). Night illumination was less than 0.1 foot candles.

Data Analysis

Each photographic frame was compared to the next by using a film strip projector. Figure 3 shows parts of two consecutive frames taken 16 minutes apart. The first two limpets show no obvious movement of the head from one frame to the next, while the third limpet shows a movement of approximately 45°, which may be associated with feeding. This kind of motion and/or movement of the whole organism from one point to another was used as an activity indicator.

If the head had moved relative to the previous frame, a plus mark was entered in that time slot, a minus if movement was not obvious. If neither determination could be made (shell facing camera), the slot was left blank. Plus marks were summed for all animals in a one-hour period and divided by the total number of all observations for that hour giving a percentage of activity. These data were plotted and compared visually with tidal and day-night cycles.

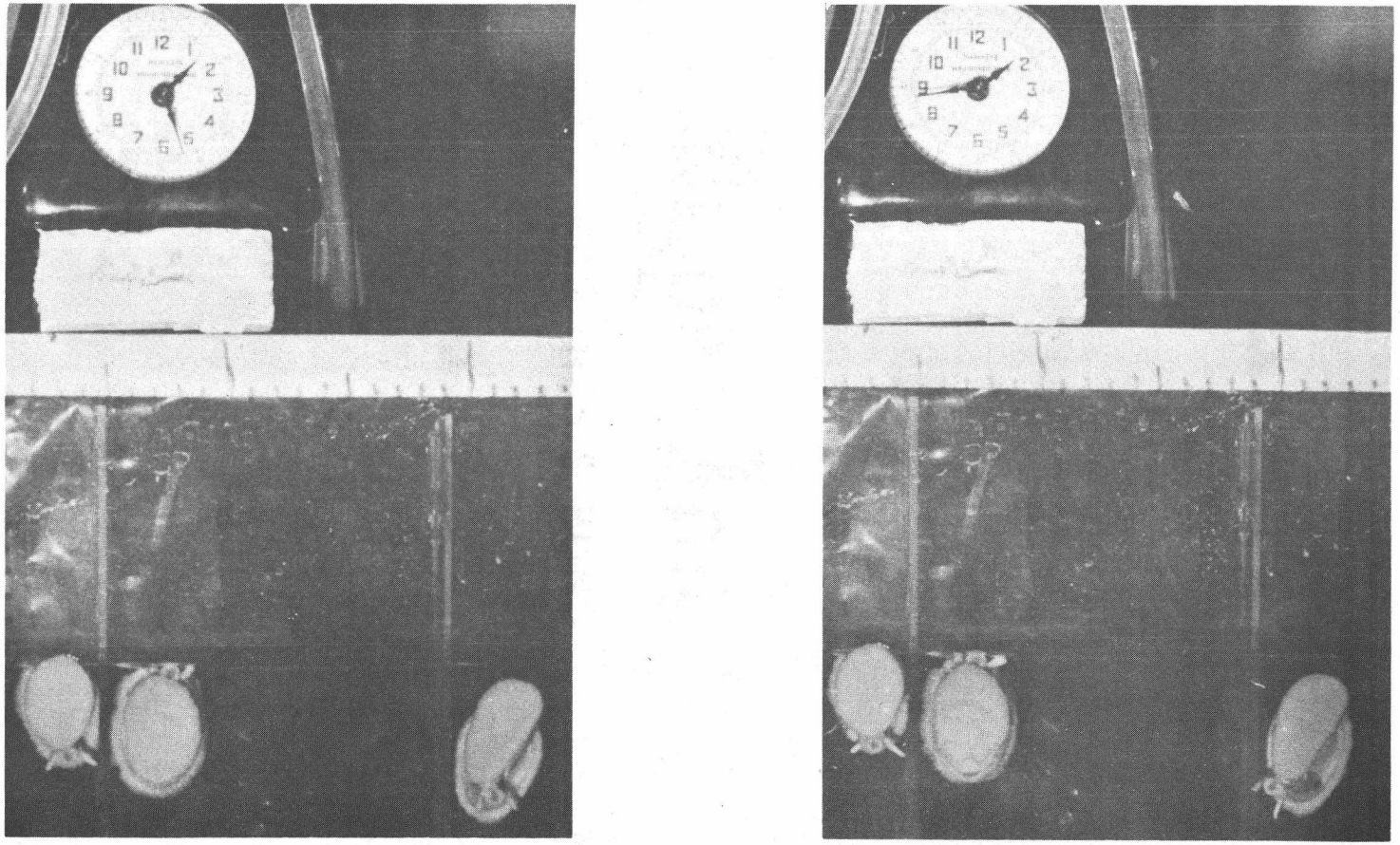

Figure 3. Two consecutive frames showing the head movement of one limpet.

Statistical Methods

Statistical methods were also used to treat the data. Periodogram analyses have been used to determine the presence of "whole-hour" frequencies (f) in activity patterns (Enright, 1965; Williams and Naylor, 1967). A time series of hourly data (X_1, X_2, X_3, ..., X_n) is ordered into groups of desired lengths (most commonly 8-30 hours). Each scan of the data gives 8-30 average hourly activity values ($\bar{X}_1$, $\bar{X}_2$, $\bar{X}_3$, ..., $\bar{X}_f$). These average hourly values were calculated by:

X_1,	X_2,	X_3,	..., X_f
$X_{f\ 1}$,	$X_{f\ 2}$,	$X_{f\ 3}$,	..., X_f
$X_{n-f\ 1}$,	$X_{n-f\ 2}$,	$X_{n-f\ 3}$,	..., X_n
$\bar{X}_1$,	$\bar{X}_2$,	$\bar{X}_3$,	..., $\bar{X}_f$

A high standard deviation in these means for a given frequency (f) indicates that a rhythm of that frequency may be present in activity.

Another method used to determine the possible presence of rhythms was the autocorrelation routine (Spectlc, O.S.U. computer center, Arand Systems). The program does an autocorrelation analysis of the experimental data. The wave form is delayed or lagged and superimposed on the original data series. The average amplitude of the two summed wave forms is plotted. This procedure is repeated using increasingly longer delay times. The plot of this autocorrelation function will tend to zero if the data are non-periodic. If the data are periodic, the autocorrelation will have the same periods that are present in the original data although the wave form will be different.

A power spectrum (measure of dominance versus frequency) is plotted. This is accomplished by generating a series of cosine functions of frequencies, 1/n, 2/n, 3/n, ..., 1/2 (n being the total number of observations in the original data set). These cosine functions are multiplied by the autocorrelation function. The average product of this multiplication will tend to zero if the period of the exploring cosine function is different from any of the periods in the data. If the cosine function is similar to the autocorrelation function, the average product will be greater than zero and some energy of that periodicity is present in the data. The results of these multiplications are plotted against the frequencies (1/period) of the cosine functions. The total number of cosine frequencies examined is termed the spectra while the results of their multiplications are usually termed spectral density (roughly equivalent to amplitude).

Since biological data usually do not follow any absolute periodicity the spectral density at any given frequency is smoothed by averaging the value with spectral densities of adjacent cosine frequencies. Smoothing also reduces the chance of random "noise" being present in the plot; however, with continued smoothing information can be lost. The extent of this smoothing is indicated by a band-width plotted on the spectral density graph.

If a logarithmic scale is used for spectral density, 95% confidence limits are drawn. Given an infinite time series the spectral density at any frequency can be expected to lie within those bounds with 95% confidence. Increased smoothing reduces the width of the confidence limits.

RESULTS AND DISCUSSION

Using time-lapse photography, it is easy to accumulate a large amount of data over a rather short period of time. For this thesis, four major experiments were done in a four-month time span. The experimental periods ranged from 107 to 677 hours. Altogether 1000 feet of film was used for 1536 total observation hours, or roughly 46,000 individual data points.

A typical data sheet (table 1) shows total number of positive activity values for each hour divided by the total number of observations for that hour to determine a percentage. The data from these hourly values plotted against time (figure 4) show the results of one experiment done in the dark (107 hours at 0.1 foot candles), and an experiment done in constant lighting conditions (400 hours at 22 foot candles). Both curves have obvious rhythmic functions which appear to be diel and suggest a day-time pattern of activity. There are obvious differences in the maximum activity values of the two curves. The curve generated in the dark has a much greater amplitude and seems to be less variable than the constant light curve. Constant light has been shown to inhibit some kinds of behavior in animals; e.g., rats show a reduction in activity (Munn, 1950). Although the reverse may be true, constant dark or dim light is less damaging to circa-rhythmic patterns than constant high-intensity light (Bünning, 1964; Harker, 1964).

In both curves (figure 4) the maximum activity occurs near noon but shifts slightly with time. This may suggest that rhythms other

Table 1

Sample Data Sheet

		Animal Number									
Date	Time	1	2	3	4	5	6	7	num.	num./hour	% active
2/5	21:03	+	+	+	+	+	+	+	7		
	:18	+	+	+	+	+	+	+	7	25	89
	:34	+	+	o	+	+	+	+	6		
	:50	+	+	o	+	+	o	+	5		
	22:06	o	+	o	+	+	o	+	4		
	:22	o	+	o	+	+	o	o	3	11	39
	:38	o	o	o	+	+	o	o	2		
	:54	o	o	o	+	+	o	o			
	23:10	o	o	o	o	+	o	o	1		
	:26	o	o	o	o	+	o	+	2	6	21
	:42	o	o	o	o	+	o	o	1		
	:58	o	o	o	+	+	o	o	2		
2/6	00:14	o	o	o	+	o	o	o	1		
	:30	o	o	o	+	o	o	o	1	5	18
	:46	o	o	o	+	o	o	o	1		
	01:02	o	o	o	o	o	o	o	0		
	:18	o	o	o	o	o	o	o	0	0	0
	:34	o	o	o	o	o	o	o	0		
	:50	o	o	o	o	o	o	o	0		
	02:06	o	o	o	+	o	o	o	1		
	:22	o	o	o	o	o	o	o	0	2	7
	:38	o	o	+	o	o	o	o	1		
	:52	o	o	o	o	o	o	o	0		
	03:08	o	o	o	+	o	o	o	1		
	:25	o	o	o	o	o	o	o	0	1	4
	:41	o	o	o	o	o	o	o	0		
	:57	o	o	o	o	o	o	o	0		
	04:13	o	o	o	o	o	o	o	0		
	:29	o	o	o	o	+	o	o	1	1	4
	:45	o	o	o	o	o	o	o	0		
	05:01	o	o	+	o	o	o	o	1		
	:17	o	o	o	o	o	o	o	0	1	4
	:33	o	o	o	o	o	o	o	0		
	:49	o	o	o	o	o	o	o	0		
	06:05	o	o	o	+	o	o	o	1		
	:21	o	o	o	o	o	o	o	0	4	14
	:37	+	o	o	o	o	o	o	1		
	:53	+	+	o	o	o	o	o	2		

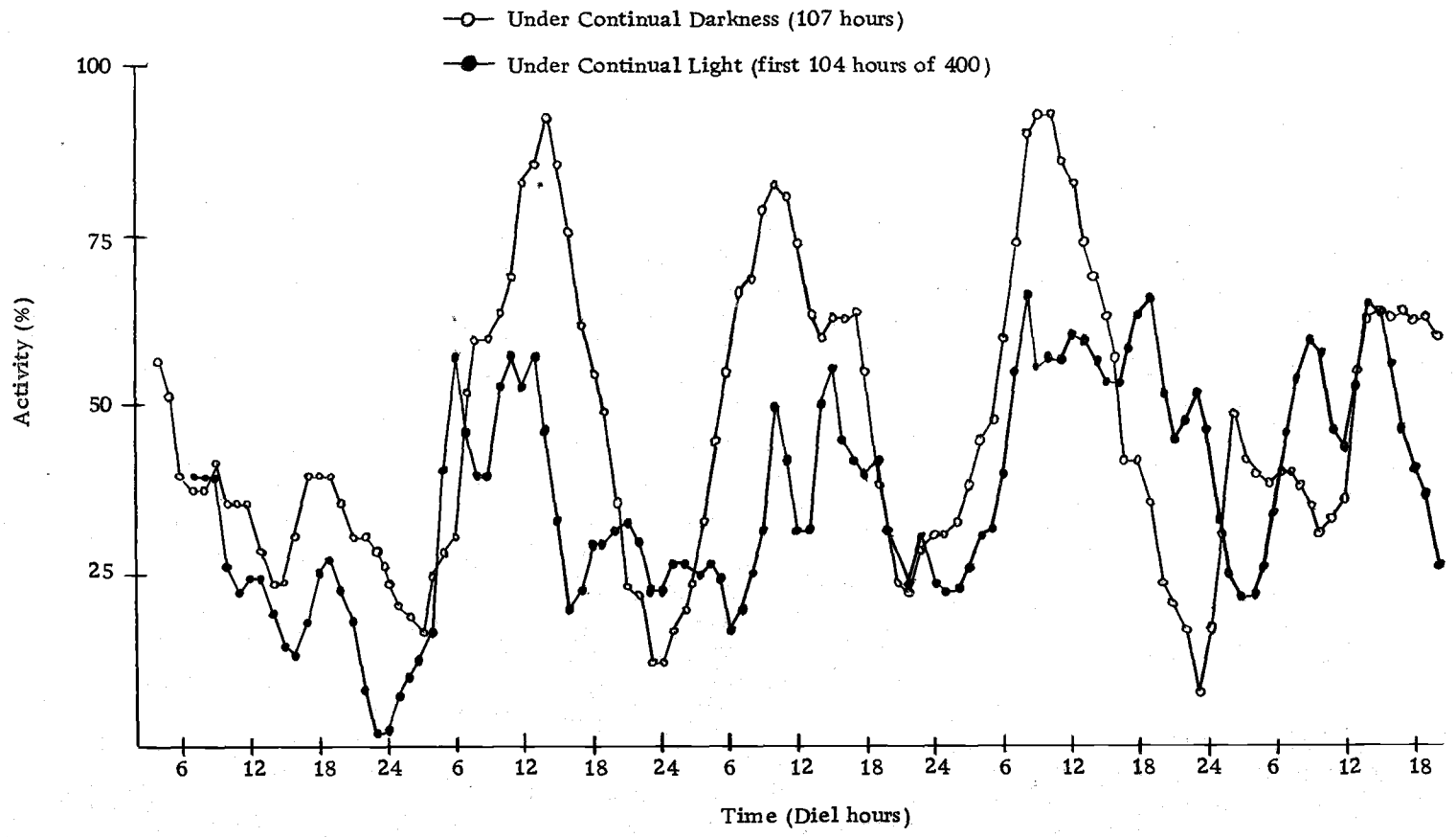

Figure 4. Activity for two groups of D. aspera

than diel are present. Fingerman (1955) attributed such skewed, sometimes biomodal curves in Callinectes sapidus to the action of a tidal (12.4 hour) rhythm.

Several time-series analysis techniques are available to determine the actual periods that may be present in these kinds of data (Steiner, 1954; Mercer, 1960). These techniques may be of questionable value when the periods of interest are not significantly shorter than the duration of the experiment. What is shown by these techniques is not the presence or absence of any one definitive frequency but of bands of frequencies of width 1/T (T total data length). Therefore in order to distinguish a 24 from a 25 hour rhythm a frequency band of width 1/24-1/25 is necessary which means an experimental length of 500 hours (Mercer, 1960).

One problem occurs when using a 16 minute data interval: every 15th hour contains only three data sets (see table 1). Whenever this occurred the last datum set from the preceeding hour was included to even out the sets. Although it is probable that no more artifacts developed than if the 3 sets alone were averaged, any activity pattern of 14-16 hours should accordingly be questioned.

Another problem often encountered with long-term biological data of this sort is that the overtness of the rhythm tends to fade or dampen out under non-periodic conditions, sometimes to the point of appearing arhythmic (Bünning, 1964; Enright, 1963). This effect is shown (figure 5) when experiments were run for 400 hours (about 17 days) in constant light (blanks in figure 5 are the results of camera malfunctions). The first few days showed a diel rhythm peaking about noon,

but after a few days this was no longer clearly evident.

The reasons for this "fading" are unknown. It is possible that in the absence of an external cue, the rhythm of each individual limpet has become free running, each individual responding with a slightly different period. This could cause loss of obvious rhythmicity. Another, more likely, possibility is that the activity indicator is effected by stress due to the unnaturalness of constant conditions and as a result it becomes unstable. Whether or not such "unstable" data can be used in statistical analysis to obtain hidden rhythmic functions has been questioned by Enright (1963) who feels that stability in period and amplitude are necessary for valid results.

One possible way to obtain long-term, overt data is to supply an external synchronizer (zeitgeber). An artificial day-night cycle for example, should keep individual activity patterns from slipping out of phase with those of others and add a stimulus that may stablize the activity indicator. If D. aspera is truly day active (as suggested in figure 4) then a day active pattern should also appear when a diurnal cycle in light is supplied. Rhythms other than diel in D. aspera should also be enhanced by a zeitgeber. Barnwell (1966) and Honegger (1973) have shown that tidal rhythms can be synchronized from diurnal rhythms in light, even though the periods are different.

Two experiments, in which an external day-night cycle was supplied, were attempted. The first was done with the same animals previously used in the 400 hour "lights on" experiment. Soon after the imposition of a day-night cycle 3 of the 7 limpets died. Starvation is one possibility but since the remaining 4 survived 30 more day until their

release, this seems unlikely. This experiment showed no overt periodicity and statistical analysis yielded no further information. Although the data were plotted the results were essentially aperiodic either due to using only 4 animals in the sample or due to physiological stresses. These data have not been included.

Fresh limpets were collected and the experiment repeated. A day-night cycle was imposed (10 hours light at 10 foot candles and 14 hours dark) which roughly corresponded to the natural day-night cycle at the time of the experiment. A portion of the results of this 677 hour experiment is shown (figure 6) and suggests a fairly precise day active cycle of 24 hour period. Auxiliary peaks other than those corresponding to a diel pattern may indicate other rhythmic functions.

A periodogram (figure 7) of the two long-term data sets shown in figures 5 and 6 suggests rhythmic function of 24-25 hours and of 12-13 hours, which may correspond to a tidal rhythm.

As a time series analysis technique, the periodogram has come under criticism because one must assume that the data are genuinely periodic since the effect of random fluctuations is not considered. Because of this, it can show rhythmic function in random data (Cole, 1957). Jenkins (1961) believes that misuse of this technique has led to the acceptance of more misinformation than any other statistical technique.

Another drawback to this technique is that harmonic peaks may occur. In figure 7 the peak at 12-13 hours may be a harmonic reflection of the 24-25 hour peak. If both peaks are real the larger height of the 24-25 hour peak could result from an additive effect.

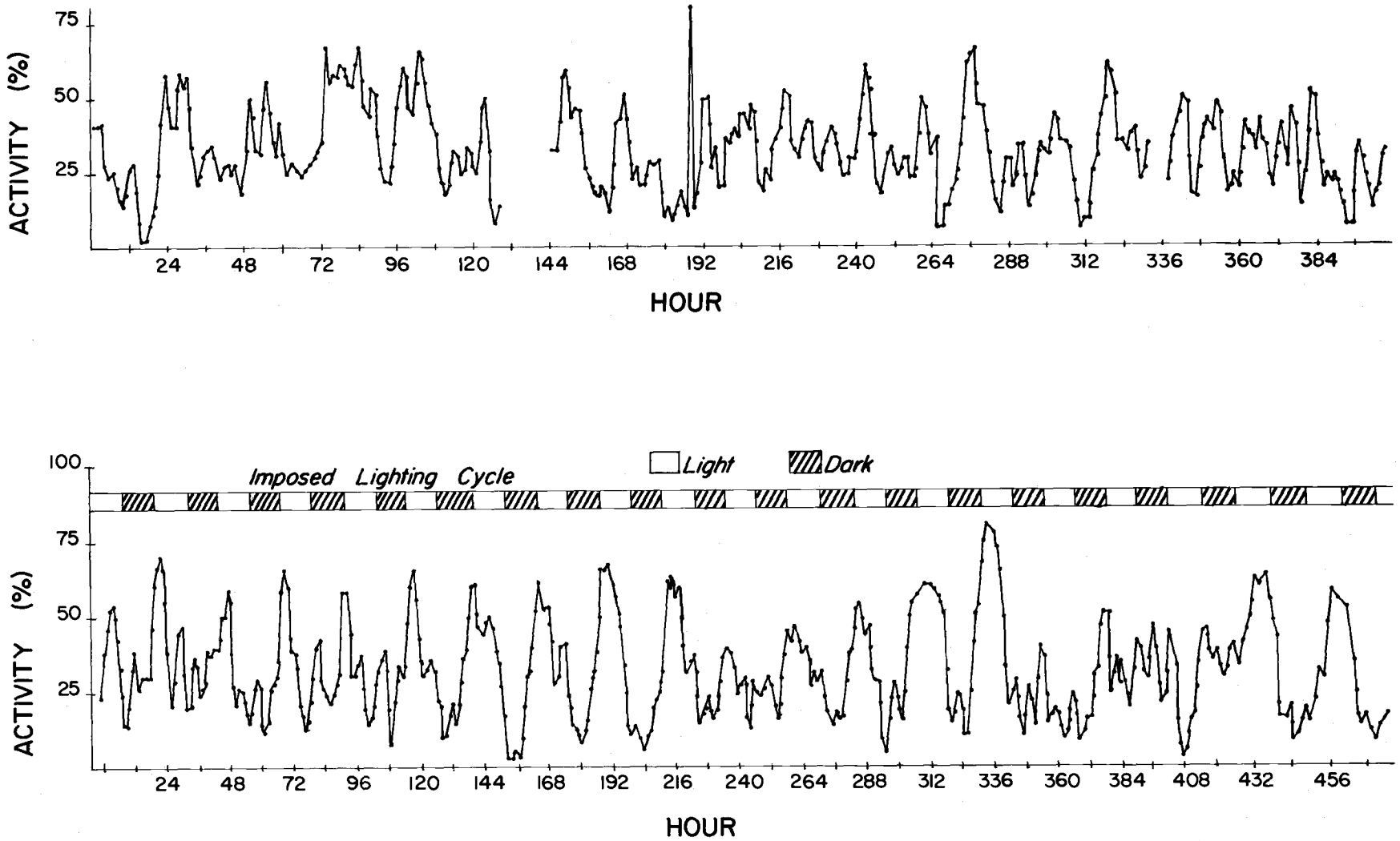

Figure 6. Percent activity of seven D. aspera for 470 hours of a 677 hour experiment done with an imposed day night cycle. (10 hr. light: 14 hr. dark)

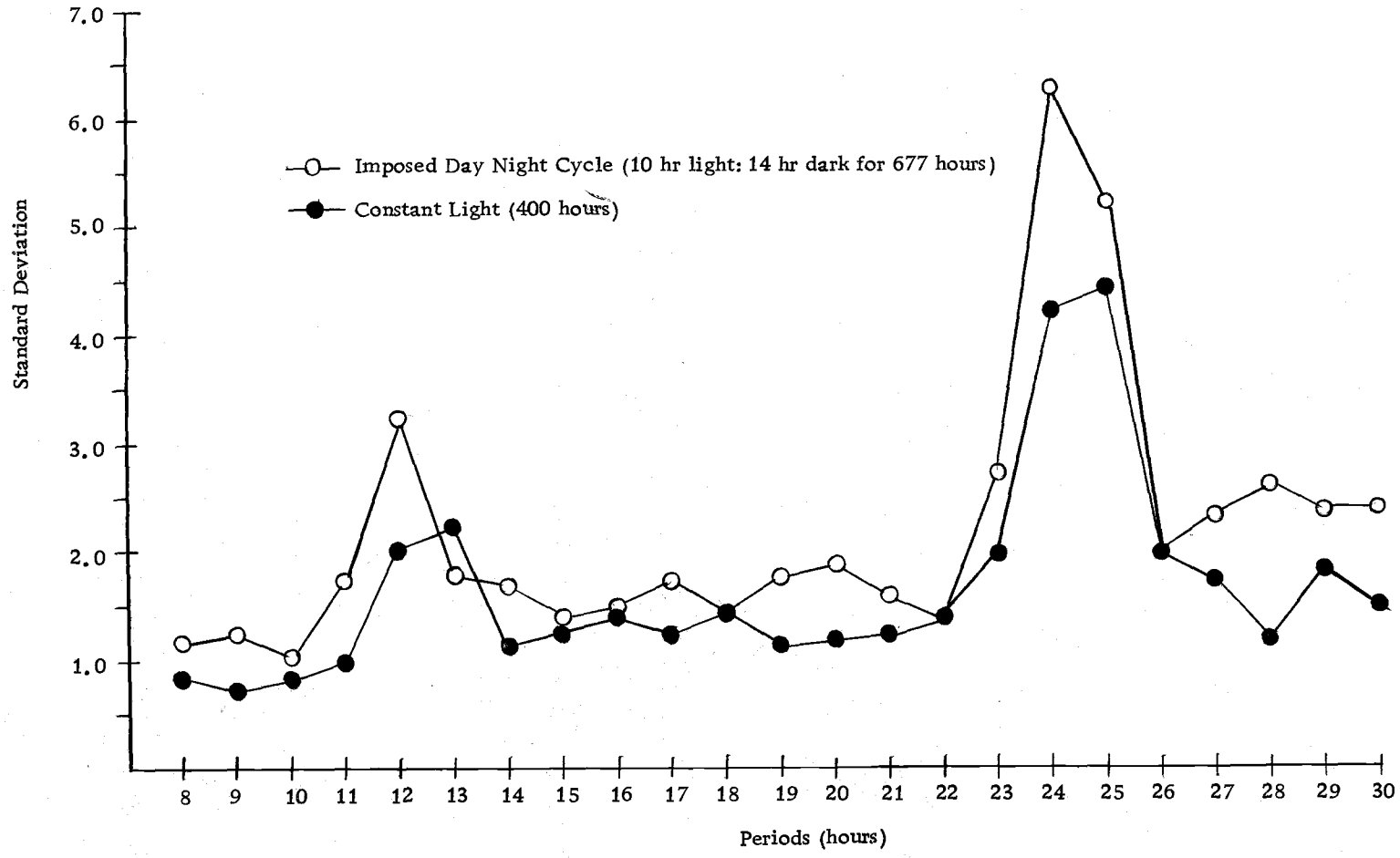

Figure 7. Periodogram of data from two experiments done under different lighting conditions.

In figure 7 there is a gradual increase in the base line of the graph. Since the time over which data are collected is finite, the number of data points decreases as frequencies of progressively longer periods are investigated. For example, for a fixed data length of 677 hours the number of data points for a period of 8 hours would be about 84, while for a period of 30 hours it would be reduced to 22. The standard deviation of the means would increase because fewer data points were used to determine those means. This effect would make investigations of long period functions difficult.

Another (more rigorous) time-series analysis technique which does reduce random fluctuations and eliminates harmonic effects was used. It estimates power spectra versus frequency. Figures 8 and 9 show spectra for a "lights-on" and day-night cycle experiment. Again some energy appears in the 12-13 hour and 24-25 hour ranges supporting the suggestion made by the periodograms. Confidence limits (95%) make the validity of the 12-13 hour rhythm questionable. Figure 8 also shows another longer period rhythm of 40-80 hours. Although resolution of this peak is poor due to the limited time length of the data, it may reflect the times when the film was changed. Spectral energy in excess of 100 hours in figure 9 may or may not be present. Part of this energy peak may also be due to the changing of the film, but since this experiment is only about half the length of the experiment shown in figure 8, the peak is not clearly discernable. Spectral energy below 10 hours was minimal in both experiments and is not shown. Spectral energy at the very low frequencies (near $f = 1/n$) becomes asymptotic and this is not shown.

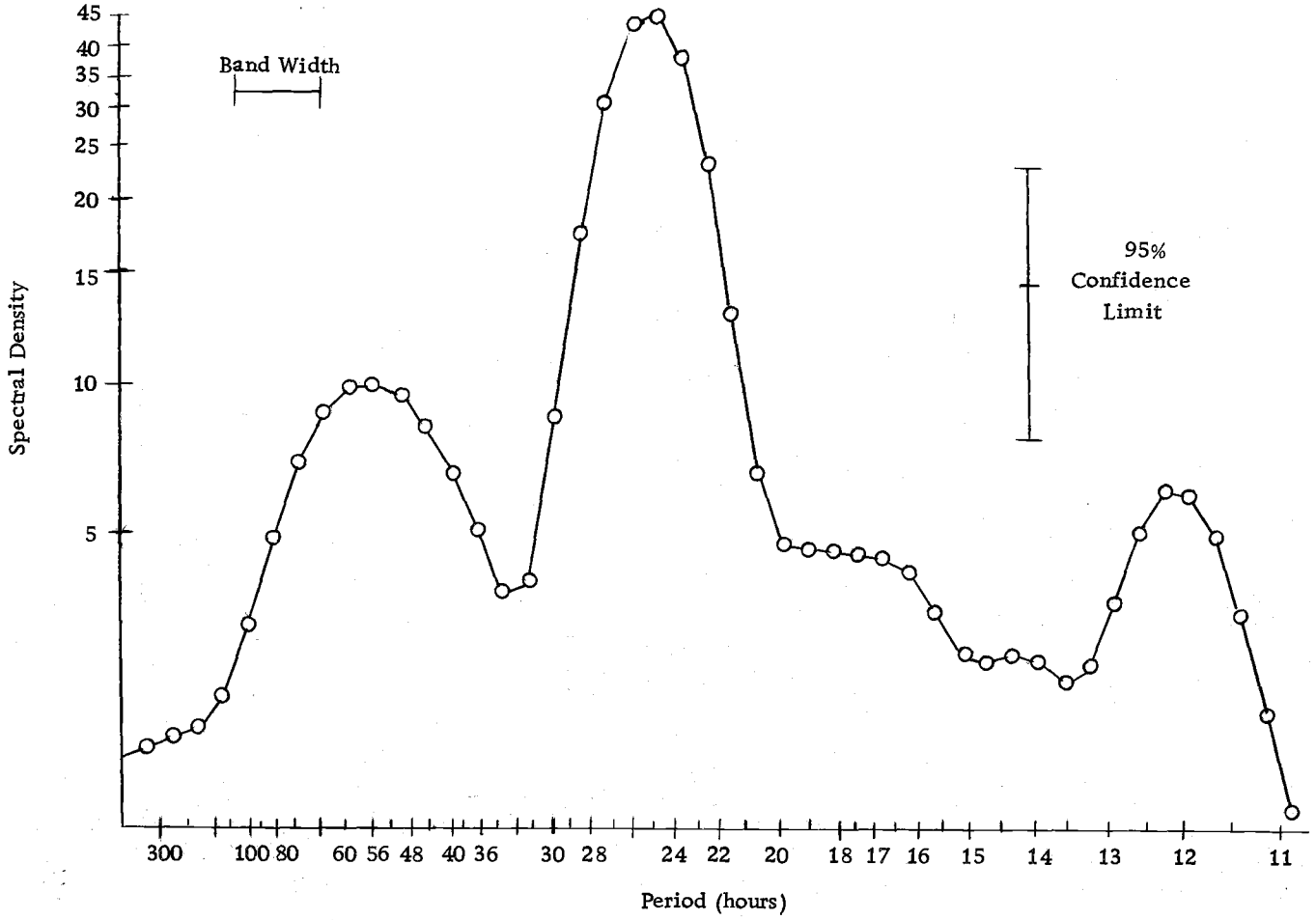

Figure 8. Spectral density at various periods for seven D. aspera under an imposed day night cycle (10 hr light: 14 hr dark for 677 hours).

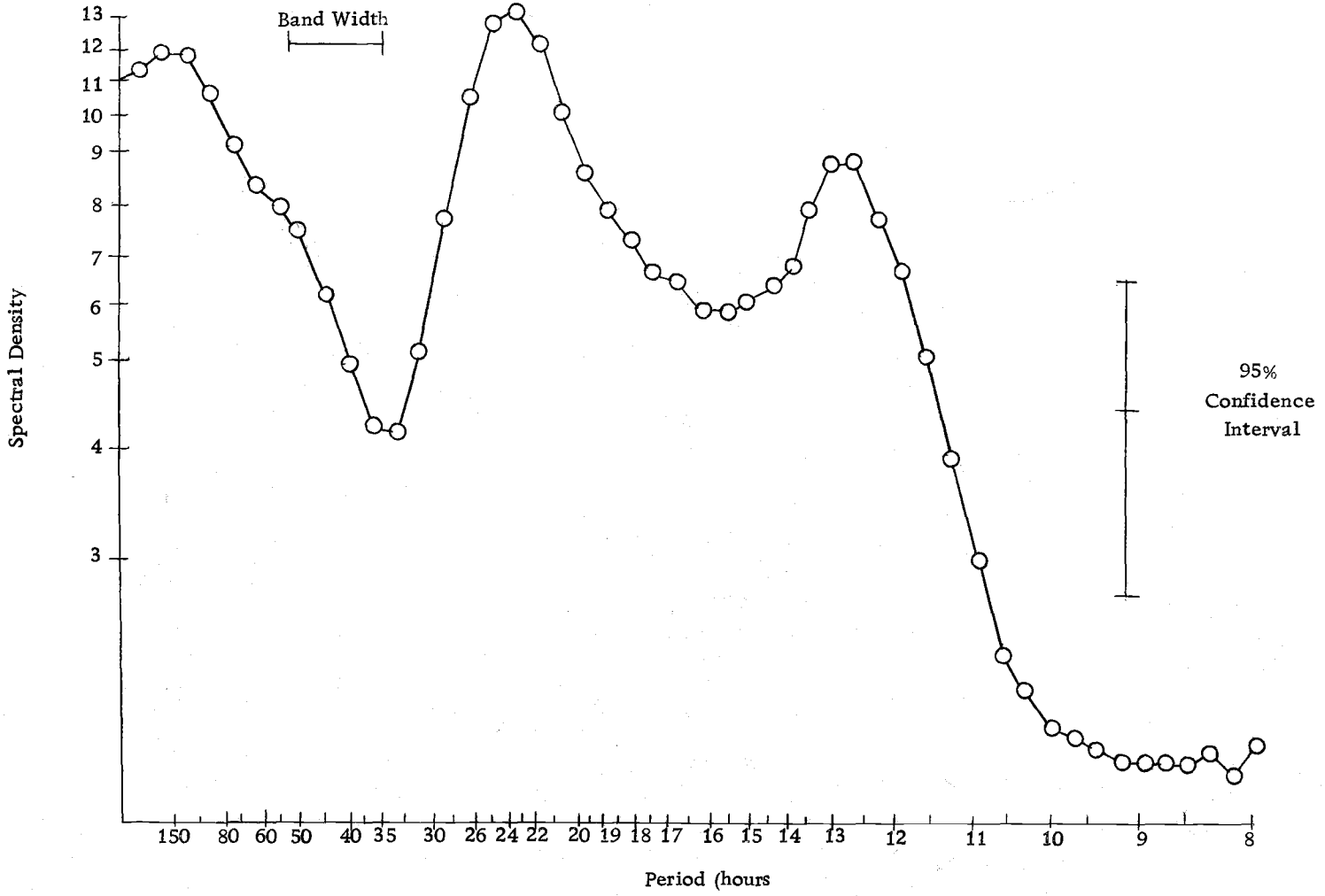

Figure 9. Spectral density at various periods for seven D. aspera under continual light for 400 hours.

Figure 10 shows plots of the smoothed mean results obtained from 10 limpets in three oxygen consumption experiments. Smoothing was done by taking the mean of three consecutive points and plotting the result as the middle value of the set. This was repeated in series down the time series. The results reveal a peak at noon and also a peak of equal amplitude occurring at variable times during the night. The data are extremely short term and the range of values obtained at any given hour is highly variable. Confidence limits (95%) are wide (±15-21% of the mean value). Conclusions based on these results are at best tenuous, but they do seem to substantiate the results of the photographic experiments by showing a noon peak. The variable night peak may correspond to the elusive tidal rhythm. In no case however did the time of maximum oxygen consumption correspond closely with the time of high or low tide. It always occurred on the incoming tide about 3-4 hours before the high. Auxiliary peaks occurring in photographic data also often seem to occur at about 3 hours before the high.

The presence of both diel and tidal rhythms in Diadora aspera is neither surprising nor unique. What is interesting is the phasing. The day-active pattern at first glance would seem to increase the danger of desiccation, but the habitat is such that desiccation is apparently no threat. At Yaquina Head, D. aspera lives low in the intertidal, exposed extensively to aerial conditions only during minus tides on calm days. Since they seem to prefer well-shaded areas (often under vegetation) as well, desiccation is probably not a significant factor.

The primary predator of D. aspera is in all likelihood the sun star, Pycnopodia helianthoides. In experimental aquaria Pisaster spp. have

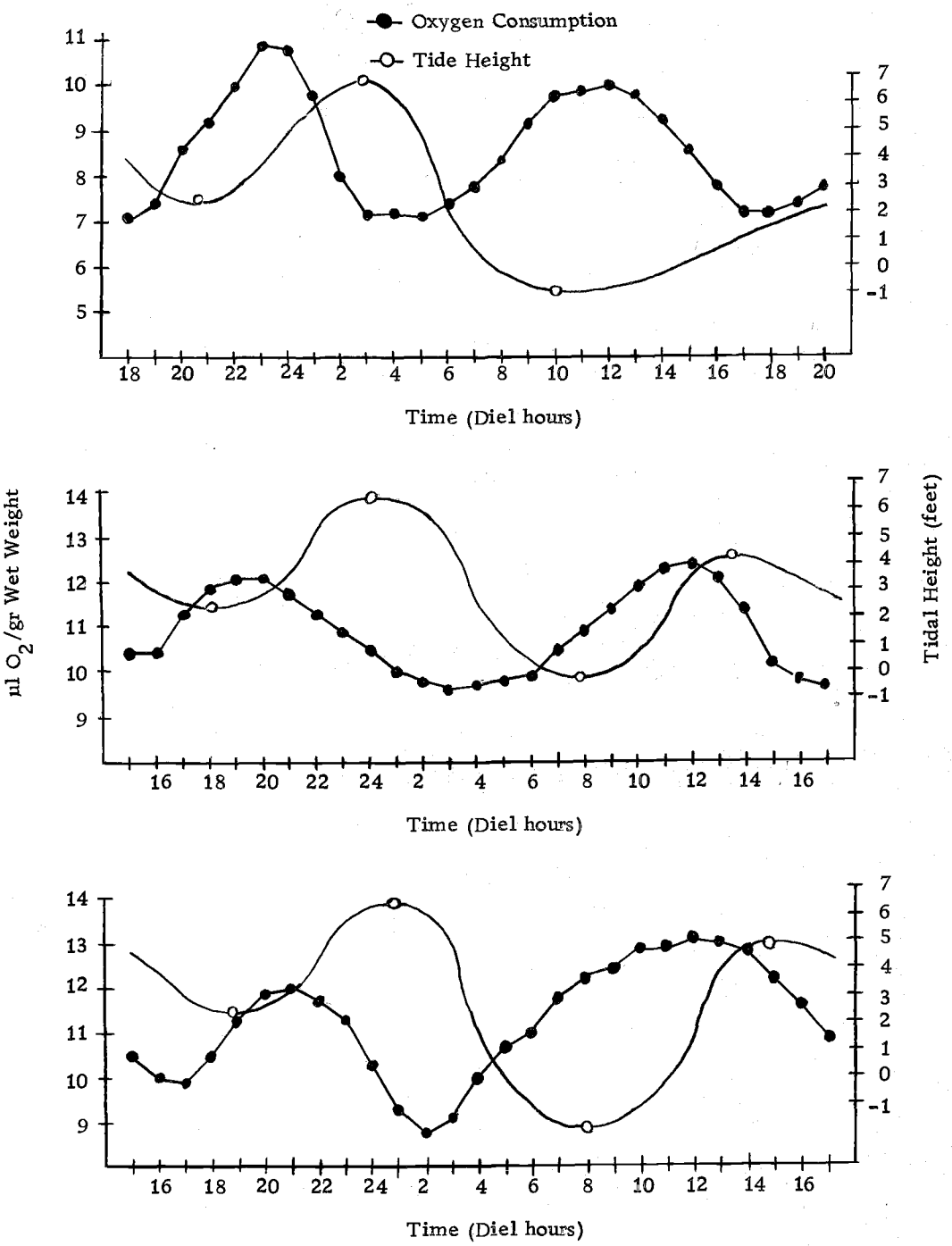

Figure 10. Three determinations of mean hourly oxygen consumption for 10 limpets at 12.5 C compared to tidal height.

been shown to be ineffective in preying upon D. aspera, while P. helianthoides has had little difficulty (Margolin, 1964). Several P. helianthoides have been observed near collection sites of D. aspera at Yaquina Head, usually in shaded tide pools beneath limpets exposed on vertical walls above. It would be interesting to know how the activity pattern of P. helianthoides correlates with that of D. aspera.

The tidal phasing is even more puzzling. Zann (1973) suggested that low intertidal organism may have circa-tidal rhythms that correspond to more subtle factors than desiccation protection, for example wave action at their perferred site or prey-predator relationships. Diadora aspera at Yaquina Head were found almost always in areas of low wave energy. The largest concentration (about 15 limpets) was found in an area surrounded on three sides by high rock walls that overhung the collection site. Waves broke on the opposite side of the rocks and water flowed around behind the rocks to the limpets.

In the aquarium, limpets spent almost all of their time at the air-water interface. When active the shell is lifted and gapes back and above the interface probably indicating utilization of both aquatic and aerial respiration. This same gaping posture was observed among limpets collected near the water surface. In the environment they may follow the air-water interface which may help explain the confusing phasing of the tidal rhythm.

To show conslusively the presence and phasing of these rhythms, one needs long term experiments with numerous limpets. The "artifact" that seems to occur at 40-80 hours (figures 8 and 9) suggests that slight disturbances may cause activity that is measurable, especially

if the disturbance is periodic. To assure unbiased results, no extraneous disturbance should be allowed. On this basis the use of a strobe light for taking photographs can be questioned. The duration and intensity is at best unnatural and it is possible that the periodic flashes could function as a zeitgeber. However, it is difficult to conceive of a 16 minute, or multiple of that, being reflected in the rhythms observed here. Some distrubance might have been avoided if infra-red strobes and film were substituted, but the effect of infra-red light is not presently known for this organism. This possibility was suggested after about half of my experiments had been completed, but the cost of changing techniques and the time lost could not be justified.

Zann (1973) has successfully used field observations of other limpet species in studying the relationship between habitat and circatidal rhythms. With D. aspera several things suggest that activity might have been impractical to measure in a field situation. Observations of limpets in a relatively unconfined aquarium space suggests that the average distance traveled was at most a few centimeters per day. In time-lapse experiments some limpets appeared not to move at all for 4 or 5 days while their heads changed position cyclically. Diving in those waters would have been dangerous due to the proximity to large often over-hanging rocks and tricky cross currents. With low concentrations of limpets and their lack of rapid mobility one would be forced to use subjective judgements of activity which would not justify the effort.

The dominance of the diel rhythm in the photographic data makes it difficult to detect the presence and phasing of a tidal rhythm. Short term experiments suggest that oxygen consumption may be a much better way to explore this rhythm. However the high variability in oxygen-consumption rates suggests the need for taking very large numbers of measurements.

SUMMARY

1. Diadora aspera has an approximately 24 hour (light) and a 12-13 hour (possibly tidal) pattern of locomotor activity in the laboratory under non-tidal conditions.
2. The phasing of the diel rhythm in locomotion is day active with the peak about noon.
3. Short-term oxygen-consumption data substantiate the noon peak and suggest that the tidal rhythm is peaked 3-4 hours before the high.
4. Phasing of circadian and circa-tidal rhythms and the habitat of D. aspera suggest that desiccation is not a selective factor in behavior.
5. Protection from predation is proposed as a possible selective factor for the phasing.

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