Chapter 14

ANALYSIS OF BIOPERIODICITY IN PHYSIOLOGICAL RESPONSES[1]

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I. BIOPERIODICITY

Bioperiodicities (rhythms) in biological phenomena have long been recognized and studied in order to gain insight into the dynamics of living organisms. Rhythms are ubiquitous phenomena which occur at all levels of biological organization and are present in subcellular units, cells, and tissues and in the organism as a whole. A rhythm has been defined as a sequence of events that repeats itself through time in the same order and at the same interval. Often, physiologic rhythms have been ignored (viewed as an epiphenomenon of homeostasis) and/or attributed to random error of measured responses. Table I compares the homeostatic view with the chronobiologic view of biology. If functional rhythmicity is present in an organism, ignoring it could impair the interpretation of physiological experiments.

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TABLE I
TWO VIEWS OF BIOLOGY

Homeostatic	Chronobiologic
Regulatory mechanisms maintain constancy of "internal milieu"	Physiologic changes recur with reproducible waveform
Time ignored as source of variability	Reference time markers are essential
Steady state	Biorhythm

Bioperiodicity is an integral part of physiological responses to nutrients and diets. Recently, Halberg has stated "In the science and practice of nutrition today, the provision of a control requires the assessment of a multifrequency rhythmic structure" (Halberg, 1989). A mathematical model, such as the cosinor model of Halberg, can be used to establish the occurrence of rhythms and quantify the rhythm characteristics of components identified by spectral analysis.

II. CHARACTERIZATION OF BIOLOGICAL RHYTHMS

According to the approach pioneered by Halberg, deterministic, biological rhythms (i.e., chronobiologic rhythms) have four measurable parameters: the mean, amplitude, acrophase, and period (Pauly, 1980). These are shown graphically in Fig. 1.

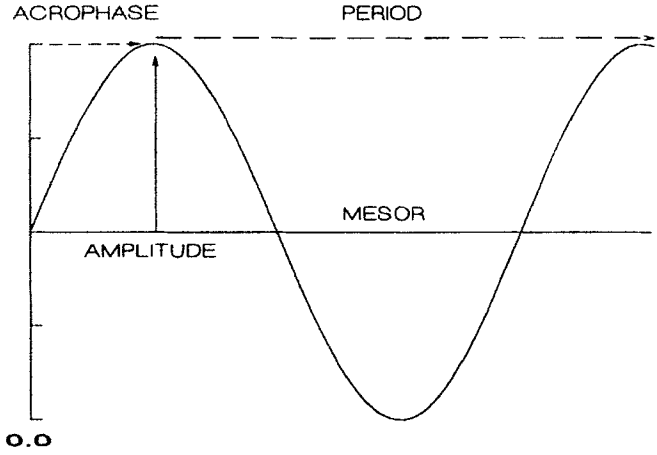

FIG. 1. A cosinor curve showing the various parameters of response.

The mean of a rhythm is the average value of a continuous variable over a single cycle. When the rhythm is described by the fitting of a cosine curve, the half way point between the peaks and the troughs is known as the MESOR. Only when the data are measured equidistantly, over an integral number of cycles, will the MESOR equal the arithmetic mean.

The amplitude refers to the magnitude of the response variable between its mean value and the (estimated) trough or peak. Such mathematical usage, however, is limited to rhythms which oscillate symmetrically about the mean value.

The phase refers to the value of a biological variable at a fixed time. The word phasing is often used to describe the shape of a curve that depicts the relationship of a biological function to time. Acrophase is a more limited term which refers to a specified reference standard or zero time and indicates the lag in the crest of the function used to describe the rhythm.

The period is the duration of one complete cycle in a rhythmic function and is equal to 1/frequency.

Haus and Halberg (1980) have further categorized rhythms (by time frame) as infradian, circadian, and ultradian. Circadian rhythms are the rhythms that have been studied most extensively and have periods in the range 20–28 hr (therefore, frequencies are about 0.04 cycles per hour). There are many examples that can be cited, including rhythms in mitotic activity, metabolic processes, and susceptibility to drugs.

Infradian rhythms have periods longer than 28 hr and therefore their frequencies are correspondingly lower than circadian. Some of the well known infradian rhythms are the human menstrual cycle and the annual reproductive cycle of salmon. Infradian rhythms have been identified in nutrient intake and metabolism of foodstuffs (Reinberg, 1983). A more specific type of infradian rhythm is the circasemiseptan (period approximately 3.5 day) found by Schweiger *et al.* (1986).

Ultradian rhythms have periods shorter than 20 hr. Examples of these rhythms are the electrocardiogram, respiration, peristalsis in the intestine, etc.

Rhythms may also be categorized as exogenous and endogenous (Pauly, 1980). The exogenous rhythm can be caused, driven, and/or coordinated by a force in the environment, but disappears when the driving force ceases. The endogenous rhythm has an intrinsic mechanism and its coordination lies at a cellular level, such as transcription of DNA. Rhythmicity of phospholipids, RNA, DNA, glycogen content, and mitosis has been demonstrated by Halberg *et al.* (1959). Endogenous rhythms have periods similar to, but statistically different from, their environmental counterparts. Those external influences (environmental factors) which are capable of entraining a rhythm are referred to as synchronizers (Minors and Waterhouse, 1981),

and their manipulation can reset the phase of rhythms. Several environmental factors, such as light/dark cycles, sleep/wakefulness, timing of energy intake, and, presumably, qualitative dietary factors, may act simultaneously or separately on a given physiologic variable. One or the other of these external synchronizers may be dominant for the timing of the rhythm of a given function, but not for others. After a change in the synchronizer schedule, the adjustment of a rhythm to the changed environmental routine will occur with a different rate for different variables (Haus and Halberg, 1980). However, if the external synchronizer disappears, the endogenous rhythm will not disappear and will take on a characteristic called "free running." Our goal in this manuscript is to demonstrate the protocols necessary for time-based analysis of weight gain in rats. The techniques can then be applied to other responses.

III. EXPERIMENTAL CONDITIONS

A typical experimental design might follow one carried out in our laboratory. Ten weanling rats, (Sprague–Dawley, Indianapolis, IN) of weight range 38–60 g, were fed a nonpurified diet (Purina rodent chow No. 5001, Purina Mills, St. Louis, MO) for 2 days to acclimate after shipping. The rats were then fed a diet containing normal levels of dietary protein (25% casein). All animals were housed and fed in the animal care facility of the Division of Laboratory Animal Resources which is fully accredited by AAALAC. The rats were singly housed in suspended, wire-bottom cages and were given water (purified by reverse osmosis) and diets *ad libitum*. A 14:10 light–dark cycle was maintained (light on 0600 to 2000 daily). Daily activities were carried out in the room by animal care personnel. Rats were weighed every day at the same time of day on a time integrated Sartorius balance (Brinkmann Instruments, Westbury, NY) interfaced to a computer (Dell, Austin, TX). Food intakes were calculated as disappearance from food cups with adjustments made when necessary for spillage. Since rhythm detection requires sampling involving (preferably) more than a few cycles, a long-term experiment was carried out (45 days) (Mercer *et al.*, 1993).

IV. EVALUATION OF RESULTS

A. DAILY WEIGHT GAIN AND DAILY FOOD INTAKE ANALYSIS

Daily weight gain rates (dW/dt) and daily food intake rates (dF/dt) for each experimental diet were calculated as

$$dW/dt = W_t - W_{t-1}$$
$$dF/dt = F_t - F_{t-1},$$

where W_t and W_{t-1} denote rat's weight at times (t) and $(t-1)$, respectively. F_t and F_{t-1} represent the food weight at times (t) and $(t-1)$, respectively. We used dW/dt and dF/dt (rather than cumulative weight gain and food intake) to observe the daily rhythms.

B. ANALYSIS OF RHYTHMS

The first step in any analysis to detect the presence of a rhythm in a response variable measured over time is to plot the data as a function of time in rectangular coordinates. This often reveals the empirical properties of a time series, including confounding features such as trend (long-term change in the mean). All time series, statistical analyses, and graphics in this study were performed using the PC-based statistics/graphics program SYSTAT (SYSTAT, Inc., 1800 Sherman Avenue, Evanston, IL 60201) and its companion time series program, MESOSAUR.

Statistical analysis, such as a two-way analysis of variance may be used to test whether differences between values at various times are significant. However, statistical analyses do not provide any information about the shape, phase, amplitude, or mean level of the rhythm; they merely indicates whether the data are different from random variation. In order to quantify rhythm parameters, other mathematical techniques, such as Halberg's cosinor model, are required. Our hypothesis assumes that the measured data follow a deterministic series model. Deterministic series are obtained when successive observations are dependent variables and any future values may be predicted from past observations (Chatfield, 1975).

The cosinor method is based upon the least-squares regression of a cosine function of the form

$$g(t) = M + A \cdot \cos(\omega t + \phi) + e(t), \tag{1}$$

where $g(t)$ is the value at time (t) of the regression function (more cosine and sine terms may be added if necessary to describe the response). M, A, ω, and ϕ denote the mean level (termed the MESOR), amplitude (half the range of oscillation), angular frequency (radians per unit time), and phase of the periodic variation, respectively. $e(t)$ is an error term assumed to be an independent random variate with mean 0 and unknown variance σ^2.

The angle $(\omega t + \phi)$ is measured in radians. Some authors refer to the frequency as the number of cycles per unit time $(\omega/2\pi)$. This form of

frequency is much easier to interpret. The period (τ) of a sinusoidal cycle is equal to $2\pi/\omega$ or $1/f$ (unit time per cycle).

A complicating factor in the analysis of growing rats is that growth is not constant but follows a trend, which must be accounted for (i.e., removed) before analysis for bioperiodicities. Figure 2 shows the data from the experiment. A definite downward trend is noted.

Trends must be removed before proceeding with the analysis. Often, a simple function (growth equation) such as a logarithmic, logistic, or parabolic equation will suffice to predict the trend. Coefficients can be estimated from logarithmic, logistic, or parabolic analyses. Observed time series data are then fitted to the trend equation, residuals are recorded, and parametric analysis (for τ, M, A, and ϕ) is carried out on the residuals of the detrended data. Trends can also be removed by "differencing" the data, i.e., replacing observed values by the difference between each value and the previous value. This approach has the advantage of not requiring an equation to "fit" the data and parametric analysis can be carried out as usual by regression. Figure 3 shows the data from Fig. 2 after being detrended by differencing.

After detrending, continuation of the analysis requires converting the observed time-dependent data to the frequency domain. This is carried out by subjecting the data to a fast Fourier transform. Then it is possible to determine a period (if it exists) for the suspected waveform. This process

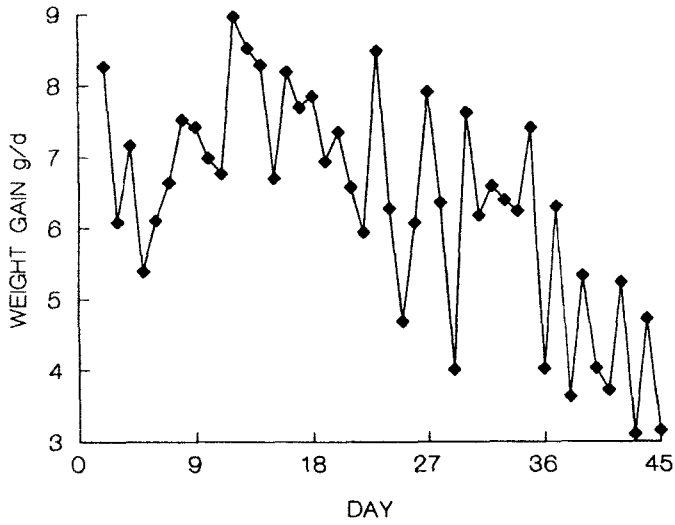

FIG. 2. Observed responses (points connected by solid line). Each point is the mean of 10 rats.

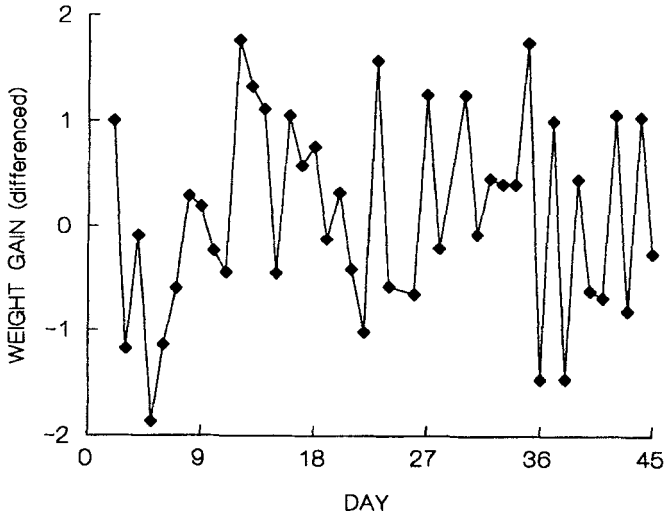

FIG. 3. Observed responses after being detrended (points connected by solid line). Each point is the mean of 10 rats.

involves spectral analysis or, more simply, a periodogram (Fig. 4) which estimates the spectral density by plotting the square of the magnitude against frequency. This can be readily done with a computer program such as MESOSAUR. After determination of the period (τ), the frequency of the periodic variation (ω) can be calculated using the equation $\omega = 2\pi/\tau$. M, A, and ϕ are estimated from regression analysis after replacing ω in Eq. (1) by a numerical value. The cosinor model assumes that the τ (period) can be anticipated *a priori* based on some knowledge of the biological system being analyzed.

One caveat about a period length. It could be a valid parameter or an artifact of the sampling rate; i.e., frequencies ($1/\tau$) higher than 0.5 can appear at lower values due to the phenomenon of aliasing. For example, a 2-day period is close to a multiple of a 1-day sampling rate. Sampling at a higher rate (i.e., weighing rats two or more times a day) would be required to determine the validity of a proposed 2-day period in rats weighed once a day.

After determination of τ from the periodogram, M, A, and ϕ can be estimated by standard nonlinear regression techniques. An equation for calculating the parameters of "daily weight gain" could take the form (based on Eq. (1)): $dwg = M + A \cdot \cos(2 \cdot \pi/\tau \cdot \text{day} + \phi)$ where dwg is the detrended data. Using $\tau = 3.64 \pm 0.089$ from the periodogram of the experimental data, nonlinear regression gives $M = 0.06 \pm 0.147$, $A =$

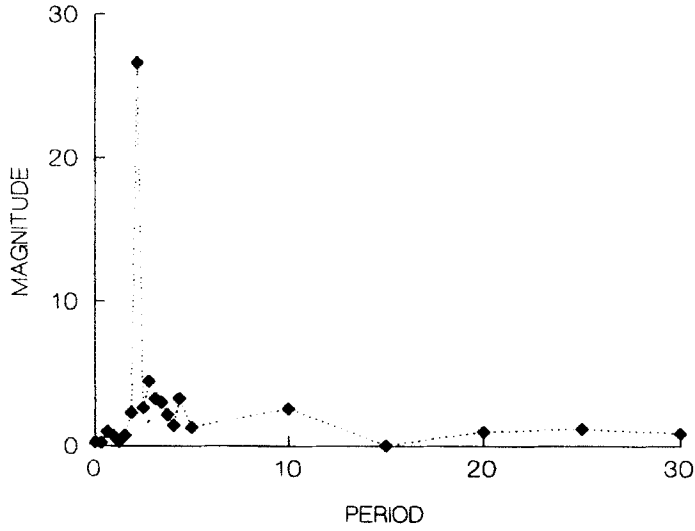

FIG. 4. Periodogram of data after fast Fourier transform. A strong peak indicates a significant period (τ) of approximately 3.5 days.

0.57 ± 0.202, and $\phi = -0.99 \pm 1.038$. M is approximately zero (as expected) since the data has been detrended. To get back to the original, observed data (with its trend), M must be stated as a function of time, $M = f(t)$, rather than carried as a constant. Therefore, the least-squares regression of a cosine function for growing animals can be represented by Eq. (1), with M replaced by a trend functions mentioned above. We then fit the exponential equation $M = (7.29/(1 + \exp(-5.92 + 0.14^* \text{ day})$. This can be done by choosing appropriate equations and accepting the one giving the lowest sum of squares. Using this equation for M produces Fig. 5.

V. DISCUSSION

Progress in the chronobiology of nutrition is impeded by the attitude "any changes around the mean physiological response should be viewed as random variability," leading scientists to consider observed variation merely as an epiphenomenon of homeostasis.

However, application of an established criterion, called the Shannon criterion, indicates the presence of an oscillation based on at least two measured points in each period (Shannon and Weaver, 1963). The results of our experiment calculated for rats either as individuals or in groups

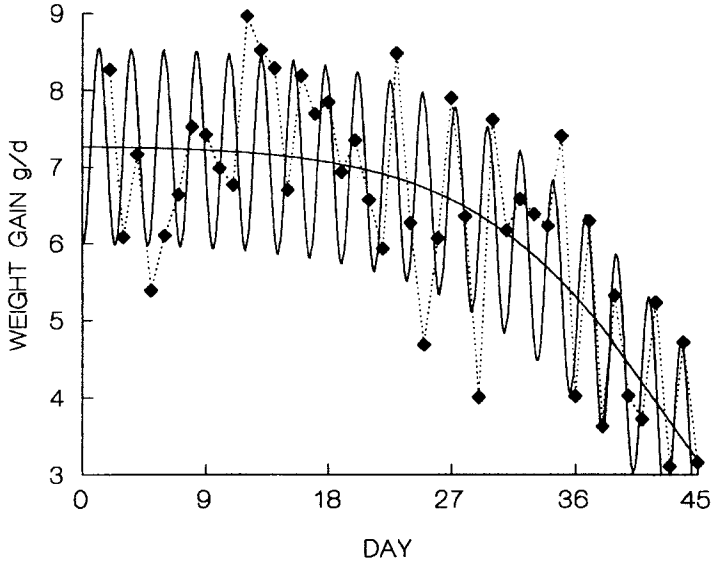

FIG. 5. Data with predicted cosinor curve.

indicate the presence of infradian rhythms (period greater than 28 hr) in growth rates. We were initially concerned that bioperiodicity might be masked by averaging responses on nonsynchronously growing rats. This did not seem to be the case in the reported experiment as indicated by analysis on individual rats compared with groups of rats.

One possible clinical application of the convergence of the disciplines of bioperiodicity and nutrition is in the area of eating disorders. Eating disorders are widespread in the United States and a clearer understanding of fundamental physiological mechanisms is essential in dealing with this problem. Single meal weight loss regimens could interact with biological rhythms of weight gain, yielding significantly different outcomes. Studies showed that when diurnally active human subjects are restricted to a single meal per day, they lose weight if the daily food (8386 kJ) was consumed as breakfast. On the other hand, when the same amount of food was consumed as an evening meal, the subject showed—on the average—either a statistically significantly smaller loss or else a weight gain (Halberg, 1989).

It appears that energy consumed at one stage of a cycle is not the same metabolically as one taken at another. Bioperiodicity of physiological responses is well documented in several species (Stolz *et al.*, 1988; Marks *et al.*, 1978; Kersten *et al.*, 1980; Schumann and Haen, 1988; Li and Anderson, 1982; de Castro, 1991). Optimal nutrition thus requires not only a consider-

ation of what food is consumed, but of when it is consumed. The timing of food intake may be helpful in controlling body weight or, perhaps more importantly, may allow to optimally utilize a limited amount of food available at times of scarcity.

In conclusion, periodicities in growth rates display infradian rhythms and these rhythms appear to be endogenous. Chronobiological analysis gives new insights into relationships between nutrition and physiological response. However, certain procedures interfere with recognition of biorhythms: use of purified rather than integrated samples, use of large samples which appear to produce synchronous activity, and the search for homeostasis rather than perturbation. Recognition of these factors will improve understanding and interpretation of many biological experiments.

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