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# The Precision of Circadian Clocks: Assessment and Analysis in Syrian Hamsters

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## ABSTRACT

Locomotor activity recordings of Syrian hamsters were systematically analyzed to estimate the precision of the overt circadian activity rhythm in constant darkness. *Phase variation*, i.e., the standard deviation of phase markers around the regression line, varied with the definition of phase. Smallest phase variation was found in the onset of wheel running activity defined by 1h running means of the raw data. Both lower and higher degrees of smoothing lead to decreased precision measured in the overt rhythm. With passive infrared recordings, the midpoint of activity defined by 3h running means was the least variable. This demonstrates that the choice of phase marker should vary between recording methods. Phase variation decreased with increasing activity and was larger in females than in males. By calculating the average *cycle variation* and serial covariance of consecutive cycles, we estimated the contribution of 'clock' and 'non-clock' related processes to the overt rhythm variability. Variance in precision between phase markers could be shown to be attributable mainly to nonclock processes. Variance in pacemaker cycle length appeared reduced in wheel running activity records compared with passive infrared sensing records, suggesting feedback from running activity onto pacemaker function.

*Key Words:* Circadian rhythms; Precision; Running wheel activity; Syrian hamster.

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## INTRODUCTION

The standard deviation of consecutive circadian cycles is not a popular parameter. Chronobiologists reporting on free-running circadian rhythms measure the average cycle length ($\tau$), but rarely its variance. Yet precision, the reciprocal of the standard deviation, possibly reveals interesting properties of the underlying rhythm generator. Precision has been proposed to reflect (a) the waveform of the endogenous pacemaker (Aschoff et al., 1971), (b) a selective premium on endogenous periods close to the zeitgeber period (Pittendrigh and Daan, 1976b), and (c) the number of neuronal elements forming the pacemaker (Enright, 1980). These are all inferences about fundamental properties of the generating oscillator. We know that the variance on cycle length is partially attributable to this oscillator but also partially to processes downstream from the oscillator. The variance can be empirically partitioned into pacemaker-related variance and ancillary (downstream) processes on the basis of the serial correlation between successive cycles (Pittendrigh and Daan, 1976a). Thus, increased variance of the overt rhythm does not necessarily reflect increased variance of the pacemaker. Different phase definitions applied to the same overt rhythm yield different standard deviations (Morin and Cummings, 1981).

The definition of phase markers is of decisive importance for the assessment of precision. Investigators have employed very different markers even in the case of the onset of activity in standard activity rhythms. Often, these are defined in a somewhat arbitrary manner, e.g., by selecting the first 5min bin in which activity attained 20% of maximal activity in a given cycle (Scarbrough and Turek, 1996) or by estimating the fifth percentile of the number of minutes with activity during 15h of a daily cycle (Morin and Cummings, 1981). Such approaches involve some sort of smoothing or bandpass filtering to reduce unwanted variance. We need to know how such definition of phase markers affects the measure of precision under study.

Selection of the onset or end of activity or measures of central tendency further affects the conclusions. Aschoff et al. (1971) have pointed out that the precision of onset and end of activity vary in opposite manner with light intensity and with spontaneous mean cycle length as affected by light intensity. Under standard conditions of constant darkness the precision of the daily onset of activity time is often greater than that of the end of activity. Researchers have therefore generally agreed on the use of activity onset as a phase marker. Little is known on several other phase markers such as midpoint of activity [promoted especially by Aschoff (1965)] or the center of gravity [introduced as a circadian phase marker by Kenagy (1980)].

A further factor potentially affecting analytical results is the recording method, e.g., running wheel revolutions, light beam interruptions, or body temperature transmission. While the high precision of activity onset seems typical for wheel running records, other methods may well lead to other maximum-precision phase markers.

We have attempted to improve our understanding of the effects of recording method and phase definition on both the cycle-to-cycle variance in the overt rhythm and on the partitioned variance in underlying pacemaker period. For this purpose we have systematically investigated 10 different markers and their dependence on circadian period, using Syrian hamsters. A large data set of freerun records in DD was available, both from running wheel and passive infrared recording. These records were all obtained under standard conditions in the process of screening the offspring of heterozygote crossings of *tau* mutant Syrian hamsters, for studies on the effects of the *tau* mutation on a series of

variables of temporal organization of behavior (e.g., Oklejewicz and Daan, 2002; Oklejewicz et al., 2001a,b,c). In this paper we solely exploit the wildtype hamster records (n = 204). These were analyzed to address four questions: (a) How is precision affected by the definition of phase markers? (b) How does recording method affect which phase marker yields maximal precision? (c) How does precision vary with the amount of activity and with sex of the hamster ? (d) Do differences in precision between markers and between methods reflect differences in pacemaker periods or in ancillary processes?

## METHODS

All Syrian hamsters used were born in the breeding colony under LD 14:10 at Zoological Laboratory, Haren, The Netherlands. They all were born from crossings between parents both heterozygote for the *tau* mutation (Ralph and Menaker, 1988). Breeding stocks were originally obtained from Dr. Andrew Loudon (University Manchester). After weaning at the age of about 40 days hamsters were placed individually in recording cages ($25 \times 25 \times 40$ cm) in a room maintained at $23 \pm 2$°C under dim red incandescent light ($<0.1$ lux). Cages were equipped with running wheels (RW; diameter 17 cm) or with Passive Infra-Red (PIR) movement detectors on top of the cage. Data were collected by a custom built Event Recording System (ERS). This stores circuit closing events accumulated in 2min bins on a computer hard disk, and transfers the data for up to 256 channels every night at midnight onto a floppy disk, which can be removed and read out in daytime. Each hamster remained for circa 10d in the recording cage. The activity rhythms were first subjected to classic periodogram analysis (Sokolove and Bushell, 1978). In total, 204 activity recordings were analyzed: running wheel data for 147 hamsters (females n = 77; males n = 57) and PIR data for 57 individuals (females n = 31; males n = 26). The effects of the *tau* mutation on precision will be published elsewhere.

In all cases the last six days of data of a hamster's activity record were used. A computer program ERSVARIA, custom-made by Leon Steijvers, and freely available from the authors, identified in each cycle the center of gravity of the activity distribution (Kenagy, 1980) and then three phase markers each for the onset, midpoint, and end of activity. The 10 different phase markers are indicated with two examples in Figs. 1 and 2. The procedure is as follows:

- C (Center of gravity): The complete actogram is split up in time slices of 24h. In each time slice the circular mean vector for activity is calculated. The direction of this vector points to the center of gravity of the distribution. In the example of Figs. 1 and 2, C's are indicated in the panel (a).

Since the center of gravity is calculated on a circular distribution, it is nearly insensitive to where in the cycle the first time slice starts. Subsequently, the phase markers for the beginning of activity per cycle are found by searching the half-cycle (12h) preceding C, while the phase markers for the end of activity are found by searching the half cycle following C:

- $B_0$ (Beginning of $\alpha$) = Earliest 2min bin with activity counts exceeding the six-day average (Figs. 1a and 2a)

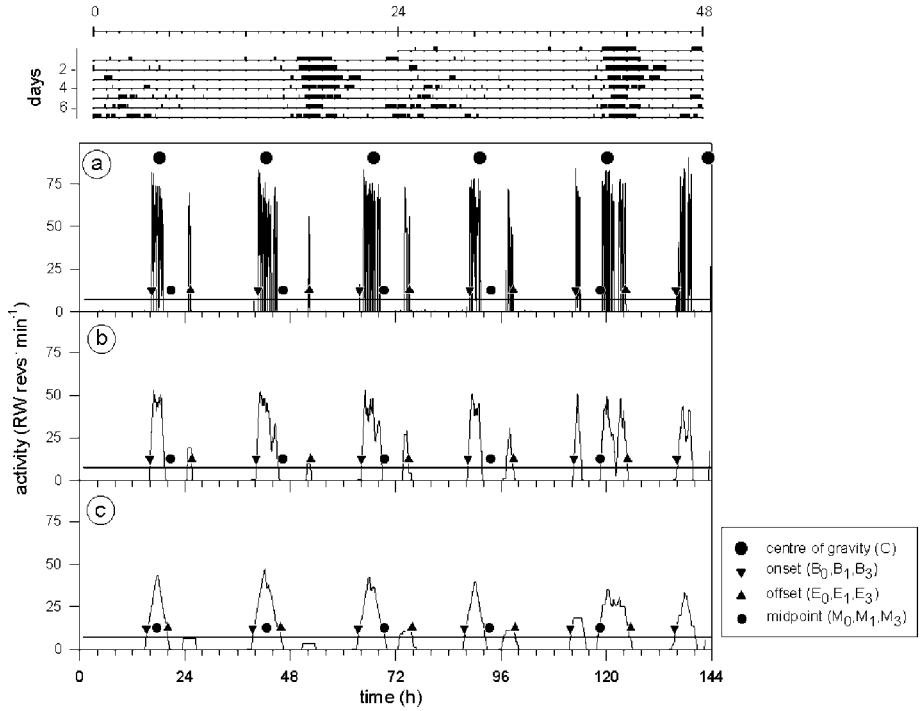


***Figure 1.*** Actogram (upper panel) and longitudinal plots (lower panel) of running wheel activity in the Syrian hamster indicated with the 10 phase markers. (a): data in 2min bins, (b): 1h running means, (c): 3h running means. For definitions of phase markers, see text.

- $E_0$ (End of $\alpha$) = Last 2min bin with activity counts exceeding the six-day average (Figs. 1a and 2a)
- $M_0$ (Midpoint of $\alpha$) = Halfway between $B_0$ and subsequent $E_0$ (Figs. 1a and 2a)
- $B_1$ = Same as $B_0$ except calculated after transforming the whole data set into 1h running means (Figs. 1b and 2b)
- $E_1$ = Same as $E_0$ except calculated after transforming the whole data set into 1h running means (Figs. 1b and 2b)
- $M_1$ = Halfway between $B_1$ and subsequent $E_1$ (Figs. 1b and 2b)
- $B_3$ = Same as $B_0$ except calculated after transforming the whole data set into 3h running means (Figs. 1c and 2c)
- $E_3$ = Same as $E_0$ except calculated after transforming the whole data set into 3h running means (Figs. 1c and 2c)
- $M_3$ = Halfway between $B_3$ and subsequent $E_3$ (Figs. 1c and 2c)

Figure 1 highlights some of the problems involved in establishing circadian phase markers. The main activity band is preceded by a tiny blip occurring 1–3h before. On days 1 and 2 this remains subthreshold; on day 3 it exceeds the threshold. Thereby $B_0$ is found at the onset of the main activity band on days 1 and 2 and 2h before it on day 3. In the 1h

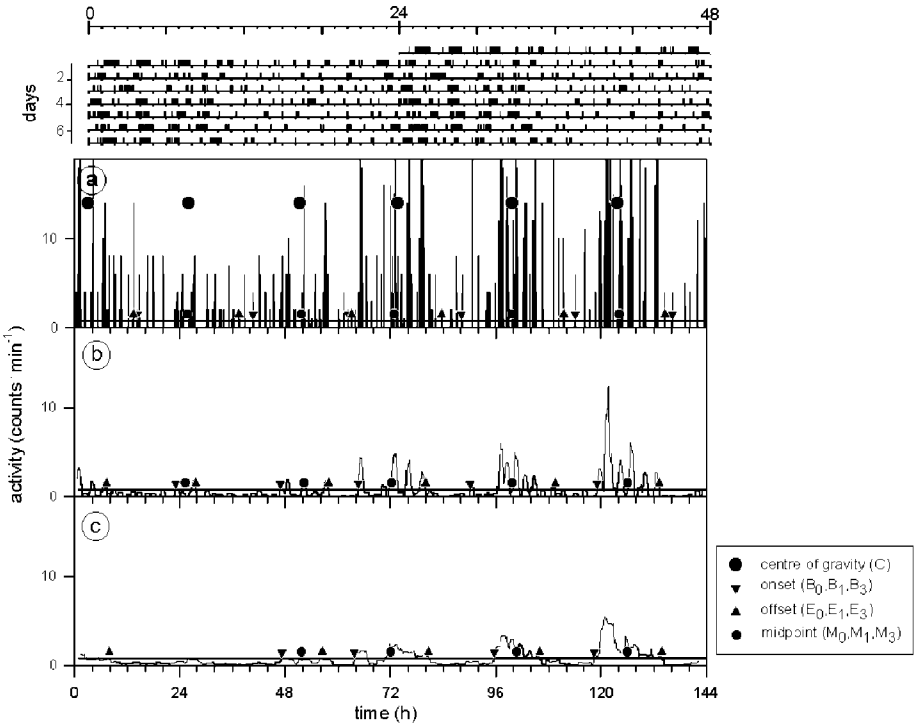


***Figure 2.*** Actogram (upper panel) and longitudinal plots (lower panel) of general activity in the Syrian hamster indicated with the 10 phase markers. (a): data in 2min bins, (b): 1h running means, (c): 3h running means.

running mean the blip is always subthreshold, rendering variance in $B_1$ smaller than in $B_0$. The variable second peak in activity determines E. This peak is usually narrow and on days 1 and 2 it remains subthreshold in the 3h running mean (Fig. 1c) increasing the variance in $E_3$ compared with $E_0$ and $E_1$. With PIR recording activity, patterns are often much more fragmented (Fig. 2) than with wheel running. Under such conditions, smoothing procedures applied greatly affect the determination of phase markers.

## RESULTS

### Effect of Phase Definition and Recording Method on Precision

We subjected 204 activity records to the program calculating these phase markers. Then for each definition, we computed the regression of the markers on sequence number. This yielded both a measure of $\tau$ (slope) and of the standard deviation of phase markers around the regression. We call the latter **phase variation**. The average values for period ($\tau$) and phase variation are presented in Table 1 (running wheel) and Table 2 (PIR = Passive InfraRed sensing) along with the interindividual standard deviations of $\tau$. These data

***Table 1.*** Mean circadian cycle length and mean phase variation in Syrian hamsters (n = 147) determined from wheel running activity rhythm according to 10 different phase markers. Smallest standard deviations are highlighted in bold type.

| Phase marker | Symbol | Mean $\tau$ | Interindividual sd of $\tau$ | Intraindividual phase variation |
|---|---|---|---|---|
| Periodogram | | 23.91 | **0.23** | |
| Center of gravity | C | 23.89 | 0.64 | 0.63 |
| 2min raw data | $B_0$ | 24.01 | 0.44 | 0.69 |
| | $M_0$ | 24.08 | 0.36 | 0.56 |
| | $E_0$ | 24.09 | 0.46 | 0.63 |
| 1h running mean | $B_1$ | 24.00 | 0.27 | **0.27** |
| | $M_1$ | 24.01 | 0.38 | 0.57 |
| | $E_1$ | 23.98 | 0.54 | 1.15 |
| 3h running mean | $B_3$ | 24.00 | 0.33 | 0.36 |
| | $M_3$ | 23.97 | 0.45 | 0.73 |
| | $E_3$ | 23.91 | 0.63 | 1.45 |

demonstrate that for wheel running the phase marker $B_1$ (upward crossing of the 1h running mean through the long-term average activity) yields the smallest average intraindividual phase variation (0.27h), and hence the highest precision (Table 1). The offset is considerably more variable on average than the onset, while the midpoint is intermediate. Interindividual variation of $\tau$ is always smallest in the periodogram analysis, possibly because it integrates all the data instead of searching for markers in a restricted part of the data set. Of course, periodogram analysis yields no measure of phase variation.

***Table 2.*** Inter- and intraindividual standard deviation of circadian cycle length in Syrian hamsters (n = 57) determined from general activity rhythm by passive Infrared sensing according to 10 different phase markers. Smallest standard deviations are highlighted in bold type.

| Phase marker | Symbol | Mean $\tau$ | Interindividual sd of $\tau$ | Intraindividual phase variation |
|---|---|---|---|---|
| Periodogram | | 24.08 | **0.52** | |
| Center of gravity | C | 24.14 | 0.65 | 1.23 |
| 2min raw data | $B_0$ | 24.21 | 0.55 | 1.28 |
| | $M_0$ | 24.18 | 0.68 | 1.03 |
| | $E_0$ | 24.08 | 0.70 | 1.17 |
| 1h running mean | $B_1$ | 24.32 | 0.81 | 1.63 |
| | $M_1$ | 24.31 | 0.64 | 1.24 |
| | $E_1$ | 24.13 | 0.77 | 1.79 |
| 3h running mean | $B_3$ | 24.36 | 0.69 | 1.21 |
| | $M_3$ | 24.21 | 0.74 | **0.92** |
| | $E_3$ | 24.13 | 0.97 | 1.58 |

In animals without running wheels, and with their activity recorded via PIR sensing, standard deviations, both inter- and intraindividual, are usually considerably higher than with running wheels (Table 2). Here it is the midpoint of activity, based on 3h rather than 1h running means, which consistently yields the smallest (intraindividual) phase variation. The smallest interindividual variation is again found in the $\tau$-estimates from periodogram analysis.

## Variation in Precision: Activity and Sex

For the phase marker leading to the most precise rhythm (smallest phase variation), we evaluated how the phase variation varies with the amount of activity, and with the sex of the hamsters. The results are plotted in Fig. 3. Clearly, increased activity leads to smaller phase variation in both sexes separately. For all levels of spontaneous activity, females had larger phase variation than males. Both sex and activity significantly increased the explained variance in phase variation.

## Partitioning of the Variance: Serial Correlation of $\tau$

Different phase markers thus yield different phase variations. However, they are derived from the same data and indeed generated by the same pacemaker. Thus we cannot conclude that differences in precision of the rhythm observed necessarily reflect

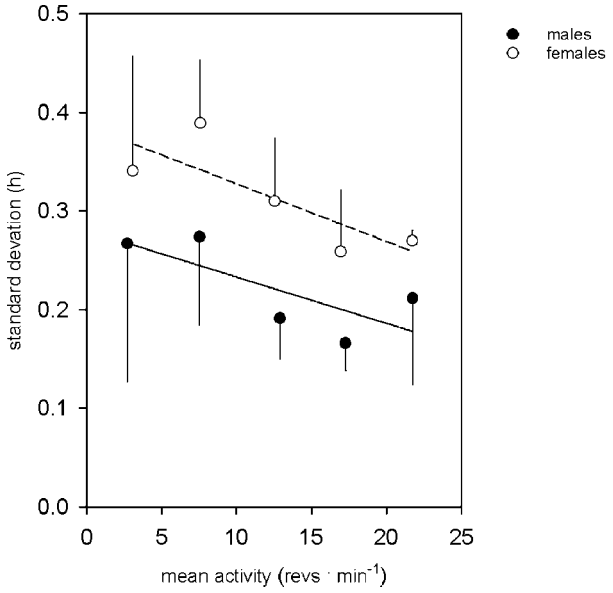


***Figure 3.*** Mean phase variation (standard deviation of onsets defined by B1 around the regression) for male and female hamsters in five classes of spontaneous mean activity level (revolutions per minute). Error bars represent s.e.m.

differences in precision of the pacemaker. There are processes involved in the control of overt behavior that may contribute to the cycle variance in the overt rhythm. Pittendrigh and Daan (1976a) have proposed a way in which variance of the observed cycle length may be partitioned into variance in pacemaker cycles and variance in processes peripheral to the pacemaker. The approach makes use of the standard deviation of cycle length and the serial correlation in cycle length. This serial correlation is the correlation between consecutive cycles of the overt rhythm. It reflects the extent to which an occasional long cycle is compensated by the following short cycle. Strong compensation, i.e., a more negative serial correlation coefficient, would indicate that most of the observed variance is due to peripheral, not clock-related, processes. Since there were two minor typographical errors in the original derivation published by Pittendrigh and Daan (1976a), we repeat the formal argument in the Appendix. The bottom line of the derivation is that the variation in pacemaker periods can be estimated by $s(\tau) = s(t)\sqrt{(1 + 2r_s)}$, where s(t) is the standard deviation of observed cycle lengths, and $r_s$ is the serial correlation between consecutive cycles. Likewise, the variation in peripheral processes is estimated by $s(w) = s(t)\sqrt{(-r_s)}$. Obviously, this partitioning of variance works only as long as $-0.5 < r_s < 0$: with positive serial correlation there is no compensation, with $r_s < -0.5$ there would be overcompensation. We emphasize that s(t), or **cycle variation**, is not the same measure as the phase variation (around the regression) used in Tables 1 and 2.

Partitioning of the variance was done for all phase markers on the basis of all actograms where at least five consecutive cycles could be measured, i.e., complete runs with at least six phase markers. Runs with fewer data were discarded. The resulting number of data sets on midpoints of activity (which require both onset and offset) was too small to retain these in the analysis. For the remaining seven phase markers (C, $B_0$, $B_1$, $B_3$, $E_0$, $E_1$, $E_3$) the average values for s(t) and $r_s$ are presented in Table 3, along with s(w) and

***Table 3.*** Partitioning of the variance in cycle length in seven different circadian phase markers and two methods of activity recording (running wheel and passive infrared). For each case, the table shows: n—number of activity records where at least five circadian cycles were recorded; s(t)—the average standard deviation in cycle length; $r_s$—the average of the serial correlation coefficients calculated in each run; s(w)—the computed standard deviation due to nonclock processes; s(τ)—the computed standard deviation due to clock cycle length. In all except one case $-0.5 < r_s < 0$, as required for variance partitioning. In the exception s(τ) is estimated to be ~0.

| | Running activity (wheel) | | | | | General activity (PIR) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Marker | n | s(t) | $r_s$ | s(w) | s(τ) | n | s(t) | $r_s$ | s(w) | s(τ) |
| C | 126 | 0.85 | −.38 | 0.52 | 0.42 | **50** | **1.36** | **−.40** | **0.86** | **0.60** |
| $B_0$ | 105 | 1.13 | −.31 | 0.63 | 0.69 | 44 | 2.27 | −.37 | 1.38 | 1.15 |
| $B_1$ | **99** | **0.45** | **−.25** | **0.23** | **0.31** | 29 | 2.84 | −.44 | 1.88 | 1.00 |
| $B_3$ | 100 | 0.61 | −.30 | 0.33 | 0.39 | 27 | 1.72 | −.32 | 0.97 | 1.04 |
| $E_0$ | 128 | 1.74 | −.40 | 1.10 | 0.77 | 51 | 1.95 | −.38 | 1.20 | 0.97 |
| $E_1$ | 121 | 1.92 | −.46 | 1.30 | 0.55 | 47 | 3.15 | −.44 | 2.10 | 1.07 |
| $E_3$ | 111 | 2.49 | −.46 | 1.69 | 0.69 | 33 | 2.57 | −.56 | 1.93 | ~0 |

the calculated estimates of $s(\tau)$. The omission of some runs because a phase could not be determined explains why the numbers reported in Table 3 are often slightly less than those in Tables 1 and 2.

The first conclusion from Table 3 is that indeed nearly all the average $r_s$ values are between the boundary values of $-0.5$ and 0. Hence there is good evidence for compensation and for the idea that non–clock-related processes contribute to the variance in cycle length. For wheel running records, the smallest variation in endogenous cycle length ($s(\tau)$) is obtained using the onset of activity in a 1h or 3h running average (Table 3). For general activity (PIR) records obtained without wheels the Center of Gravity method yields the most precise estimates of pacemaker cycle length. This corresponds with the results obtained in Table 2 in the sense that in non-wheel running activity records a measure of central tendency of the activity—either activity midpoint or center of gravity—yields the most precise phase marker.

In Fig. 4a, we have plotted the average $r_s$ values against the average s(t) for each combination of recording method/phase reference, yielding 14 data points. The figure reveals a clear negative association between s(t) and $r_s$. Fig. 4b demonstrates that the variation attributable to non–clock-related processes (s(w)) increases steeply with increasing overall variation (s(t)), in fact more steeply than expected on the basis of proportionality (a regression through the origin would be proportional). The variation attributable to the underlying pacemaker ($s(\tau)$) increases much less steeply with s(t) as a consequence of stronger negative serial correlation ($r_s$). Thus, phase markers leading to large variance in cycle length apparently overestimate the variance in endogenous pacemaker cycle and underestimate its precision.

## DISCUSSION

The analysis presented here underscores the importance of selecting the phase marker yielding the most precise rhythm. In countless chronobiological studies, the overt rhythm is used to unveil properties of the underlying pacemaker. As demonstrated in Fig. 4b, variation in precision of the overt rhythm is mainly due to peripheral processes. With phase definitions leading to small variance in cycle length, this variance is much closer to that of the pacemaker. This result gives an empirical reason why phase markers leading to the most precise measure should be preferred. The most precise rhythms most closely reflect the pacemaker. Hence a systematic search for the phase marker yielding the most precise rhythm is far from futile.

For any particular species or recording method one phase marker may be optimal (i.e., yielding maximal precision). This need not be the same, however, for all methods and species. We compared the behavior of 10 phase markers for groups of hamsters with two methods: wheel running and general activity by PIR sensing. Consistently, in wheel running the onset yielded the most precise measure (Table 1). With general, non-wheel running activity, measures of central tendency (center of gravity and midpoint) perform better (Table 2). Aschoff (1965) used to propagate the midpoint of activity as the appropriate phase marker, albeit without much success. When running wheels became standard tools in chronobiology, researchers have usually preferred the onset of activity as

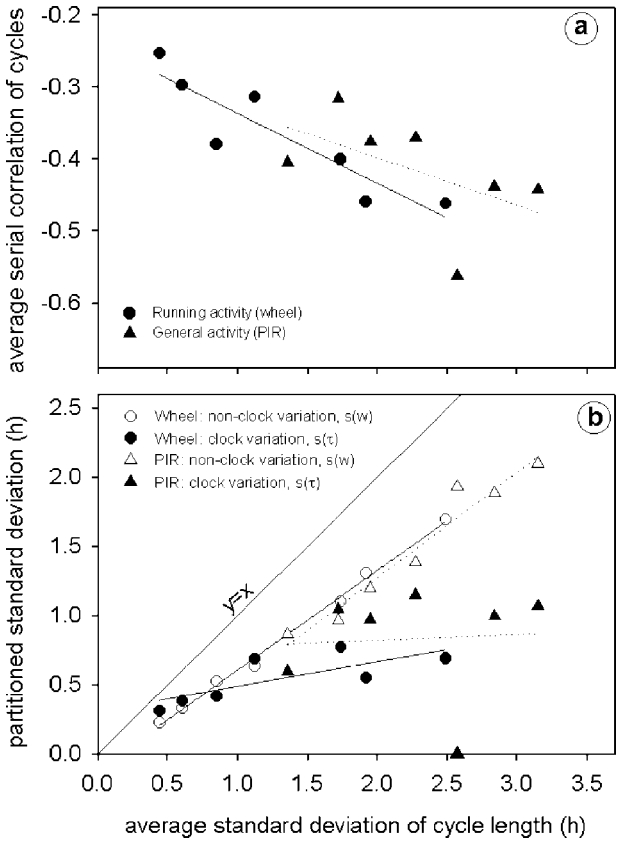


***Figure 4.*** (a) The relation between mean standard deviation of cycle length and mean serial correlation for each phase markers, and with both methods of activity recording. (b) The dependence of clock and nonclock contributions to variation in the cycle length. Solid lines in both panels are regressions for wheel running data, stippled lines for PIR data. Data correspond to Table 3.

the phase marker of choice. Correctly so. For other means of recording Aschoff may well have been right.

Smoothing clearly affected the precision. With wheel running records the most precise rhythm was obtained with an intermediate degree of smoothing the raw data: running means over 1h rather than 3h. The less-precise overt rhythms of PIR records required smoothing over a somewhat longer period (3h running means) to attain highest precision. Such analysis is useful prior to the decision on optimal phase markers for a rhythm.

We observed that precision increases with increasing level of activity (Fig. 3). This is hardly surprising in view of the statistical law of large numbers (variance of central tendencies decreases with increasing n). The difference between the sexes, with males having higher precision or less phase variation, is also expected, but for

a biological reason. The effect of the estrous cycle leading to scalloping (Fitzgerald and Zucker, 1976) increases the variance in consecutive cycles and has led mammalian chronobiologists to prefer working with males over females.

The onset of wheel running in hamsters produced the highest precision of circadian cycles in our data set, with an average cycle-to-cycle standard deviation of 0.45h (based on 1h running means). From the average serial correlation of $-0.25$ in this case the variation in pacemaker cycles is estimated at 0.31h (Table 3). With PIR records obtained without wheels these estimates are about twice as large. The data thus suggest that the use of a wheel by hamsters affects the precision not only of the overt rhythm, but indeed of the circadian pacemaker itself. This would not be astonishing given the extensive evidence for feedback from wheel running onto the pacemaker (Edgar et al., 1991; Mrosovsky et al., 1992). A study directed at this issue comparing general activity rhythm precision in the same individuals with and without a wheel would be required to firmly settle this issue.

The main message emerging from our analyses is that different recording methods do lead to different optimal phase markers. The use of the onset of activity may be preferred in wheel running records but is unlikely to be optimal for other means of recording rhythms.

## APPENDIX (AFTER PITTENDRIGH AND DAAN, 1976a, WITH MINOR CORRECTIONS)

If pacemaker periods are denoted as $\tau_1$, wake-up times as $w_i$, and observed periods of the rhythm as $t_i$, it is clear that:

$$t_i = \tau_i - w_i + w_{i+1}$$

and

$$t_i + t_{i+1} = \tau_i + \tau_{i+1} - w_i + w_{i+2}$$

Independent variations in w and $\tau$ would then lead to:

$$\sigma^2(t) = \sigma^2(\tau) + 2\sigma^2(w) \tag{1}$$

and

$$\sigma^2(t_i + t_{i+1}) = 2\sigma^2(t) + 2\mathrm{cov}_s(t) = 2\sigma^2(\tau) + 4\sigma^2(w) + 2\mathrm{cov}_s(t) \tag{2}$$

where $\mathrm{cov}_s$ (t) indicates the covariance of successive observed periods of the rhythm.

Also,

$$\sigma^2(t_i + t_{i+1}) = 2\sigma^2(\tau) + 2\sigma^2(w) \tag{3}$$

because of independence of $\tau_1$, $\tau_{i+1}$, $w_1$, $w_{i+2}$.

From Eqs. (2) and (3), it follows that

$$\sigma^2(w) = -\mathrm{cov}_s(t)$$

If in a sample $\mathrm{cov}_s(t)$ is estimated by $r \cdot s(t)$, with s(t) denoting the standard deviation of t, then the variances of w and $\tau$ are estimated by:

$$s^2(w) = -r_s s^2(t)$$

$$s^2(\tau) = (1 + 2r_s)s^2(t)$$

thus,

$$s(w) = s(t)\sqrt{(-r_s)}$$

$$s(\tau) = s(t)\sqrt{(1 + 2r_s)}.$$

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