

# A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents

## II. The Variability of Phase Response Curves

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**Summary.** 1. Phase response curves for 15' bright light pulses of four species of nocturnal rodents are described. All show delay phase shifts early in the subjective night, advance shifts in the late subjective night, and relative insensitivity during the subjective day.

2. The broad scatter in measured phase-shifts is largely due to error of measurement: the response of the pacemakers to light stimuli is more accurate than we observe.

3. Indications are found that the response to a resetting stimulus at a given phase of the rhythm is correlated with the individual  $\bar{\tau}$  (freerunning period). Fast pacemakers (short  $\bar{\tau}$ ) tend to be more delayed or less advanced by the light than slow pacemakers (long  $\bar{\tau}$ ).

4. Within individual mice (*Mus musculus*) the circadian pacemaker adjusts its resetting response to variations in its frequency: when  $\tau$  is long (induced as after-effect of prior light treatment) light pulses at a defined phase of the oscillation (ct 15) produce smaller delay phase shifts than when  $\tau$  is short.

5. Among species there are conspicuous differences in the shape of the phase response curve: where  $\bar{\tau}$  is long, advance phase shifts are large and delay phase shifts small (*Mesocricetus auratus*); where  $\bar{\tau}$  is short, advance shifts are small, and delay shifts are large (*Mus musculus*; *Peromyscus maniculatus*).

6. The functional meaning of the interrelationships of  $\tau$  and PRC is briefly discussed.

## I. Introduction

As long as the concrete mechanism and the localization of a circadian pacemaker remains unknown, and we are limited to observing its action on some peripheral rhythm driven by it, only two properties can be measured, and confidently be

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considered pacemaker properties: (i) the free-running period ( $\tau$ ), which in steady-state is necessarily identical for pacemaker and driven rhythm, and (ii) the phase response curve for some standard perturbation, at least when measured from steady state-phase shifts of the rhythm, i.e., after allowing the driven rhythm to regain its original steady-state relationship with the driver. This prerequisite of awaiting steady-state motion after each single perturbation, makes the measurement of phase-response-curves a time-consuming operation, especially so in organisms studied individually.

Phase response curves have proved a useful tool in analyzing the mechanism of the entrainment by light-dark cycles of the circadian clocks involved in pupal eclosion of insects (Pittendrigh and Minis, 1964; Pittendrigh, 1966, 1967, in preparation; Ottesen et al., in preparation). These rhythms are entrained by a zeitgeber consisting of a series of light pulses with period  $T$  in such a way that the phase ( $\phi$ ) hit by the light pulse in any cycle is that phase where an isolated light pulse would produce a phase shift  $\Delta\phi(\phi)$  which equals the difference between the freerunning period ( $\tau$ ) and  $T$ :

$$\Delta\phi(\phi) = \tau - T.$$

The analytical consequences of this statement have been elaborated elsewhere (Ottesen et al., in preparation). The model derives much of its strength from the observation that such light pulses to some extent truly simulate natural light-dark cycles. In nature  $T$  always equals 24 h, the period of the earth's rotation. Hence in reality the model depicts a relationship between  $\Delta\phi$  and  $\tau$ : In every cycle the phase shift elicited by the light stimuli, adjusts the period of the rhythm from  $\tau$  to  $T$ . As we described earlier (Pittendrigh and Daan, 1976a) considerable differences in  $\tau$  exist, not only between species of nocturnal rodents, but also between individuals of the same species, and in some species within individuals, depending on such factors as age, prior conditions, and unknown sources of variation leading to what one labels "spontaneous changes". Two questions then emerge: 1. do species differences exist in the response to light pulses which are functionally related to the known differences in  $\tau$ ; and 2. is there also a dependence of  $\Delta\phi$  responses on the differences in  $\tau$  known to exist both between and within individuals? This latter question clearly cannot be asked in experiments where the rhythm measured is exclusively a population phenomenon in which  $\tau$  variation between and within individuals is never directly measured.

Phase response curves for short light pulses have already been measured in single animals of several rodent species: *Mesocricetus auratus* (Burchard, 1958; DeCoursey, 1964; and recently Elliott, 1974); *Glaucomys volans* (DeCoursey, 1960, 1961); *Ammospermophilus leucurus* (Kramm, 1971). But in these species the sample sizes were never large enough to make it feasible to look for a relationship between an individual's freerunning period and its phase response curve. The question has been addressed, however, by Natalini (1972) in his study of the activity rhythm in the kangaroo rat, *Dipodomys merriami*. The purpose of the present report is to compare the phase responses to brief light pulses in four species of nocturnal rodents, both intra- and interspecifically. Our results are in sharp contrast with Natalini's (1972) findings in *Dipodomys* as we shall see below (Section IV).

## II. Experimental and Analytical Procedures

The general procedures for maintenance of the experimental animals and recordings of their activity rhythms have been described earlier (Pittendrigh and Daan, 1976a). The phase response curves for 15' fluorescent light in DD were obtained in two series of experiments employing essentially the same technique. In the first series, 13 golden hamsters (*Mesocricetus auratus*), 5 white-footed mice (*Peromyscus leucopus*) and 7 deer mice (*Peromyscus maniculatus*), isolated in light-proof boxes, were allowed to freerun in DD for two months following prior entrainment to LD 12:12. Interspecific and inter-individual differences in  $\tau$  rapidly led them to become out of phase with each other. Once every two weeks a single light pulse was administered via signal clocks connected to the fluorescent light tubes in each box, and set so that the succession of pulses would scan the whole circadian cycle. The times of the pulses were recorded and the phase of the cycle where the light had fallen calculated afterwards. In the second series, involving 32 mice (*Mus musculus*; C57BL/6S) the same protocol was used, except that circadian times where the lights should fall were calculated before setting the clocks. Thus in this experiment we obtained the phase responses to 15' light pulses falling exactly at ct (= circadian time)

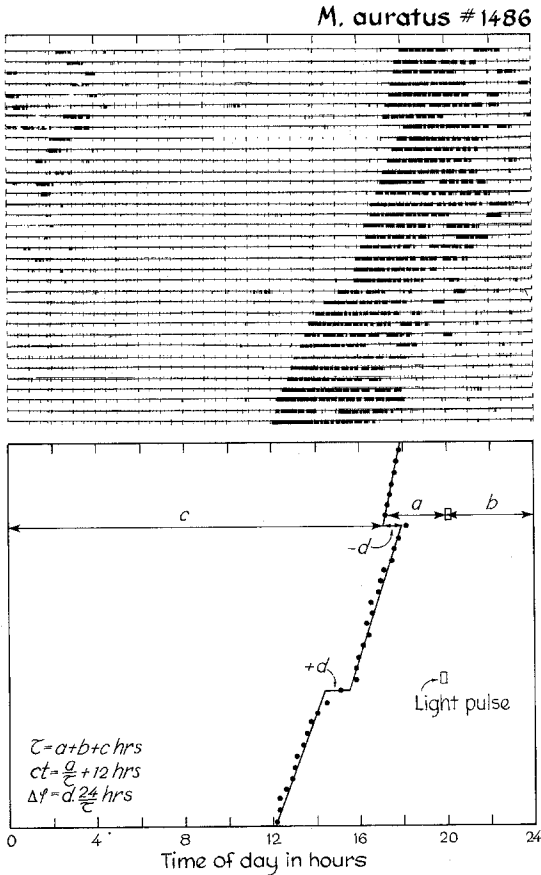
12 (= at the expected onset of activity), ct 14  $\left(2 \cdot \frac{\tau}{24} \text{ h after the onset}\right)$ , ct 16  $\left(4 \cdot \frac{\tau}{24} \text{ after onset}\right)$ , ct 18  $\left(6 \cdot \frac{\tau}{24}\right)$ , ct 20, 22, 24, 3, 6 and 9. In the *Mus musculus* experiment, lasting seven months, all animals received 10 light pulses. The other species were exposed to 26 light pulses altogether in the course of 13 months, although due to deaths and replacements several animals had less than 26 pulses. 15 of the 32 *Mus musculus* continuously had 25% D<sub>2</sub>O (heavy water) in their drinking water. The results from these animals are discussed elsewhere (Daan and Pittendrigh, 1976).

Figure 1 illustrates the kind of data obtained and the methods of computing freerunning period ( $\tau$ ), phase ( $\phi$ ) of the rhythm hit by the light pulse and phase shift ( $\Delta\phi$ ) due to that pulse.  $\tau$  was measured by calculating the linear regression through the times of activity onset on their sequence number. The slope of this regression gives the departure of  $\tau$  from 24 h. The first three onsets of activity following a light pulse were not taken into account, since they are usually characterized by transients. Three intercepts of the regression line are further used: (i) its value estimating the last activity onset before the light pulse, to measure the time distance [(a) in Fig. 1] from this value to the onset of the light pulse and compute the phase (ct = 12 + a/ $\tau$ ) hit by the pulse; (ii) its value on the next day as the predicted next onset in the absence of a light pulse; (iii) the intercept of the next regression line on this same day, to obtain the difference  $\Delta\phi$ , which is given a negative sign for phase delays, a positive sign for phase advances. The phase shifts obtained are expressed either in hours of real time, or normalized into degrees of arc by multiplication with a factor 360/ $\tau$ . The  $\Delta\phi$  values thus obtained were in the first experiment collected in bins, two circadian hours wide, around ct 0, 2, 4, etc. Values in each bin were treated as a random sample of  $\Delta\phi$ -values at ct 0, 2, 4, ..., and the usual statistics calculated (Fig. 2). Smoothed curves were obtained by eyefitting a curve through bin averages.

## III. Phase Response Curves for Brief Light Pulses

### a) Species Comparison

The experimentally obtained values of  $\Delta\phi$  for a given phase of the circadian rhythm show more scatter for these individual animals than we had become used to in populations of thousands of *Drosophila* pupae (Pittendrigh, 1966). They present a cloud of points moving up and down along the circadian time scale (Fig. 2). Some of this variation is due to our way of measuring the phase shifts: Even without administering a light pulse, two consecutive regression-lines used to measure  $\Delta\phi$  may of course have measurably different extrapolations to the same day, due to scatter in the raw data and to spontaneous changes in  $\tau$ . When no light pulse was given, the standard deviation of " $\Delta\phi$ ", measured in the same way

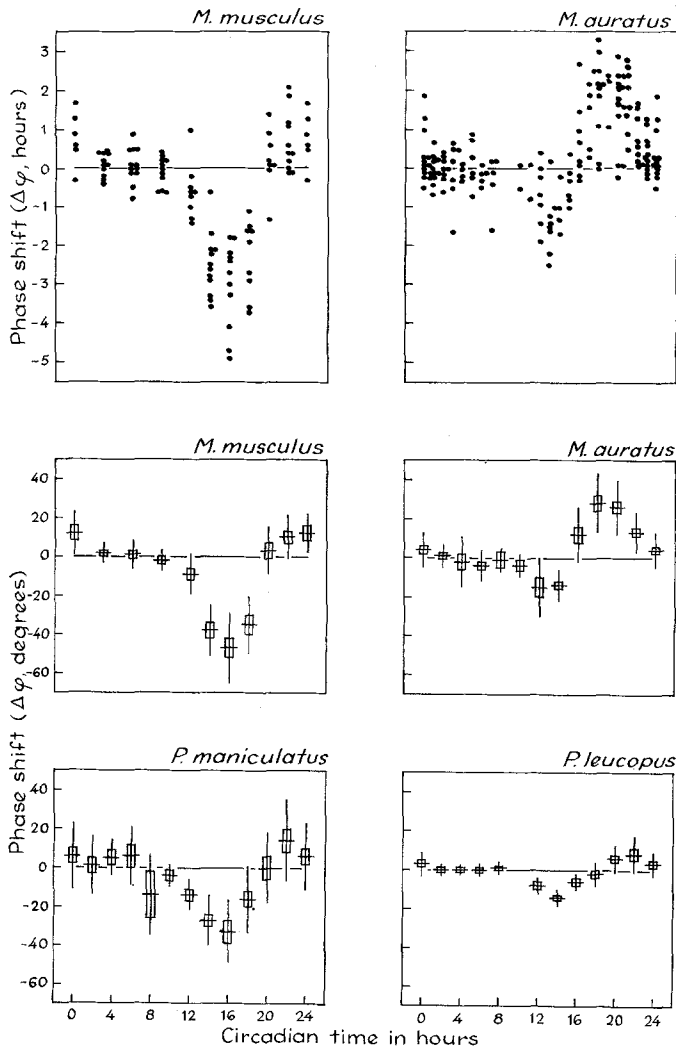


**Fig. 1.** Measurement of phase shifts of the activity rhythm caused by 15' light pulses. Upper panel: raw data. Lower panel: Computation of freerunning period ( $\tau$ ), circadian time ( $ct$ ) at which the light pulse was given, and phase shift ( $\Delta\phi$ ) produced by this pulse

as real phase shifts, was 0.45 h in hamsters ( $n=13$ ), 0.22 in *P. leucopus* ( $n=5$ ), 0.68 h in *P. maniculatus* ( $n=7$ ) and 0.35 h in *M. musculus* ( $n=11$ ). Compared with the average standard deviations in the two-hour bins of real  $\Delta\phi$ -values (0.67; 0.32; 0.94; 0.70 h respectively), these figures suggest that between 1/2 and 2/3 of the variation in  $\Delta\phi$  is due to error of measurement.

In the PRC-experiment in *Mus musculus* we included two animals which received a light pulse every two weeks at the same phase ( $ct$  15). The average phase shifts observed in these animals were  $-2.75$  h ( $\pm$ s.d. 0.45;  $n=7$ ) and  $-2.61$  h ( $\pm$ s.d. 0.49;  $n=8$ ). Thus for this species we have three estimates of the variation in  $\Delta\phi$ :

- (i) s.d. " $\Delta\phi$ " in the absence of light pulses (error of measurement): 0.35 h.
- (ii) s.d.  $\Delta\phi$  within individuals, for light pulses at fixed  $ct$ : 0.47 h.
- (iii) s.d.  $\Delta\phi$  among individuals for light pulses at fixed  $ct$ : 0.70 h.



**Fig. 2.** Phase response curves for 15' light pulses in four species of rodents. Two panels show all phase shifts measured in hours of real time, as a function of circadian time, in mice (*Mus musculus* C 57) and hamsters (*Mesocricetus auratus*). In the two panels in the middle these data are normalized to degrees phase shift (i.e., multiplied by  $360/\text{preceding } \tau$ ), and averaged over 2-hourly bins of circadian time. Rectangles indicate 1 standard error, lines 1 standard deviation on both sides of the mean. In the two bottom panels, the phase shifts measured in *Peromyscus maniculatus* and *P. leucopus* are represented in the same way

Or, if one would assume that the three sources of variation give independent contributions to the total variation (iii), their variances are additive, and one would obtain as estimates for the standard deviations due to:

error of measurement only: 0.35 h;

intra-individual variation only: 0.32 h ( $=\sqrt{0.47^2 - 0.35^2}$ );

inter-individual variation only: 0.52 h ( $=\sqrt{0.70^2 - 0.47^2}$ ).

In the other species, where we have no information on intra-individual variation in the response, only a maximum estimate for both intra- and inter-individual variation alone can be obtained along these lines:

*M. auratus*      0.50 h      ( $=\sqrt{0.67^2 - 0.45^2}$ );

*P. leucopus*      0.22 h      ( $=\sqrt{0.32^2 - 0.22^2}$ );

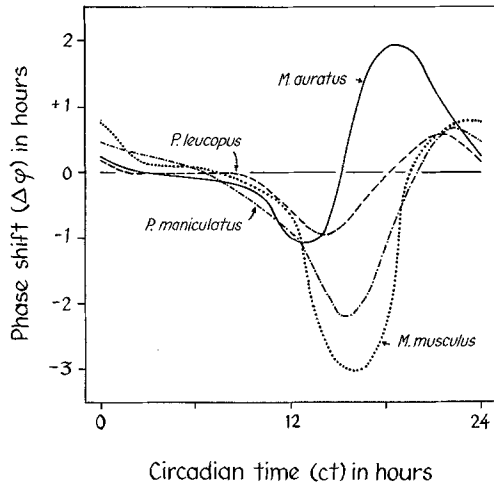
*P. maniculatus*    0.65 h      ( $=\sqrt{0.94^2 - 0.68^2}$ ).

The variation in the response of individual animals to a light pulse at a fixed phase (ii) is only slightly larger than what we would expect on the basis of the error of measurement (i). We have seen earlier in the discussion of precision of periods of the pacemaker and of the observed rhythm (Pittendrigh and Daan, 1976a) that the pacemakers' periods must be considerably more precise than the activity rhythm we use for their assay. Similarly, its response to brief light pulses is apparently more accurate than our best estimate based on the overt rhythm. Among individuals, the average standard deviation at fixed phase points (iii) is clearly larger than within individuals (ii). Therefore, although the major part of the scatter is due to error of measurement, some of it is apparently caused by interindividual differences in the response to light pulses. We shall discuss one aspect of this interindividual variation below (Section IIIb).

Nevertheless, even in the unreduced data, the familiar general features of circadian PRC's are immediately clear. Phase delays are effected by light pulses falling late in the subjective day and early in the subjective night. Phase advances are effected by light pulses falling in the late subjective night and early subjective day. The greater part of the subjective day (ct 2-10) is characterized by a "dead zone" where the pacemakers are virtually unresponsive to light.

There are, however, significant differences among the species (Fig. 3). The hamster's PRC is characterized by large advance phase shifts, and small delay phase shifts. Its shape is in good agreement with the PRC for 10' light pulses reported for a single hamster by DeCoursey (1964, Fig. 2). Responses to light are generally small in *P. leucopus*. *Mus musculus* and *P. maniculatus* had small advances and large delays. The differences between the species have been quantified by integrating the area under the phase response curves, i.e. summing the products of bin width (in circadian hours) and bin average  $\Delta\phi$  (in degrees). The area under the advance part ( $+\Delta\phi$ ) is called *A*; the area under the delay part ( $-\Delta\phi$ ) of the curves is called *D*. The difference ( $D-A$ ) is negative in *M. auratus*, small positive in *P. leucopus*, and large positive in *M. musculus* and *P. maniculatus* (Table 1). Thus the two species (*P. maniculatus* and *Mus musculus*) with the smallest average  $\tau$  in *DD* have the largest delay phase shifts, the species with longest  $\bar{\tau}$  has the largest advance phase shifts. We believe this species sequence in both  $\tau$  and PRC shape, as expressed by ( $D-A$ ) is of functional significance. In fact, the relationship was initially detected by comparing hamsters with the two *Peromyscus* species. Since we knew that the average  $\tau$  in *Mus musculus* was considerably shorter than 24 h (Pittendrigh and Daan, 1976a), we set out to measure its PRC, among

**Fig. 3.** Phase response curves for 15' light pulses, obtained by drawing a smooth curve through bin average  $\Delta\phi$ 's



**Table 1.** Areas under delay (*D*) and advance (*A*) sections of the phase response curves (in h × degrees)

Species	<i>N</i>	$\bar{\tau}$	<i>A</i>	<i>D</i>	<i>D</i> - <i>A</i>
<i>M. auratus</i>	13	24.04	155	82	-73
<i>P. leucopus</i>	5	23.99	44	66	22
<i>M. musculus</i>	13	23.43	62	268	206
<i>P. maniculatus</i>	7	22.92	64	207	143
<i>M. auratus</i> long $\tau$	6	24.12	217	72	-145
<i>M. auratus</i> short $\tau$	7	23.97	133	126	-7
<i>P. leucopus</i> long $\tau$	3	24.09	37	58	21
<i>P. leucopus</i> short $\tau$	2	23.85	58	94	36
<i>M. musculus</i> long $\tau$	8	23.60	81	272	191
<i>M. musculus</i> short $\tau$	5	23.17	52	264	212
<i>P. maniculatus</i> long $\tau$	4	23.14	78	177	99
<i>P. maniculatus</i> short $\tau$	3	22.64	58	234	176
<i>M. musculus</i> 25% D <sub>2</sub> O	14	25.21	33	250	217
<i>A. leucurus</i> <sup>a</sup>	?	24.28	5	138	133
		h	circadian hours × degrees of arc		

<sup>a</sup> Data on the antelope ground squirrel derived from Kramm (1971; Table 2: average  $\tau$  from day 30 in DD onwards; Fig. 38)

other reasons, in order to test the prediction that it would have a large *D* and a small *A* area.

The dependence of PRC-shape on  $\tau$  does not hold in fine detail, since *D* in *Mus musculus* slightly exceeds *D* in *Peromyscus maniculatus*, although the average  $\bar{\tau}$  is shorter in the latter species. The general trend, however, is conspicuous enough, and of enough importance for considerations on the entrainment mechanism, to ask whether indications of the same trend can be found in a comparison of individuals within a species.

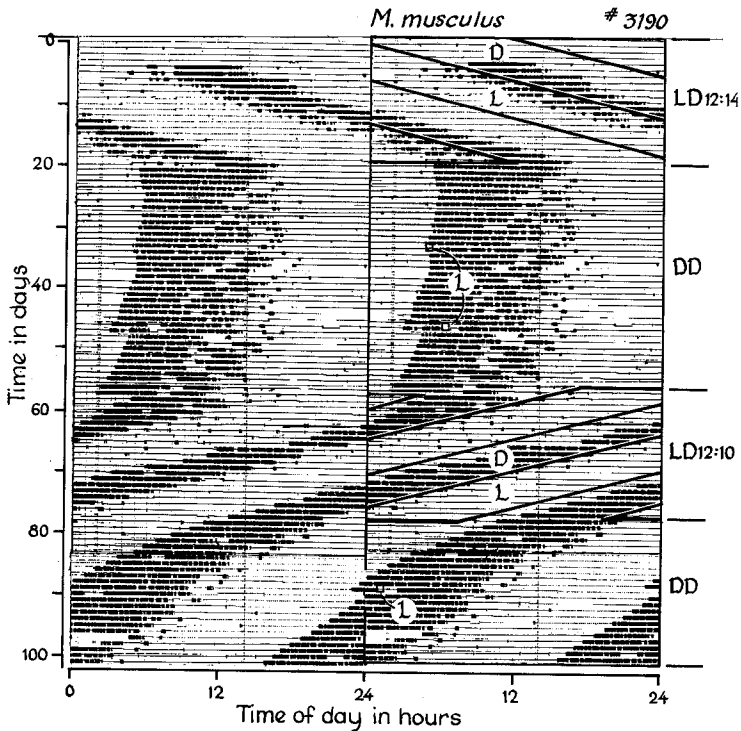
b) *Differences between Individuals: The Relation with  $\tau$*

In order to investigate the relation between freerunning period and phase response curve among individuals, we have divided each species in two groups: a "short- $\tau$  group", defined as those animals having an average  $\bar{\tau}$  over the duration of the experiment that was smaller than the average  $\bar{\tau}$  of all animals; and a "long- $\tau$  group", comprising those animals with  $\bar{\tau} > \bar{\tau}$ . We then proceeded to treat the  $\Delta\phi$ -values measured in both groups in the same way as before. In the hamster, average  $\Delta\phi$ 's were more positive in the long- $\tau$  group in 8 out of 10 bins, in *Peromyscus leucopus*, in 6 out of 9, in *Mus musculus* in 6 out of 10, in *P. maniculatus* in 6 out of 9. Hence in the total of 38 bins where a qualitative comparison could be made, there were 26 cases (or 65%) where long- $\tau$  animals had more positive (or less negative) average  $\Delta\phi$ . This is significantly more (sign-test  $p < 0.05$ ) than 50%. Thus there is indeed a general trend that the short- $\tau$  groups tend to have larger phase delays and/or smaller phase advances than the long- $\tau$  groups. This is also apparent when we compare ( $D - A$ ) in the two groups (Table 1), which is always larger in the short- $\tau$  groups, although the difference is small in *P. leucopus*. We have looked at this relationship in yet another way; by measuring in each animal, individually, what fraction of the  $\Delta\phi$ -values for each bin was more positive (or less negative) than the species average  $\Delta\phi$  for that bin. The coefficient of correlation of this fraction (percentage) with the animals's  $\bar{\tau}$  was then computed. It was 0.33 ( $n = 12$ ) in *M. auratus*; 0.54 ( $n = 5$ ) in *P. leucopus*; 0.23 ( $n = 7$ ) in *P. maniculatus* and 0.48 ( $n = 13$ ) in *M. musculus*. There is no statistical significance to any of these coefficients alone. Yet the fact that they are positive in all four species is another indication of the slight tendency in these rodents that short- $\tau$  animals have more negative (less positive)  $\Delta\phi$ -values than long- $\tau$  animals. Clearly, the scatter in  $\Delta\phi$ -measurements, mainly due to the estimation technique as we have seen, would make it necessary to study a much larger number of animals to establish this point firmly.

c) *Within the Individual: Does the Phase Response Change with  $\tau$ ?*

Thus, species with  $\tau$  considerably shorter than 24 h, respond to brief light pulses with large phase delays and small phase advances; within a species, the phase delays tend to be larger and/or the advances smaller, the shorter  $\tau$  is. The question obviously arises whether the same relationship holds within individuals when  $\tau$  changes. In other words; does the individual pacemaker compensate for changes in  $\tau$  by changing its response to light pulses?

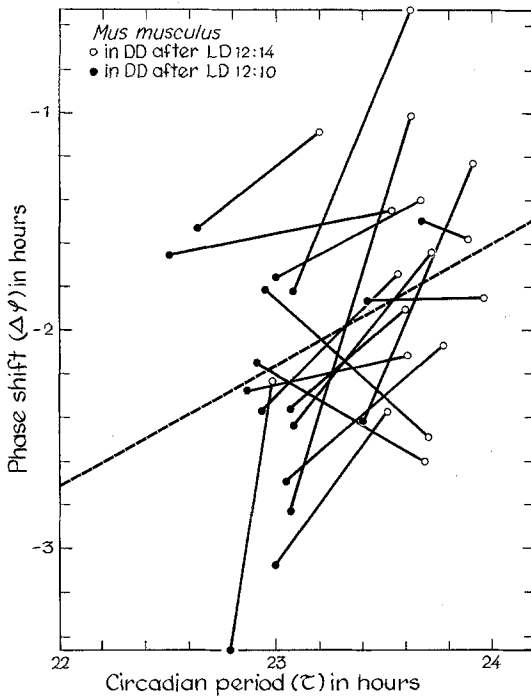
To answer this question, one has to modify  $\tau$  in a predictable manner. This can be done either permanently by altering the experimental conditions during the test period, or temporarily, by giving different pretreatments and make use of the after-effects on  $\tau$  (Pittendrigh and Daan, 1976a) created thereby. We have applied both techniques. In one experiment complete phase response curves were measured in two groups of mice (*Mus musculus*); one group with and one group without D<sub>2</sub>O in their drinking water, as  $\tau$  is known to be strongly increased by D<sub>2</sub>O in rodents (Suter and Rawson, 1968; Dowse and Palmer, 1972). The results of this experiment are reported elsewhere (Daan and Pittendrigh, 1976) since they obviously have a bearing on the action of D<sub>2</sub>O on the pacemaker.



**Fig. 4.** Activity record of *Mus musculus* #3190, showing the protocol used to investigate the dependence of  $\Delta\phi$  on  $\tau$ . Differences in  $\tau$  in DD were created by 3 weeks of entrainment to both a long (T26) and a short (T22) light-dark cycle. (Note the difference, comparing the two light cycles, in the phase-relation between activity and light-pulse). After release into DD the phase shift due to a light pulse administered at ct 12 (see Fig. 1) and one at ct 15 was measured. The pulse at ct 12 was not repeated in the second freerun, since the phase shifts it produced were too small.  $\tau$ -values, ct-times, and phase shifts were calculated from linear regressions through the onsets of activity

It should suffice here to say that the general shape of the PRC obtained was not different between mice drinking 25% D<sub>2</sub>O and mice drinking tap water. The difference  $D - A$  clearly remained the same, even though  $\bar{\tau}$  was increased by 1.70 h due to the D<sub>2</sub>O treatment (Table 1). Thus, the pacemaker in no way compensates for change in  $\tau$  when this is created by heavy water. In the other experiments,  $\tau$  was changed by prior exposure to different light cycles ( $T = 22$  and  $T = 26$  h). The duration of the after-effects created in this protocol does not permit the measurement of a complete phase-response curve, and we have to be satisfied with the  $\Delta\phi$ -measurement at one or two specified phase points of the circadian cycle. On the other hand, it allows us to make the comparison between identical situations *within* individuals.

We have done this experiment twice. In the first series, light pulses were administered at ct 15 in each of two freeruns. In the second series, light pulses were administered both at ct 15 and ct 18 in each of two freeruns. The experimental protocol used in the first experiment, together with one sample of raw data is illustrated in Figure 4. Ten mice (*Mus musculus*) were for three weeks entrained



**Fig. 5.** Phase shifts due to 15' light pulses at *ct* 15 as a function of  $\tau$  in *Mus musculus*. Experimental protocol as in Figure 4. Lines connect two observations in the same individual, in DD free-runs after entrainment to  $T=26$  (○) and after  $T=22$  (●). Phase shifts are expressed in real time, not, as in Figure 2 normalized in circadian time. (Such normalization would depress the points farthest to the left of  $\tau=24$ , and increase the positive slope.) The linear regression through all points ( $\Delta\phi=0.56$ ,  $\tau=15.05$  h,  $r=0.364$ ;  $p>0.05$ ) is shown as a dashed line. The mean slope of the individual lines is  $1.13 (\pm \text{s.e.} = 0.41)$

to an LD 12:14 cycle, then freerun in DD for 37 days, were entrained to an LD 12:10 cycle, and again released into DD for 25 days. During the first DD freerun two 15' light pulses were given, at *ct* 12 (activity onset) and at *ct* 15 ( $3/24 \times \tau$  h after activity onset). During the second DD freerun only the *ct* 15 pulse was repeated. The *ct* 12 pulse was not repeated since it resulted in phase shifts not larger than ca 1 h. Ten other animals had the same protocol but with reversed order: LD 12:10; DD; LD 12:14; DD.

The phase shifts due to the light pulses at *ct* 15, measured according to Figure 1, are shown as a function of  $\tau$  (slope of the regression through activity onsets on the previous 10 days) in Figure 5. The regression line through the 34 points has a slope of 0.56 h, the coefficient of correlation between  $\Delta\phi$  and  $\tau$  is 0.364 ( $p < 0.05$ ). Here again is the tendency that small  $\tau$ 's are positively correlated with large delaying phase shifts.

This relationship becomes much more striking, however, if we look at the individuals rather than at the pooled data. All 17 animals had shorter  $\tau$  after LD 12:10 than after LD 12:14, as expected, and 14 out of 17 had larger delaying

phase shifts when  $\tau$  was short than when  $\tau$  was long. The average slope for the individual changes of  $\Delta\phi$  with  $\tau$  was 1.13 ( $\pm$ s.e. = 0.41). This implies that each minute of decrease in  $\tau$  within the individual, resulting from prior treatment, is offset by about one minute of increase in the delaying phase shift brought about by a light pulse at ct 15. This initial result gives a strong affirmative answer to the question whether  $\tau$ -changes within the individual are compensated for by changes in the individual's phase response ( $\Delta\phi$ ) to light, at least when the changes in  $\tau$  are created as after-effects, and when  $\Delta\phi$  is measured at ct 15.

The important implications of this experiment made us decide to repeat it with the same protocol, except that light pulses were administered both at ct 15 (on the down-slope of the PRC) and at ct 18 (on the upward slope of the PRC). This time, the average slope for the individual changes with  $\tau$  of  $\Delta\phi$  at ct 15 had precisely the same value as in the experiment a year earlier: 1.13 ( $\pm$ s.e. = 0.48;  $n=15$ ). However, the pulses at ct 18 tended to cause larger phase delays when  $\tau$  was long. The average slope ( $(\Delta\phi_1 - \Delta\phi_2)/(\tau_1 - \tau_2)$ ) was  $-0.62$  ( $\pm$ s.e. 0.65;  $n=13$ ), not significantly different from zero. The difference in the results obtained at ct 15 and at ct 18 shows that the compensatory response is not present at all phases of the circadian cycle.

#### IV. Discussion

Two general results emerge from this study of phase response curves. First, there is a sequence among the four species in their PRC-shape which is related to their average freerunning period ( $\bar{\tau}$ ); as  $\bar{\tau}$  shortens delay responses increase and advance responses decrease. Second, within each species there is a comparable dependence of PRC-shape on  $\bar{\tau}$  of individual animals. Meaning in these clear empirical regularities can be sought in two contexts: (i) do they tell us anything about the structure of the pacemaker involved, and (ii) what is their functional meaning? Functional meaning is to be sought in the mechanism of the entrainment process, which cannot be pursued in detail here until we have explicitly developed the principles of entrainment, including natural photoperiod and the consequences of seasonal change in two-pulse interactions which govern the entrained steady-state (Pittendrigh and Daan, 1976b).

Our evidence on the intraspecific correlation of  $\tau$  and PRC derives from two wholly different experiments, one where PRC's between individuals were compared, and one where  $\tau$  was manipulated within individuals and we measured the response to light at a fixed phase. It is likely that the interindividual differences are at least partly brought about by the intraindividual changes in  $\Delta\phi$  with  $\tau$ . Both of these types of variation are buried in a great deal of noise. It was shown that this is to a large extent due to the error of measurement involved. We went through the trouble of calculating two linear regressions through about 10 activity onsets each for every single  $\Delta\phi$  estimation, and we feel that no analytical improvement is possible to reduce the error further. This noise is partly due to instabilities in  $\tau$ : spontaneous changes in  $\tau$  will clearly affect the "apparent"  $\Delta\phi$  (Lohmann, 1966). It is significant in this context that the greatest amount of noise was found in *Peromyscus maniculatus*, which is the species with the most labile  $\tau$  of all four

(Pittendrigh and Daan, 1976a). The same source of noise may explain why in comparing a few individuals belonging to "short- $\tau$ " and "long- $\tau$ " groups within each species, we only found an indicative trend of what became impressively clear when  $\tau$  was deliberately modified by a major amount within an individual.

There are no data in the literature to support our results. Although phase response curves for short light pulses have been published for several other rodents (DeCoursey, 1960, 1964; Kramm, 1971), the number of animals in these studies has been too small to search for a relationship with  $\tau$ . The problem was, however, addressed by Natalini (1972) in a study of the kangaroo rat, *Dipodomys merriami*. He found in long- $\tau$  (>24 h) animals phase delays near activity onset, and phase advances near activity end, while in short- $\tau$  (<24 h) animals the PRC was a mirror-image with phase delays near activity onset and advances near activity end. Natalini's phase shifts were obtained with 1 h light pulses (35 Lux) against an LL-background (1 Lux). Apart from a possible species difference, it is not excluded that the behaviour of the *Dipodomys* pacemaker would have been different in DD. There is now a clear precedent, in the luminescence rhythm of *Gonyaulax polyedra*, for a shift from delay to advance phase responses at the same phase of the cycle, related to an increase in the background illumination (Christianson and Sweeney, 1973). However, Natalini's comment (1972, p. 156) that the responses he measured were usually transient, and that the original phase was commonly re-established by the third onset after the pulse, leaves the meaning of his data unclear. It suggests that there was no resetting of the pacemaker at all following the—relatively weak—light pulses.

Our own results, though indicating a clear trend, are not sufficient to firmly establish how  $\Delta\phi$  depends on  $\tau$  at all phases. In two experiments, we observed a difference in  $\Delta\phi$  at ct 15 when different  $\tau$ -values were generated by two prior light treatments. In contrast, pulses given at ct 18 did not lead to a significant difference in  $\Delta\phi$ ; if anything the trend was towards larger delay  $\Delta\phi$  at ct 18 when  $\tau$  was increased. The PRC for *Mus musculus* (Fig. 3) has a downward slope at ct 15, an upward slope at ct 18. Hence, these results may indicate that the PRC is shifted over "to the right" relative to the circadian time scale when  $\tau$  is lengthened, and to the left when  $\tau$  is shortened. The phase response curve is a *pacemaker* property, while the circadian time scale refers to phases in the observed *rhythm* (ct 12  $\equiv$  activity onset). Thus while our data may well reflect the expected change in the pacemaker's PRC shape as its  $\tau$  is changed, they are also interpretable in terms of an equally predictable change in the phase-relation between pacemaker and the rhythm it drives—if that rhythm is itself based on a slave oscillation comparable to the *B*-oscillation postulated in the *Drosophila* system (Pittendrigh et al., 1958; Pittendrigh, 1974). On this hypothesis, the activity rhythm would phase lead the pacemaker in long- $\tau$  after-effects, and it would phase lag it during short  $\tau$  after-effects. This interpretation, while conceivably correct, would require the measurement of more phase shifts during after-effects than we produced.

The interpretation would not account for the general trend in the inter-individual comparison of  $\Delta\phi$ : If  $\tau$ -changes merely effect a change in phase relationship between driving and driven rhythm, the total delay (*D*) and advance (*A*) areas of the PRC should remain the same. Yet in all species, there is a tendency for (*D*−*A*) to increase as  $\tau$  decreases. It is of no statistical significance in any

one species, perhaps due to the extent of the measurement error, but nevertheless suggestive by its occurrence in all four (Table 1). Experiments on a large scale would be needed to establish this trend firmly in a single species.

The correlation between  $\tau$  and PRC shape ( $D-A$ ) is unequivocally clear in comparing species, and in our judgment is also real both between and within individuals. If it does indeed obtain in all three cases it would suggest a remarkable feature of pacemaker structure whereby change in  $\tau$  automatically entails a change in PRC shape. We should seek functional meaning in this interdependence of pacemaker properties.

Its detailed consequences in the entrainment phenomenon turn out to be complex (Pittendrigh and Daan, 1976b) and different in the inter- and intra-specific cases. There is, however, a clear general point that is independent of those complexities. When selection either sets  $\bar{\tau}$  further from 24 h or tolerates such a departure it must at least assure that the daily light pulse can generate the phase-shift (positive or negative) necessary to compensate for the difference between  $\tau$  and  $T$  ( $=24$  h). It is then not surprising that as  $\tau$  gets shorter and further from 24 h, that the delay section of the PRC becomes greater. From Figure 3 we can derive (admittedly for the artificial case of entrainment by single pulses of 15 min duration) the range of  $\tau$ -variation which each species PRC could tolerate in the sense of assuring entrainment to  $T=24$  h. As the following listing shows those ranges are in remarkably close agreement with the observed range of  $\tau$  variation in species (Pittendrigh and Daan, 1976a, Fig. 2).

	range of $\Delta\phi + 24$	observed range of $\tau$
<i>M. auratus</i>	22.9–25.9	23.5–24.5
<i>P. leucopus</i>	23.1–24.6	22.7–24.8
<i>P. maniculatus</i>	21.8–24.7	21.7–24.7
<i>M. musculus</i>	21.0–24.8	22.1–24.9

The only other vertebrate species for which a comparable short lightpulse PRC is known, *Ammospermophilus leucurus* (Kramm, 1971), does not fit into this picture. (DeCoursey's 1960 phase response curve for *Glaucomys* was obtained by measuring the transient phase shifts directly following the light pulses, and presumably underestimates advance shifts). In the antelope ground squirrel,  $\tau$  is usually longer than 24 h, and the range of  $\tau$  (Kramm, 1971, Table 2) is 23.6–24.6. The PRC for 10' light pulses (Kramm 1971, Fig. 63) consists almost exclusively of phase delays, and, when analyzed like our own data, would allow entrainment to  $T=24$  only for a  $\tau$ -range from 22.7–24.1. For a diurnal animal, short light pulses may not be as biologically relevant as for nocturnal creatures. Response curves for long light signals in a group of taxonomically and ecologically related species are not known. The PRC of the antelope ground squirrel for six-hour light pulses (Kramm, 1971, Fig. 39) showing large advance phase shifts and small delays, is quantitatively very different from its 15' PRC with nearly only delays. With longer signals, some "parametric" action of the light presumably becomes noticeable, at least in this day-active mammal. Yet in our nocturnal rodents, the entrainment by such long, natural signals can to some extent be adequately simulated

by two light pulses (Pittendrigh and Daan, 1976b). It is in the interaction of both short stimuli, one at dawn and one at dusk, where we shall seek, in a later paper, the functional meaning of the correlation between a species' freerunning period and its system of response to light.

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