university of groningen

University Medical Center Groningen

University of Groningen

A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents. III. Heavy Water and Constant Light

Daan, Serge; Pittendrigh, Colin S.

Published in:
Journal of Comparative Physiology A

DOI:
10.1007/BF01417856

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
1976

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Daan, S., & Pittendrigh, C. S. (1976). A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents. III. Heavy Water and Constant Light: Homeostasis of Frequency? *Journal of Comparative Physiology A*, *106*(3), 267-290. https://doi.org/10.1007/BF01417856

Copyright
Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment.

Take-down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

J. comp. Physiol. 106, 267–290 (1976)

Journal of Comparative Physiology · A
© by Springer-Verlag 1976

A Functional Analysis of Circadian Pacemakers in Nocturnal Rodents

III. Heavy Water and Constant Light: Homeostasis of Frequency?

Serge Daan* and Colin S. Pittendrigh

Department of Biological Sciences, Stanford University, Stanford, California 94305, USA

Received November 7, 1975

Summary. 1. In a preceding paper (Pittendrigh and Daan, 1976a) differences in the lability of the freerunning circadian period (τ) in constant darkness (DD) were described among four species of rodents. This lability (i) is strongly correlated with the responses of τ to (ii) D_2O-administration and to (iii) constant light (LL) of various intensities. The question is raised whether these are three reflections of the action of the same mechanism conserving circadian frequency.

2. A number of qualitative differences exist in the responses to D_2O and LL:

(i) D_2O always decelerates, while LL may decelerate (as in nocturnal rodents) or accelerate circadian rhythms.

(ii) D_2O does not affect the pattern of activity or cause aperiodicity or "splitting" as sometimes observed in LL.

(iii) The magnitude of the response to D_2O is independent of τ in DD (*Mus musculus*); the response to LL is negatively correlated with τ in DD (*Peromyscus maniculatus*).

(iv) The response to D_2O appears subject only to the time constants of the processes of deuteriation and dedeuteriation of body tissues; the response to LL involves long time constants, gradual approach to equilibrium frequency, and "after-effects" upon return to DD.

3. Phase response curves for 15′ light pulses are virtually identical in mice drinking D_2O (25%) and in mice drinking tap water, although their τ's differ by as much as 1.8 h. This is seen as evidence that D_2O-action is not restricted to a specific phase of the circadian cycle.

4. Serum concentrations of D_2O, 12 days after onset of deuteriation of the drinking water are 8.6% in hamsters and 13.9% in C57 mice. The difference accounts for the difference in pacemaker response (change in τ by 20% D_2O: 3.8% and 6.6%, respectively). Thus the response to D_2O is

* Present address: Zoologisch Laboratorium, Rijksuniversiteit Groningen, Haren (Gr.), The Netherlands

related to the characteristics of water metabolism, and species differences do not reflect differences in the homeostatic mechanism conserving frequency.

5. Concerning the action of constant light, no firm conclusion can be made. The long time constants in the response to LL suggest that τ *is* homeostatically protected in the face of alterations in LL-intensity.

6. On the other hand, the strong correlation both among and within species between LL-response and shape of the phase response curve (PRC; linearly transformed in the analysis to a "velocity response curve", VRC) suggests that the change of τ with LL is best explained as an artifact caused by the daily curve of light sensitivity, which itself is necessary for entrainment. PRC-shape and the lability of homeostatic conservation of frequency are believed to be functionally interrelated.

7. "Aschoff's Rule", concerning the differences in response of τ to LL between nocturnal and diurnal animals, is given new support by a literature survey of pertinent data (Table 2). It is again most readily understood as an artifact reflecting different light response curves involved in different strategies of entrainment (Pittendrigh and Daan, 1976b) in nocturnal and diurnal animals.

I. Introduction

In a previous paper we described conspicuous differences between four species of nocturnal rodents (*Mesocricetus auratus*, *Peromyscus leucopus*, *P. maniculatus* and *Mus musculus*) in the long-term stability of their circadian frequency (Pittendrigh and Daan, 1976a). This stability appears to depend on how close $\bar{\tau}$ is to 24 h. Our conclusions were based solely on measurements of τ of the activity rhythm in constant darkness (DD). The question was raised whether these differences in stability reflect differences in the general homeostatic protection of frequency. Is the extent to which τ can be modified directly by certain agents in any way related to the extent of its spontaneous lability? Although the invariance of frequency with a multitude of environmental factors is one of the remarkable adaptive properties of circadian rhythms (Pittendrigh, 1960), there are two classic ways of modifying τ: by varying the light intensity under continuous illumination, and by the administration of heavy water (D_2O). The effects of both agents are analyzed in the present paper.

There are strong indications of an interdependence of several pacemaker parameters: frequency, stability of frequency and shape of the phase response curve for brief light pulses. The differences in phase response curves found among species (Daan and Pittendrigh, 1976) make it possible to pursue an old suggestion (De Coursey, 1959), that the proportionality of delay and advance responses to brief light pulses would dictate how a circadian oscillator responds to constant light.

For procedures of maintenance of the experimental animals and recording of their activity rhythm we refer to the first paper of this series (Pittendrigh and Daan, 1976a).

II. Heavy Water

To measure the effect of D_2O on the frequency of the circadian pacemaker we have kept 73 animals of all four species on either 20% or 25% D_2O, administered via the drinking water. No attempt was made to establish dose response curves for every species. The results of Suter and Rawson (1968) indicate that there is a virtually linear relationship between the change in τ ($\Delta\tau = \tau_{D_2O} - \tau_{H_2O}$) and the concentration of D_2O (from 0 to 30%) administered. In our measurements of $\Delta\tau$, a prolonged DD-freerun (>30 days) always preceded deuteriation, permitting assay of τ_{H_2O} for each individual. The animals remained on D_2O for at least one month, and in some cases for over four months.

a) The Time Course of D_2O Action

Figure 1 illustrates the effect of 20% D_2O on the freerunning circadian rhythm in mice. The animal (*Mus musculus* C-57) in the left panel was subjected to prolonged (4 months) deuteriation, the other animal (*Peromyscus leucopus*, right panel) was returned to tap water after 38 days of D_2O treatment. A pronounced increase in τ was observed immediately upon D_2O administration. After removal of D_2O the white-footed mouse returned gradually to its original freerunning period. τ-estimates from eye-fitted lines through 10 successive activity onsets in prolonged freeruns of mice (Figure 2, upper panel) show that the increase in τ attributable to D_2O is essentially completed by day 10. Also in hamsters most of the change in τ due to deuteriation of the drinking water is completed within ten days (Figure 7).

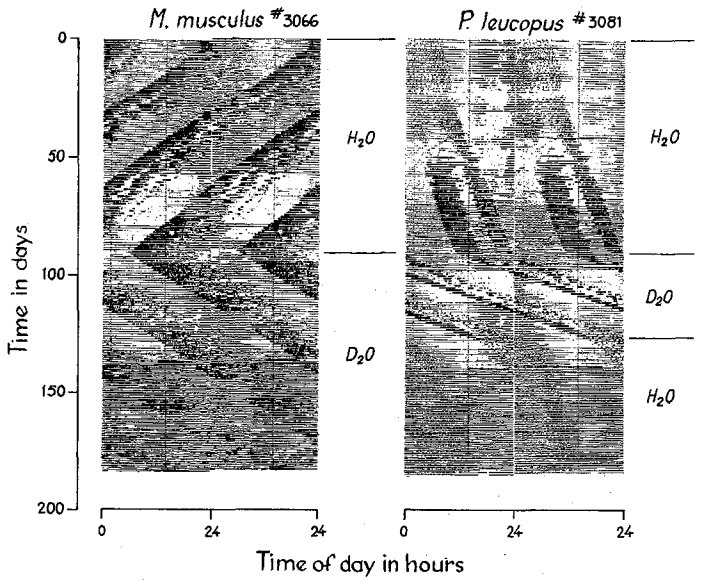

Fig. 1. Examples of raw data showing the effect of 20% D_2O in the drinking water on the circadian rhythm of activity in a mouse (left) and a deermouse (right)

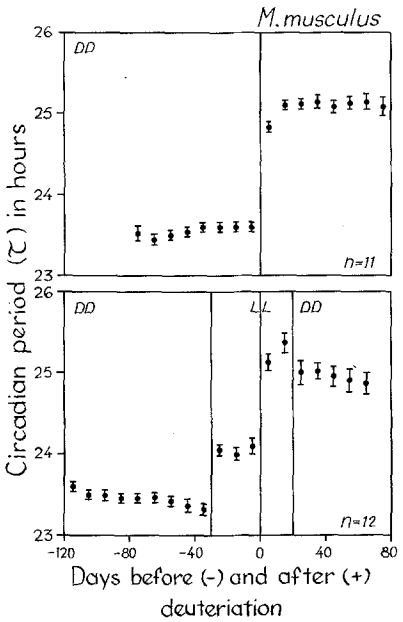

Fig. 2. Effects of deuteriation of the drinking water on τ in *Mus musculus* (DBA and C57 strains). Day 0 = day of administration of 20% D_2O in the drinking water. Upper panel: mice deuteriated in DD. Lower panel: mice deuteriated in LL (1 Lux). For every 10-day interval mean τ values and standard errors are shown, as estimated from eye-fitted lines through successive onsets of activity

To detect the time course of the effect during the first ten days following deuteriation we can only use the length of single cycles of the activity rhythm, i.e., the intervals between consecutive onsets of activity. It is uncertain that these adequately represent pacemaker periods, since (i) day-to-day variations in observed period involve more than variation of pacemaker periods only, as shown by their usually negative serial correlation coefficient (Pittendrigh and Daan, 1976a) and (ii) there may be transients, i.e., cycles of varying lengths due to the overt rhythm slowly regaining a stable steady-state phase relationship with the pacemaker. The average periods for 18 mice (*Mus musculus* 10 DBA; 8 C57) that had rhythms precise enough for this analysis (Figure 3, upper panel) show that most of the D_2O effect is actually completed within the first four cycles. After that the curve levels off at about 106.6% of the $\bar{\tau}$ value expressed when the animals were drinking H_2O.

The lower panel in Figure 3, based on the data of Katz et al. (1962) shows the time course of changing D_2O concentration in mouse urine after heavy water is added to the drinking supply. The approach to equilibrium is similar to the time course of the response in τ. Thus it appears that in the first week after deuteriation we are not looking at transient cycles in the usual sense, as τ steadily increases following D_2O administration: the pacemaker's period, from the outset, appears to be a direct function of the D_2O concentration in body fluids.

The time course of $\bar{\tau}$ change when the animals are returned to pure H_2O is somewhat less prompt as Figure 4 indicates for *Peromyscus leucopus*, which is the only species for which we have the necessary data. The slightly reduced rate of return is probably due to a slow and continuing release of deuterium as it is re-substituted in body tissues by hydrogen.

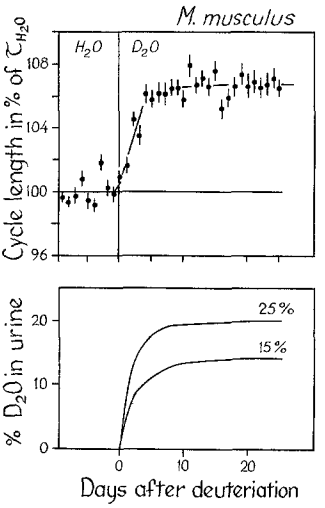

Fig. 3. The time course of deuterium effects on circadian cycle length in *Mus musculus* (DBA and C57 strains). Upper panel: sample means and standard errors of single period lengths measured from onset to onset of activity, and expressed in % of the pre-deuteriation τ. Lower panel: the approach to equilibrium in the urine D_2O concentration of mice following administration of 25% (upper line) and 15% (lower line) D_2O via the drinking water; data from Katz et al. (1962)

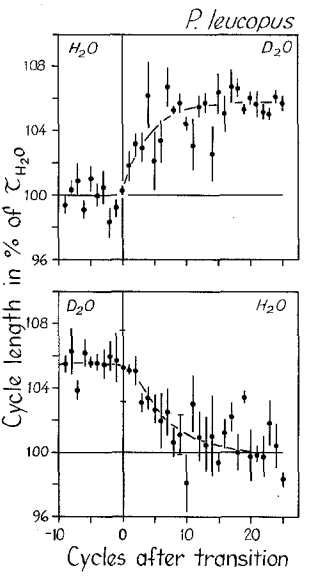

Fig. 4. The time course of deuterium effects on the circadian cycle length in *Peromyscus leucopus*. Sample means and standard errors of single period lengths (measured from onset to onset of activity, and expressed in % of the pre-deuteriation τ) are shown. Upper panel: transition from H_2O to D_2O. Lower panel: transition from D_2O to H_2O

b) Species Differences in the Response to D_2O

In comparing the effect of a standardized (20%) concentration of D_2O on the pacemakers of the four species, we based our estimates of τ_{D_2O} on the pacemaker's behavior during cycles 11–20 after the H_2O/D_2O transition. τ_{H_2O} is estimated from the last 10 cycles prior to the transition. Each τ-value was derived by linear regression through 11 onsets of activity.

Table 1 summarizes the response of all species to 20% D_2O. We include earlier data from Suter and Rawson (1968) on *P. leucopus*, and from Dowse and Palmer (1972) on *M. musculus*. Our values for *P. leucopus* are larger (mean 5.36%)

Table 1. The effect of 20% D_2O on τ in four species of rodents. τ-values were measured by linear regression through onsets of activity in the last 10 days before deuteriation (τ_{H_2O}) and days 11–20 after deuteriation of the drinking water (τ_{D_2O}). Arrows connect significantly (t-test) different sample mean $\Delta\tau$ (expressed in % of τ_{H_2O}) among the upper five samples

Species	Conditions	n	τ_{H_2O} ±s.d.	τ_{D_2O} ±s.d.	$\overline{\Delta\tau}$ ±s.d.	$\frac{100\cdot\overline{\Delta\tau}}{\tau_{H_2O}}$ ±s.d.	$p<0.01$
M. auratus	DD	9	24.09±0.16	25.00±0.21	0.92±0.20	3.80 ±0.82	↑↑↑↑
P. leucopus	DD	4	23.70±0.58	24.98±0.62	1.27±0.07	5.36 ±0.23	↓
P. maniculatus	DD	8	24.09±0.28	25.43±0.40	1.33±0.14	5.53 ±0.53	↑
M. musculus C-57	DD	10	23.42±0.33	24.98±0.33	1.55±0.22	6.63 ±0.95	↓
M. musculus DBA	DD	11	23.52±0.18	25.12±0.27	1.60±0.19	6.80 ±0.83	↓
M. musculus C-57	LL[c]	10	24.25±0.18	25.68±0.30	1.43±0.27	5.91 ±1.10	
M. musculus DBA	LL[c]	6	23.87±0.34	25.28±0.44	1.41±0.28	5.89 ±1.18	
P. leucopus	DD[a]	5	23.74±0.29	24.65±0.39	0.91±0.15	3.81 ±0.59	
M. musculus CF-1	DD[b]	4	23.54±0.36	25.03±0.13	1.49±0.38	5.67 ±0.93	
			h	h	h	%	

[a] Data from Suter and Rawson (1968)
[b] Data from Dowse and Palmer (1972)
[c] In constant light ca 1 Lux

than those reported by Suter and Rawson (1968) for 20% D_2O (mean 3.81%). The same is true for *Mus musculus*, when we compare the effect in our strains (6.80% in DBA and 6.63% in C57) with the $\Delta\tau$ (mean 5.67%) reported by Dowse and Palmer (1972) for CF-1 mice. In both reports τ was measured from intervals shorter after deuteriation, although Suter and Rawson (1968) allowed at least two cycles for transients to subside. Hence the difference of our results with those previously published can be accounted for by the time constants involved in attaining steady state frequency.

The differences between the species are pronounced. The increase in τ caused by 20% D_2O in the drinking water of mice (DBA: 6.8%; C57: 6.6%) exceeds the increase realized in the golden hamster (3.8%) by almost a factor of 2. The two *Peromyscus* species are between these extremes, both significantly different from hamsters and DBA mice.

Table 1 includes results of deuteriation of *Mus musculus* in both constant darkness and constant illumination (1 Lux). This light intensity increases the circadian period of mice to ca. 24 h (Figure 2). In two strains of mice the response to D_2O was slightly less in animals deuteriated in LL ($\overline{\Delta\tau}=5.91$% in C57; 4.89% in DBA) than in animals in DD (6.63% in C57; 6.80% in DBA). In neither strain alone is the difference statistically significant (t-test, $p>0.05$). It is significant ($p<0.02$) when results from the two strains are pooled (average $\Delta\tau$ in DD: 6.72%±s.e. 0.19; in LL: 5.90%±s.e. 0.27). When the DD and LL groups are pooled there is a slight dependence of $\Delta\tau$ on τ_{H_2O}, but that regression is not detectable within either the DD or LL groups separately (Figure 5).

c) The Effect of D_2O on the Pacemaker's Motion

We have asked whether D_2O has a differential effect on the pacemaker at different phases of its cycle: it could conceivably slow only a fraction of the

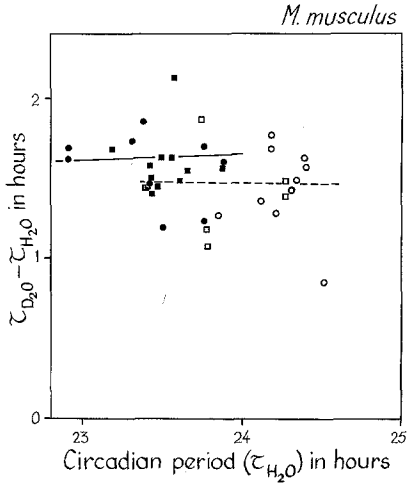

Fig. 5. Changes in circadian period (τ) resulting from deuteriation (20% D_2O) of the drinking water in mice (*Mus musculus*; circles: C57; squares: DBA), plotted as a function of τ before deuteriation. Lines are linear regressions through points measured in DD (slid symbols) and in LL (1 Lux; open symbols)

cycle (even accelerate others) and still effect a net increase in τ. The only assay of the pacemaker's motion other than measurement of the period is its phase response curve (PRC) to resetting stimuli. A PRC measures the steady-state phase shift of a rhythm induced by a single external perturbation, as a function of the time in the circadian cycle when the perturbation is applied. When enough time is allowed for transients to subside, the overt rhythm is phase shifted by the same amount as the pacemaker. Hence, the observed phase shifts characterize points in the cycle of the pacemaker.

We have measured PRC's for 15′ light pulses in *Mus musculus* C57 with and without 25% D_2O in their drinking water. The experimental procedures were detailed in the previous paper (Daan and Pittendrigh, 1976). Phase shifts observed in deuteriated and undeuteriated animals are plotted as a function of circadian time in Figure 6.

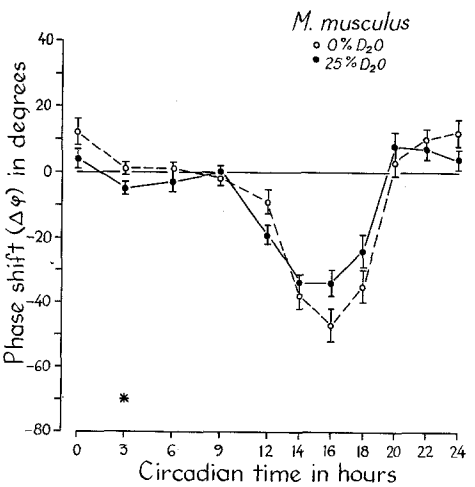

Fig. 6. Phase response curves for 15′ light pulses in mice drinking 0% and 25% D_2O. Mean phase shifts (± 1 standard error) are shown for pulses administered at ten phases (ct) of the circadian rhythm. The asterisk indicates the phase (ct 3) where a significant ($p < 0.01$) difference between the phase shifts of deuteriated and non-deuteriated mice was found

The overall average τ for the D_2O (25%) group during the experiment was 25.21 h (s.d.=0.31; $n=14$), for the H_2O group $\tau=23.43$ (s.d.=0.26; $n=13$), a difference of 7.6% of τ_{H_2O}. The difference between mean $\Delta\phi$'s in the two groups was only significant (*t*-test; $p<0.01$) for the light pulses administered at ct 3. Since this is only one significant result in 10 comparisons, observed moreover in that part of the cycle where the pacemaker is virtually insensitive to light, we do not attach importance to the difference. The total amplitude of the PRC seems slightly less with D_2O than without D_2O. This is partly due to the expression of $\Delta\phi$ in degrees of the whole cycle, since τ is longer with D_2O. If anything, the D_2O has shifted the phase response curve a little to the left, or, since activity onset is used as the phase reference point, D_2O has caused the activity rhythm to slightly phase-lag its pacemaker. On the whole, however, the results are compatible with the hypothesis that the average phase response curve for brief light pulses remains unaffected by deuteriation. Hence, the results contain no indication that D_2O acts differentially on different parts of the pacemaker cycle, or that the net slowing action might involve differential acceleration and deceleration.

III. Constant Light

Constant light (LL) is almost as reliable an agent as D_2O for the experimental manipulation of pacemaker frequency. If LL does not cause arrhythmicity, as in some cases, it usually changes τ from its value in DD. But in contrast with heavy water, light either accelerates or decelerates the rhythm, depending on the organism studied. It has been noted that this effect is correlated with the ecological type, diurnal or nocturnal, to which animals belong (Aschoff, 1960, 1964). In nocturnal animals τ increases with light intensity, in diurnal animals τ decreases ("Aschoff's Rule": Pittendrigh, 1960). Although several exceptions to the rule are known, all twelve nocturnal rodents studied in this respect indeed obey the rule (see Table 2). Of the six diurnal rodents in which the relation is known, only the squirrel *Funambulus palmarum* showed a slight increase of τ with light intensity (Pohl, 1972a). We have done experiments in three species to establish whether they differ in the extent to which τ depends on the light intensity in LL, in a way related to other aspects of their homeostatic protection of frequency: spontaneous lability, susceptibility to age and after-effects, and response to D_2O.

a) The Time Course of LL Action

Figure 7 gives a first indication of significant differences between the mode of action of D_2O and LL on the pacemaker. It shows the time course of change in τ following the transitions from H_2O (DD) to D_2O (DD), and from H_2O (DD) to H_2O (LL) in golden hamsters, which is the only species for which we have strictly comparable data. The rapid response to D_2O (20%) was noted earlier. In contrast, change in τ caused by LL (100–200 lux) continues for at least 60 days, and in some individuals we have found τ continuing to increase for many months (see, e.g., Figure 2A in Pittendrigh and Daan, 1976c).

Table 2. Summary of literature data on the dependence of freerunning circadian period (τ) in various species on the intensity of constant illumination. Symbols indicate whether τ in DD or low light intensity (<1 lux) was longer (+) or shorter (−) than 24 h. $\Delta\tau$ and $\Delta\alpha$ indicate the change in τ and α due to raising the light intensity: 0 = no effect, + = increase, − = decrease, ∪ = intermediate minimum, ∩ = intermediate maximum. The sources of the data in this table will be supplied by the authors on request

	Day-active species	τ	$\Delta\tau$	$\Delta\alpha$	Night-active species	τ	$\Delta\tau$	$\Delta\alpha$
Mammals:	*Homo sapiens*	+	0		*Rousettus aegyptiacus*	+	0	
	Pan troglodytes	−	+		*Glis glis*	−	+	
	Macaca mulatta		∪		*Mus musculus*	−	+	−
	Tupaja belangeri	−	+		*Peromyscus leucopus*	+	+	−
	Funambulus palmarum	+	+	+	*Peromyscus maniculatus*	−	+	−
	Eutamias sibiricus	−	0		*Perognathus intermedius*	−	+	
	Tamias striatus	+	−		*Mesocricetus auratus*	+	+∪	−
	Ammospermophilus leucurus	+	−	0	*Clethrionomys rutilus*	+	+	
	Spermophilus columbianus	+	−		*Microtus oeconomus*	+	+	
	Spermophilus undulatus	+	−		*Glaucomys volans*	−	+	−
					Rattus norvegicus		+	
					Sigmodon hispidus	−	+	
					Microtus ochrogaster		+	
Birds:	*Fringilla coelebs*	+	−	+	*Tyto alba*	+	∪	∩
	Carduelis chloris	+	−	+				
	Carduelis spinus	+	−					
	Acanthis flammea	+	−	+				
	Pyrrhula pyrrhula	+	−	+				
	Carpodacus mexicanus	+	−	+				
	Sturnus vulgaris	0	−	+				
Reptiles:	*Lacerta sicula*	+	−	+				
	Lacerta agilis	+	−					
	Sceloporus olivaceus	+	−					
Fishes:					*Gymnorhamphichthys hypostomus*		+	−
					Hypopomus spec.	−	+	+
Arthropods:	*Geotrupes sylvaticus*	+	+	∩	*Euscorpius carpathicus*	−	+	
	Geotrupes vernalis	−	+		*Admetus pumilio*	+	+	
	Iridomyrmex humilis		+		*Leucophaea maderae*	−	+	
					Byrsotria fumigata		+	+
					Blaberus craniifer	+	+	+
					Gryllus domesticus	−	+	
					Velia caprai	+	+	
					Carabus problematicus	−	∩	
					Carabus cancellatus	−	∩	
					Carabus nitens	−	∩	
					Tenebrio molitor	+	+	
					Periplaneta americana	−	+	

b) Species Differences in the Response to LL

There are some major obstacles in designing an experiment to assay the dependence of τ on the level of constant illumination. The changes of τ with any environmental parameter are notoriously small (Lohmann, 1967). This is one reason to study them intraindividually, thereby removing inter-individual variation from the

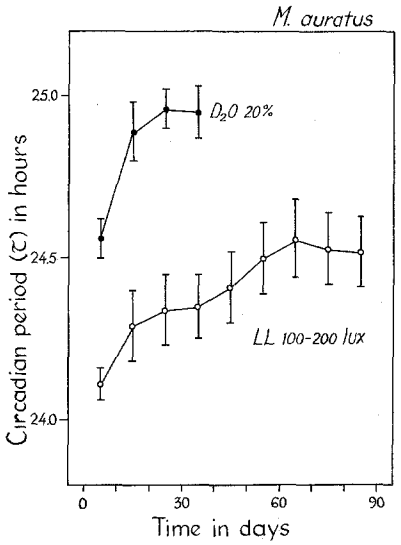

Fig. 7. The time course of the effects of LL (100–200 Lux) and of 20% D_2O on $\bar{\tau}$ in the golden hamster. For every 10-day interval mean $\bar{\tau}$ values and standard errors are shown, as estimated from eye-fitted lines through successive onsets of activity. The LL-data are a representative sample of 12 hamsters that did not show "splitting" in LL. In the D_2O experiment, nine animals were involved

data. Further, one wants to measure steady-state values of τ rather than transient behavior reflecting both the effect of current environmental conditions and of previous treatments. At least the effect of extreme photoperiodic conditions is still traceable in τ-measurements after the animals (*Mus musculus*) have been in constant darkness for well over fifty cycles (Pittendrigh and Daan, 1976a). Figure 7 shows that steady state values of τ in hamsters subjected to LL (100–200 Lux) require some sixty days to reach steady state levels. Long-lasting changes in freerunning period of the circadian rhythm are now documented for

Table 3. Circadian period (τ) and activity time (α) in different intensities of constant light (LL). $\Delta\tau$ is the difference between $\tau_{(DD)}$ and τ (400 Lux)

Light intensity		0	0.005	0.05	0.5	5	400	Lux		$\Delta\tau$
Sequence		1	5	4	3	2	6			
Duration		33	39	51	40	41	24	days	h	% of τ_{DD}
Species										
M. auratus	$\bar{\tau}$	24.14	24.23	24.21	24.28	–	24.44	h	0.33	1.37
	s.d.	0.08	0.24	0.23	0.24	–	0.09	h	0.02	0.07
	n	5	3	3	3	–	3		3	3
	$\bar{\alpha}$	10.7	10.7	10.7	8.8	–	9.5	h		
P. leucopus	$\bar{\tau}$	23.90	23.72	23.96	24.47	24.60	24.88	h	0.96	4.03
	s.d.	0.10	0.61	0.52	0.27	0.28	0.42	h	0.38	1.59
	n	8	7	7	6	8	5		5	5
	$\bar{\alpha}$	11.9	13.8	14.8	13.3	11.6	9.5	h		
P. maniculatus	$\bar{\tau}$	23.19	23.76	23.89	24.23	24.80	24.94	h	1.68	7.28
	s.d.	0.40	0.30	0.38	0.38	0.37	0.15	h	0.59	2.70
	n	7	7	7	7	7	5		5	5
	$\bar{\alpha}$	14.4	16.2	15.2	15.7	10.5	7.2	h		

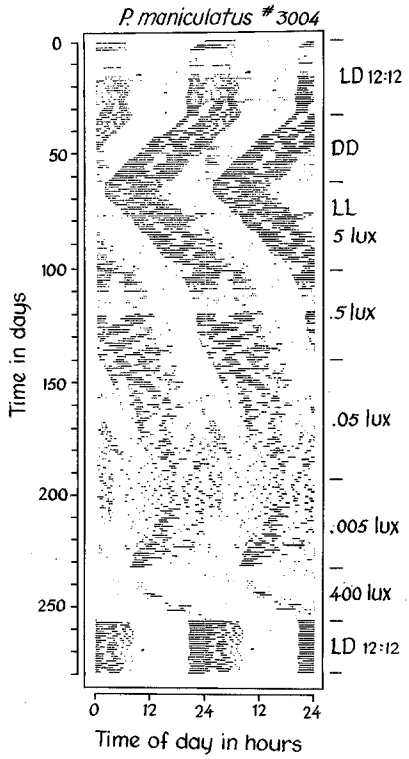

Fig. 8. Example of raw data, showing the experimental protocol used to assess the dependence of τ on the level of constant illumination. Values for τ for each section of the record are obtained from the slope of a line eye-fitted through the onsets of daily activity

several species (Eskin, 1971; Kramm, 1971). Such data imply that a real steady-state frequency for any condition can only be reasonably estimated after several months. In shorter freeruns, the after-effects of previous conditions, even of LD 12:12 entrainment and LL or DD, will modify the results. However, even if one is willing to embark on the tedious and expensive task of studying animals for two to four months in every set of conditions, there are problems. Aging of the experimental animals could not be avoided; and age has a systematic effect on the freerunning period (Pittendrigh and Daan, 1974). A valuable compromise in optimizing experimental design would be to return the pacemaker to a standard entrainment (e.g. LD 12:12) before assaying the effect of any new light intensity on τ freerunning.

In the experiments yielding the data summarized by Table 3 and Figure 9 this was not done. To the best of our knowledge, this is a shortcoming in the design of all other published experiments from which the dependence of τ on light intensity has been derived. In any case, since all three species we studied were subjected to the same sequence of intensities we can derive valid inter-specific comparisons from the experiments. The whole protocol, together with a sample of raw data, is shown in Figure 8.

In choosing the present experimental design as one among many possible compromises, we have introduced one minor artifact. The range of variation of τ_{DD} in *P. leucopus* and *M. auratus* is very small (Figure 9). Both have values

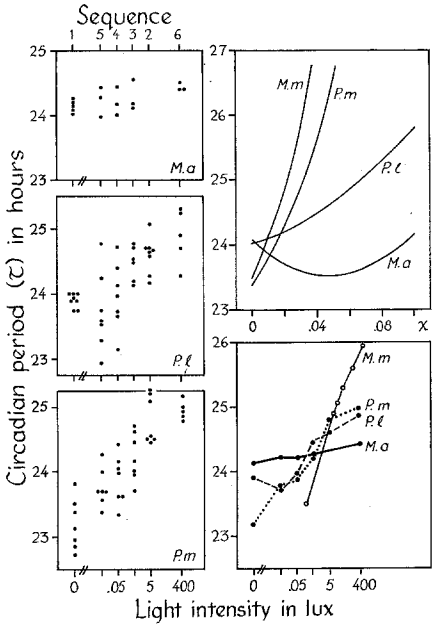

Fig. 9. The dependence of τ on light intensity. Left panels: individual values of τ in DD and in five intensities of constant illumination. Lower right panel: mean values of τ in DD and LL. The data for *Mus musculus* are those of Aschoff (1960, Fig. 4). Upper right panel: change of the circadian period with increasing values of the PRC-transformation factor (X) in four species of rodents, derived as in Fig. 10. M.a. = *Mesocricetus auratus*, P.m. = *Peromyscus maniculatus*, P.l. = *Peromyscus leucopus*, M.m. = *Mus musculus*

clustered slightly below (*P. leucopus*) or above (*M. auratus*) 24.0 h. This is probably partly due to an after-effect of the prior LD12:12 treatment. We have already demonstrated that this after-effect is significant in *P. leucopus*, but less clear in *P. maniculatus* (Pittendrigh and Daan, 1976a, Figure 17). When towards the end of the experiment the animals were returned to very low light intensity (0.005 Lux), interindividual variation in τ had greatly increased in *P. leucopus*.

Although such after-effects are not excluded from the results, we do not hesitate to conclude that there are real differences in the dependence of τ on light intensity in the three species. τ increases most rapidly with increasing light intensity in *P. maniculatus*, slightly less in *P. leucopus*, and only very weakly in hamsters. The lower right panel of Figure 9, summarizing the results, includes Aschoff's (1960) data for *Mus musculus*. These data, though not strictly comparable, indicate that τ is more affected by constant light in this than in any of the other species. In short, the pacemakers of the four species show the same trend in the extent of τ change by LL as they did in the case of D_2O. This correlation seems to suggest that both responses reflect the same aspect of species differences in the "tightness" of homeostatic control. However, there is an entirely different logic, by which the facts in Table 3 and Figure 9 may be understood, as we shall see in the next section.

c) The Effect of LL on the Pacemaker's Motion: Velocity Response Curves?

The action of light in constant conditions can be described as an effect on the angular velocity of the oscillator: it is either speeded up or slowed down by constant light. If one had a system in which the velocity of the clock, as distinct from an overt rhythm driven by it, could be continuously monitored, one might

detect that the rate of acceleration or deceleration is different at different phases, and that the observed effect on τ is the net result of decelerations and accelerations integrated over the whole cycle.

The effect of constant illumination is, by definition, a "parametric" effect (Aschoff 1960; Daan and Aschoff, 1975). The comparatively large phase shifts due to brief light pulses against a background of total darkness are attributed to "nonparametric" effects of the dark-light and light-dark transitions. There is no *a priori* reason to assume that there is a relationship between parametric and nonparametric effects of light. However, it has been suggested (De Coursey, 1959) that the shape of the phase response curve gives a hint of how continuous illumination would affect velocity at each phase. Pittendrigh (1960, p. 176) concluded for nocturnal animals in LL "that this increase in τ is related to the delay section of the phase response curve greatly exceeding the advance section, both in range and especially amplitude". Swade (1969) developed the theoretical construct of a "velocity response curve" (VRC) which describes the change in angular velocity effected by continuous illumination as a function of the phase of the cycle.

The hypothesis that, in constant conditions, light (parametrically) speeds up or slows down the oscillation, dependent on its phase, in a manner qualitatively comparable to its (non-parametric) effect in otherwise total darkness (DD), cannot be tested as long as angular velocity is not experimentally measurable. Yet we can investigate a corollary of the hypothesis. When a species has a phase response curve for brief light pulses characterized by a large delay section (D) and a small advance section (A) we expect a steeper increase of τ with increasing light intensity, than in a species with small D and large A. The major differences among our four species in the shape of their PRC's provides an opportunity to explore this prediction using Swade's concept of a VRC.

We have derived velocity response curves simply by applying an amplitude transformation factor (X) to every point in the PRC (see Appendix). That factor can be thought of as representing the ratio of the light-adapted response (to light) to the dark-adapted response of the system. X must obviously be small (<0.1): were $X=1$, i.e. no transformation of the PRC, then the clock would stop (velocity = zero) where $\Delta\phi = -15'$; if continuous light had exactly the same effect as a 15′ light pulse in DD it would cause zero velocity at this phase point, and the rhythm would never proceed from there as long as the lights remained on. This phase point, where $-\Delta\phi$ equals the duration of the pulse causing this phase shift has been called the "fixed point" (Johnsson and Karlson, 1972). Because the rhythm does persist in constant light of the same intensity, the effect of constant light on the velocity at this fixed point must be smaller than the effect of a light pulse in otherwise constant darkness. Since the photoreceptors involved in entrainment presumably adapt to light, it would indeed be difficult to expect otherwise.

Figure 10 illustrates the principle used in obtaining the predictions. Phase points in the circadian cycle of a hamster which are two hours apart in DD are shown in the uppermost line. The lines below show, for six values of X, how these phase points would be distributed in time if the cycle were accelerated or decelerated as dictated by the phase response curve (above), the amplitude of

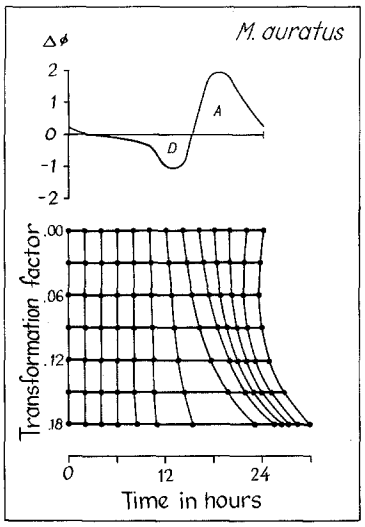

Fig. 10. Hypothetical effect of constant light, using various values of the transformation factor (X) on the circadian rhythm in the golden hamster. Above, the phase response curve for 15′ light pulses is shown (from Daan and Pittendrigh, 1976). Positive values of $\Delta\phi$ are advance phase shifts, negative values are delay phase shifts. Below, the expansion and compression in time of 12 equal parts (30°) of the full (360°) cycle are shown, assuming that the angular velocity is affected by an amount $= X \cdot \Delta\phi(\phi)$. Thin lines connect times at which identical phases of the circadian cycle are reached for various X. Maximal slowing down occurs at phases with large delay phase shifts ($-\Delta\phi$), maximal speeding up at phases with large advance phase shifts ($+\Delta\phi$)

which is transformed by X (see Appendix). Phases where light has a delaying action become expanded, those where light accelerates are compressed. Since the effects are exponential (the more a phase is slowed down, the longer it will receive the light), the expansion between ct 10 and ct 16 rapidly dominates over the compression between ct 16 and ct 24, and τ lengthens with increasing X.

Clearly, a large phase-delay (D) and small phase-advance (A) section in the phase response curve (such as found, e.g., in *Peromyscus maniculatus*; Daan and Pittendrigh, 1976) would produce a greater overall slowing effect due to constant illumination than a small D and a large A (as in the hamsters, Figure 10). This difference is found indeed between deermice and hamsters when transferred from DD to LL at 100–200 Lux (Figure 11).

The dependence of τ on the transformation factor X computed from these velocity response curves and supposedly reflecting the dependence of τ on light intensity in LL is included in the top right panel of Figure 9. Historically, the experiments on τ and light intensity were undertaken only after these "predictions" had been obtained in the three species for which pulse response curves were available at that time. Among these three species, the VRC-computations display the same sequence in the slope of $\tau(X)$ as was later found in the real experiment: the slope is flattest in hamsters (where A exceeds D), steepest in deermice (where D exceeds A). Also the still steeper increase in *Mus musculus* in Aschoff's (1960) data is qualitatively predicted by the very large delay phase shifts in the response curve of this species.

The computed curve for the golden hamster includes a minimum value of τ at some non-zero light intensity. This feature was not found in the range of intensities exploited experimentally. We do not wish to press this point strongly because several simplifications went into the predictions, and minor details in them may not be relevant. Yet it is interesting to note that a $\tau(L)$ curve for hamsters was recently reported (Aschoff et al., 1973, Fig. 5) where τ indeed shortens with increasing low light intensity and then lengthens again. In our

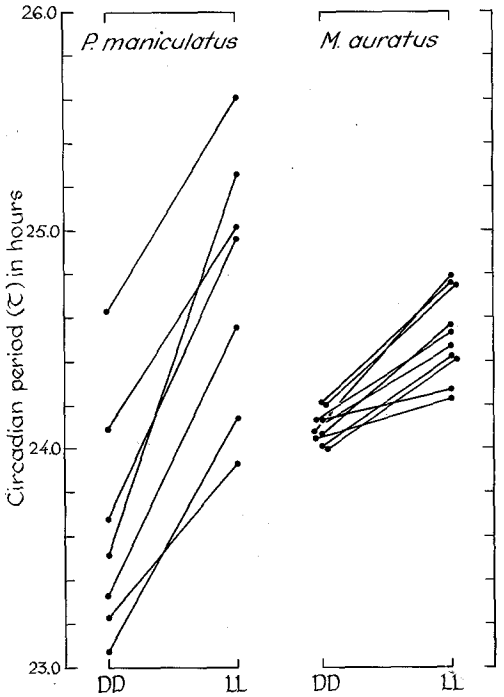

Fig. 11. Changes in circadian period (τ) due to transfer from DD to LL (100–200 Lux) in 7 deermice and 9 golden hamsters

data, τ_{DD} may have been depressed as an after-effect of the previous LD 12:12 entrainment.

There exist also *intraspecific* differences in the phase-response curves for light pulses (Daan and Pittendrigh, 1976). Individual animals with a long τ_{DD} tend to have a PRC with larger advance phase shifts and smaller delay phase shifts than individuals with a short τ_{DD}. Obviously, this would lead to different slopes of $\tau(L)$ if our assumptions were approximately correct. Predictions based on $\tau(X)$ are shown in Figure 12 for *P. maniculatus*, the only species where the inter-individual variation in τ was large enough, both in the PRC data and in the LL results, to warrant such a comparison. The prediction (Figure 12, left panel) is that in animals with long τ_{DD}, τ will increase less with light intensity than in animals with short τ_{DD}. Comparing *P. maniculatus* with short τ_{DD} (<23.00 h) with those individuals with long τ_{DD} (>23.00 h), we find that this is indeed the case (Figure 12, right panel). Long-τ animals in DD have become short-τ animals in 400 Lux and vice-versa.

The hypothesis that the change in τ with light intensity can be understood from the phase response curves is thus upheld both in the interspecific and the interindividual comparison.

IV. Discussion

The analysis of variations in a considerable sample of freeruns in constant darkness (DD) showed that our four species of rodents displayed conspicuous

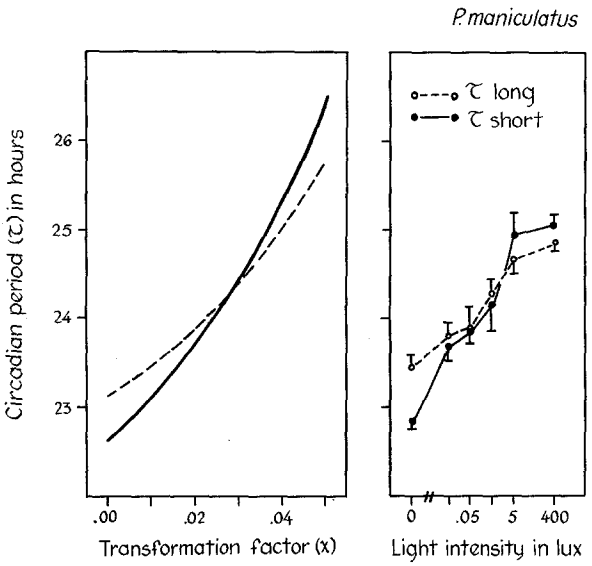

Fig. 12. Intra-specific comparison of τ as a function of light intensity in *Peromyscus maniculatus*. Left panel: predictions of $\tau = f(X)$ based on the pulse response curves for *P. maniculatus* with $\tau_{DD} > 23.0$ (dashed curve) with $\tau_{DD} < 23.0$ (solid curve). Right panel: circles indicate the three animals with long τ_{DD}-values; dots indicate the three animals with short τ_{DD}-values. Mean τ and one standard error are shown for each light intensity

differences in the lability of their circadian pacemakers (Pittendrigh and Daan, 1976a). The variations of τ, both intra- and interindividually, whether attributable to age, prior conditions or unknown ("spontaneous") causes, is small in *Mesocricetus auratus* and *Peromyscus leucopus*, large in *Mus musculus* and *Peromyscus maniculatus*. The results reported above show the same trend among the species in their response to two external agents long known to affect circadian frequency: Both constant light (LL) and heavy water (D_2O) evoke the smallest changes in τ (relative to τ in DD, H_2O) in hamsters, the largest changes in *P. maniculatus* and *Mus musculus* (Table 4).

This is a rather astonishing correlation. Does it mean that the response to LL and D_2O, as well as the lability of frequency in DD, reflect the same aspect of homeostatic control of the pacemakers? Such interpretation would imply that the mechanism in the hamster responsible for the conservation of its τ within narrow limits would also compensate for the effect of a wholly unnatural agent like deuterium. Various considerations and qualitative differences between the action of D_2O and LL invoke further suspicion.

(i) While D_2O has a universal slowing effect on circadian rhythms[1], LL can either accelerate or decelerate, depending on the organism studied.

[1] The slowing effect is known in circadian rhythms of a variety of organisms (*Protozoans*: Bruce and Pittendrigh, 1960; McDaniel et al. 1974; *Plants*: Bünning and Baltes, 1963; Brenner and Engelmann, 1973; Maurer and Engelmann, 1974; Engelmann et al., in prep.; *Arthropods*: Enright, 1971; Pittendrigh et al., 1973; Pittendrigh and Caldarola, 1973; Caldarola, 1973; Caldarola and Pittendrigh, 1974; *Birds*: Palmer and Dowse, 1969; Snyder, 1969; *Mammals*: Suter and Rawson, 1968; Palmer and Dowse, 1969; Dowse and Palmer, 1972) as well as in several biological rhythms of higher frequency (Enright, 1971; Brogårdh and Johnsson, 1974)

Table 4. Interspecific differences in pacemaker properties

	Species:	*M. auratus*	*P. leucopus*	*P. maniculatus*	*M. musculus*	
Period	$\bar{\tau}$	24.04	23.99	22.92	23.43	h
Lability	s.d. τ	0.08	0.09	0.30	0.15	h
Phase response curve	D-A	−73	22	143	206	h x degrees
LL-effect	$\Delta\tau$ (400 Lux)	1.37	4.03	7.28	(largest)	%
D_2O-effect	$\Delta\tau$ (20%)	3.80	5.36	5.53	6.63	%

(ii) While high light intensity LL often causes aperiodicity, D_2O has never been observed to abolish circadian rhythms. A hamster drinking up to 50% D_2O shows a normal rhythm, except that its period ($\tau = 25.5$ h) is far out of the known species range (Richter, in prep.).

(iii) While LL (100–200 Lux) elicits "splitting" of the activity rhythm into two components in one out of every two hamsters (Pittendrigh and Daan, 1976c), this has never been observed in connection with D_2O.

(iv) There is no evidence of a change in the distribution of activity ($\alpha:\rho$ ratio; total amount of activity) with D_2O as there is with LL.

(v) While our evidence suggests that individuals with short τ_{DD} show the steepest increase ($\Delta\tau$) in τ with increasing LL-light intensity (Figure 12) no dependence of $\Delta\tau$ on the previous τ in H_2O is found when animals are exposed to D_2O (Figure 5).

(vi) The phase-response curves for brief light pulses of mice drinking tap water and mice drinking 25% D_2O are statistically indistinguishable (Figure 6). This result is compatible with the hypothesis that all phases of the oscillator's motion are equally slowed down by D_2O. At least one interpretation of the slowing action of light (see Section III, c) suggests that it may be the net effect of differential deceleration and acceleration.

(vii) The time constants of D_2O action on the circadian rhythm are similar (Figure 3) to published figures (Katz et al., 1962) on the time course of deuteriation of body fluids in mice. Hence, in the course of attaining equilibrium, the average circadian period observed seems to be a linear function of the instantaneous D_2O concentration in the body. Similarly, the small after-effect of D_2O treatment in *P. leucopus* (Figures 1, 4) is explainable by the gradual removal of deuterium atoms from body tissues. In contrast, LL produces long-lasting after-effects in the same species (Pittendrigh and Daan, 1976a); the approach to steady-state τ-values is much slower in LL than for D_2O (Figure 7), even though the "instantaneous light intensity" stays of course constant, while the "instantaneous D_2O concentration" in the body does not.

a) Tissue D_2O Concentration and Pacemaker Response

How, then, are we to understand the difference between the responses of hamsters and mice to the same D_2O concentration in the drinking water? One possibility is that hamsters had smaller tissue D_2O concentrations than mice. Metabolic water from stored resources and undeuteriated nutrients keeps the equilibrium

Table 5. Serum concentrations of D_2O in mice and hamsters drinking 20% D_2O. Average values ± standard deviation; number of animals in parentheses. p values are based on two-tailed t-tests

Days of D_2O *administration*	*Mesocricetus auratus*	*Mus musculus*	p
12	8.65 ± 1.24 % (6)	13.91 ± 0.52 % (4)	< 0.001
33	12.81 ± 0.15 % (6)	–	< 0.05
40	–	13.07 ± 0.18 % (4)	
p	< 0.001	< 0.02	

concentration of D_2O in body fluids and urine (Figure 3, lower panel) significantly below that in the drinking water. Enright (1971) has already noted that differences between animals in the amount of metabolic water might have a significant effect on the change in τ caused by a standardized concentration administered. After termination of the experiments we therefore explored the possibility of species differences in the equilibrium concentration of D_2O in body fluids. The infra-red spectographic method described by Crespi and Katz (1961) was used to measure D_2O concentration in serum.

Table 5 shows that there are indeed large differences between hamsters and C-57 mice in these concentrations, most significantly so among the samples investigated after 12 days of deuteriation. The time course of the approach to equilibrium concentrations is apparently different between the species. The few samples analyzed leave much to clarify in the details of this time course, as compared to the time course of pacemaker response. However the results from the samples analyzed after 12 days completely account for the species differences in $\Delta\tau$ measured between days 10 and 20. Normalizing the $\Delta\tau$-values from Table 1 on the basis of these serum-concentrations, we find that the change in τ per 10% serum D_2O is 4.4% in hamsters and 4.8% in C57 mice.

Thus the results contain no indication that the homeostatic mechanism involved in conserving τ within narrow – though specifically different – limits, also reduces the effect of D_2O. There is no disagreement with the hypothesis that in all species the departure from τ_{H_2O} is a straight linear function of the instantaneous tissue D_2O concentration. The results further support the view of various other authors (Bruce and Pittendrigh, 1960; Suter and Rawson, 1968; Enright, 1971) that the time constants in the action of D_2O on circadian rhythms exclude mechanisms involving secondary isotopic substitution. Deuterium incorporation as organically bound hydrogen is a slow process, especially in the brain (Katz et al., 1962), where accumulating evidence suggests the circadian pacemaker for activity is located (see Rusak and Zucker, 1975). On the other hand, deuterium rapidly crosses the blood-brain barrier (Bering, 1952), and presumably has virtually instantaneous access to the tissue fluids in the pacemaker.

In conclusion, the discrepancies between the D_2O response of various species are related to differences in "effective doses". They are in agreement with Enright's (1971) view that the virtual identity of dose-response curves for D_2O among different organisms may reflect some fundamental similarity in the mechanism of their rhythms. On the other hand, our estimates of $\Delta\tau$ per 10% D_2O serum

concentration (*M. auratus*: 4.4%; *M. musculus*: 4.8%) considerably exceed the 2.11% Enright (1971) reported for *Excirolana chiltoni:* and by the same argument, the difference could be taken as an indication of different fundamental properties of crustacean and mammalian circadian pacemakers, until actual body concentrations of D_2O in the isopod have been measured.

b) Constant Light: the Significance of Aschoff's Rule

The magnitude of the response to constant illumination is correlated (i) with the lability of τ in DD and (ii) with the phase response curve to short light pulses. Both correlations exist when species are compared, and are indicated in the interindividual comparison in deermice (*Peromyscus maniculatus*) where variability among individuals is large enough. There is a suggestion that the correlation of the LL-response with PRC-shape is better than the correlation with lability: while *Peromyscus maniculatus* has the most labile τ, *Mus musculus* in Aschoff's (1960) data had a steeper increase of τ with LL than in any of our three species, exactly as predicted by the large delay part in its phase response curve. The match between observation and prediction based on the VRC approach (Figures 9 and 12) is evidence that PRC's are indeed a valuable guide to the action of continuous light, perhaps the more so because of the simplifying assumptions that were adopted in making the simulations. They surely involve some elements that are incorrect and, collectively, are incomplete.

First, we assumed that we were looking at the effect of sensory adaptation when deriving τ_{LL} from pulse response curves, implying that the same receptor system was involved. Alternatively, one might presume that the large phase shifts by short light pulses are mainly due to phasic input from the retina, while the small changes of τ with the intensity of constant light are undoubtedly produced by tonic input. Recent work in lizards, where extraretinal photoreception is involved in the entrainment process, suggests that parametric and nonparametric effects may be mediated by different photoreceptors (Underwood and Menaker, in preparation).

Second, the assumption of a constant "habituation factor" may be far from realistic. Nothing is known on circadian rhythms of vertebrate retinal function in constant conditions. Findings in invertebrates of freerunning rhythms of retinal pigment migration (Jahn and Crescitelli, 1940; Wada and Schneider, 1968; Aréchiga, 1974) and of the ERG-response to short light stimuli (Aréchiga and Wiersma, 1969; Page and Larimer, 1975) make us cautious in expecting constant ratios of dark-adapted versus light-adapted photoreceptor response.

Third, LL clearly effects other phenomena, like splitting, a change in amount of activity, in activity time (α), which do not follow from the phase response curves. Especially the gradual approach to a new steady state τ after a change in LL and the after-effects when a system is returned to DD probably are not related to photoreceptor adaptation. If there is differential acceleration and deceleration of parts of a single oscillator's motion, it is clearly not the whole story, and other, qualitatively different effects must be involved.

These other effects, which we shall address in an attempt to develop a general

qualitative model of the pacemakers (Pittendrigh and Daan, 1976c) should, however, not distract from the notion that much of the action of constant light is related to an animal's phase response curve. The phase response for short light pulses has clear functional significance, and several PRC-characteristics can be considered adaptive to different modes of entrainment of organisms to the external world (Pittendrigh and Daan, 1976). Is, then, the general difference between the response of diurnal and nocturnal animals to constant light, summarized in "Aschoff's Rule", indeed an artifact related to different strategies of entrainment in these groups? A conclusive answer awaits further experimentation, especially in a day-active group of animals. Of course, exceptions to the rule have been observed, but in the majority of diurnal species studied, τ is negatively correlated with light intensity and virtually all nocturnal species have a positive correlation (Table 2). While some of the exceptions are certainly real, in other cases little is known about long-term changes and after-effects of prior treatments, which may have contributed to unexpected results. As Eskin (1969) has demonstrated in the sparrow (*Passer domesticus*), the effect of a change in background illumination may be superimposed on a spontaneous trend in τ.

While we feel confident that there is some general validity, despite the exceptions, to Aschoff's Rule, the available facts limit us to a much less general statement about the light phase response curves of diurnal and nocturnal animals. Two phase response curves, using six-hour light pulses, were published, for *Passer domesticus* (Eskin, 1971) and the ground squirrel *Ammospermophilus leucurus* (Kramm, 1971). Both show, to different extents, a dominance of advance phase shifts over delay phase shifts, and would lead us indeed to expect a decrease in τ with increasing LL-light intensity. On the other hand, although Kramm (1971) saw only few significant phase shifts when he used 15′ (100 Lux) pulses, most of these were phase delays.

In summary, the remarkable agreement of species difference in their pacemaker's response to D_2O and constant illumination with the general lability of τ in DD initially suggested to us that they all are related to the same homeostatic mechanism of τ-conservation. In the case of D_2O this is certainly a coincidence, as different responses are due to different concentrations of D_2O in the body. In the case of LL the homeostasis explanation is certainly not excluded. On the other hand, the effect of light intensity, condensed in Aschoff's Rule, which in some models of circadian rhythms is considered a prerequisite for entrainment (Wever, 1965) is most readily understood as an artifact related to the sensitivity of the pacemaker to stimuli of the light dark cycle, necessary for entrainment.[2] In the next paper in this series (Pittendrigh and Daan, 1976b) we will discuss how far phase response curves indeed adequately describe the daily resetting process in nature, and how various properties of the circadian pacemaker are functionally interrelated in entrainment strategies.

---

[2] The hypothesis of velocity response curves corresponds closely to the "autophasing" hypothesis (Brown, 1972, Palmer, 1974) proposed to reconcile external timing-theory with the fact that circadian rhythms typically have periods different from 24 h, and is therefore prone to misinterpretation. While different circadian periods may indeed be related to different environmental conditions, and to varying sensitivity to these conditions in the course of the pacemaker's motion, such phenomena would add nothing to the nonexisting evidence for external driving of circadian oscillations.

Appendix: The Derivation of Velocity Response Curves

We have assumed, for simplicity, that the effect of constant light on the angular velocity is a constant fraction X of the effect of a 15′ light pulse in constant darkness (DD). We define angular velocity as the part (in degrees of arc) of a full cycle (360°) passed through per hour. In constant darkness (DD), where the period is τ,

$$V_{DD} = 360/\tau \text{ degrees/h}.$$

In 15′, the oscillation moves forward over

$$90/\tau \text{ degrees}.$$

A light pulse of 15′ administered at phase ϕ produces a phase shift of $\Delta\phi\,(\phi)$ circadian hours or $15 \cdot \Delta\phi(\phi)$ degrees; or, the oscillation moves forward during this pulse over

$$90/\tau + 15 \cdot \Delta\phi(\phi) \text{ degrees}$$

i.e. with an angular velocity of

$$V(\phi) = 360/\tau + 60 \cdot \Delta\phi(\phi) = V_{DD} + 60 \cdot \Delta\phi(\phi) \text{ degrees/h}.$$

The assumption made for the velocity response in constant light (LL) is then:

$$V_{LL}(\phi) = V_{DD} + X \cdot 60 \cdot \Delta\phi(\phi) \text{ degrees/h},$$

where X is a monotonic positive function of the light intensity, and $X = 0$ when the light intensity is zero.

It is clear that $V_{LL}(\phi) \leqq 0$ when

$$X \cdot 60 \cdot \Delta\phi(\phi) \leqq -360/\tau,$$

or

$$\Delta\phi(\phi) \leqq -6/X \cdot \tau.$$

For $X = 1$ and $\tau = 24$ h, this happens when $\Delta\phi(\phi) \leqq -0.25$ h. Hence, when constant light acted in the same way ($X = 1$) as a light pulse in DD the clock would stop ($V_{LL} = 0$) at the phase where a 15′ light pulse creates a 15′ phase delay (Johnsson and Karlson's "fixed point"). Constant light would "fix" the pacemaker at this point. For any level of constant illumination where the rhythm persists,

$$V_{LL}(\phi) > 0 \quad \text{for all } \phi,$$

i.e.

$$X < -\frac{6}{\tau \cdot \Delta\phi(\phi)} \quad \text{for all } \phi,$$

or

$$X < -\frac{6}{\tau \cdot \Delta\phi_{min}},$$

where $\Delta\phi_{min}$ is the minimum $\Delta\phi$ or the maximum phase delay.

Assuming the independence of X from ϕ, and given an empirical pulse phase response curve for 15′ light pulses we can calculate for various X the duration of

increments on the circadian cycle. The time needed for an increment of 3.75 degrees (=0.25 circadian h) is:

$$\delta t(\phi) = 3.75/V(\phi),$$

i.e.

$$\delta t(\phi) = 3.75 \Big/ \left(\frac{360}{\tau} + X \cdot 60 \cdot \Delta\phi(\phi)\right).$$

The prediction on $\tau(X)$ is then easily derived by computer-integration of $\delta t(\phi)$ over the whole circadian cycle:

$$\tau(X) = \int_0^{360} 3.75 \Big/ \left(\frac{360}{\tau} + X \cdot 60 \cdot \Delta\phi(\phi)\right) d\phi.$$

The period of the rhythm, thus derived, is a *convex* function of X. This is shown by the fact that the second X-derivative of the integrant is always positive:

$$\frac{d^2}{dX^2}\left(3.75 \Big/ \left(\frac{360}{\tau} + X \cdot 60 \cdot \Delta\phi(\phi)\right)\right)$$
$$= 7.5(60 \cdot \Delta\phi(\phi))^2 \Big/ \left(\frac{360}{\tau} + X \cdot 60 \cdot \Delta\phi(\phi)\right)^3,$$

which is positive as long as $\frac{360}{\tau} + X \cdot 60 \cdot \Delta\phi(\phi) > 0$, which is true unless the clock stops ($V_{LL} \leqq 0$). Hence, regardless of the shape of the pulse response curve, one will never obtain an intermediate maximum in τ over a range of X-values.

When the area under the advance part of the PRC is denoted as A, the area under the delay part as D, it can easily be shown that the function $\tau(X)$ has a minimum:

at $X = -\infty$	if $0 = A < D$	
at $-\infty < X < 0$	if $0 < A < D$	(as in *P. leucopus*, *P. maniculatus* and *M. musculus*)
at $X = 0$	if $0 < A = D$	
at $0 < X < \infty$	if $0 < D < A$	(as in *M. auratus*)
at $X = \infty$	if $0 = D < A$	

Hence, for positive X, only the $\tau(X)$ curve in the hamster goes through a minimum (Fig. 9, top right panel).

References

Aréchiga, H.: Circadian rhythm of sensory input in the crayfish. In: The neurosciences: Third study program. Vol. 44 (eds. F. O. Schmitt, F. G. Worden), pp. 517–523. Cambridge, Mass.: MIT Press, 1974

Aréchiga, H., Wiersma, C. A. G.: Circadian rhythm of responsiveness in crayfish visual units. J. Neurobiol. **1**, 71–85 (1969)

Aschoff, J.: Exogenous and endogenous components on circadian rhythms. Cold Spr. Harb. Symp. quant. Biol. **25**, 11–28 (1960)

Aschoff, J.: Die Tagesperiodik licht- und dunkelaktiver Tiere. Rev. suisse Zool. **71**, 528–558 (1964)
Aschoff, J., Figala, J., Pöppel, E.: Circadian rhythms of locomotor activity in the golden hamster (*Mesocricetus auratus*) measured with two different techniques. J. comp. physiol. Psychol. **85**, 20–28 (1973)
Bering, E.A.: Water exchange of central nervous system and cerebrospinal fluid. J. Neurosurg. **9**, 275–287 (1952)
Brenner, W., Engelmann, W.: Heavy water slows down the photoperiodic timing of flower induction in *Chenopodium rubrum*. Z. Naturforsch. **28c**, 356 (1973)
Brogårdh, T., Johnsson, A.: Oscillatory transpiration and water uptake of Avena plants. III. Action of heavy water on the oscillations. Physiol. Plant. **31**, 112–118 (1974)
Brown, F.A.: The "clocks" timing biological rhythms. Amer. Scientist **60**, 756–766 (1972)
Bruce, V.G., Pittendrigh, C.S.: An Effect of Heavy Water on the Phase and Period of the Circadian Rhythm in *Euglena*. J. cell. comp. Physiol. **56**, 25–31 (1960)
Bünning, E., Baltes, J.: Zur Wirkung von schwerem Wasser auf die endogene Tagesrhythmik. Naturwissenschaften **50**, 622 (1963)
Caldarola, P.C.: The interaction of temperature and heavy water on circadian pacemakers. Ph.D. thesis, Stanford Univ. 186 pp. (1973)
Caldarola, P.C., Pittendrigh, C.S.: A test of the hypothesis that D_2O affects circadian oscillations by diminishing the apparent temperature. Proc. nat. Acad. Sci. (Wash.) **71**, No. 11, 4386–4388 (1974)
Crespi, H.L., Katz, J.J.: The determination of deuterium in biological fluids. Analyt. Biochem. **2**, 274–279 (1961)
Daan, S., Aschoff, J.: Circadian rhythms of locomotor activity in captive birds and mammals: their variations with season and latitude. Oecologia **18**, 269–316 (1975)
Daan, S., Pittendrigh, C.S.: A functional analysis of circadian pacemakers in nocturnal rodents. II. The variability of phase response curves. J. comp. Physiol. **106**, 253–266 (1976)
DeCoursey, P.J.: Daily activity rhythms in the flying squirrel, *Glaucomys volans*. Ph.D. Thesis, University of Wisconsin 162 pp. (1959)
Dowse, H.B., Palmer, J.D.: The chronomutagenic effect of deuterium oxide on the period and entrainment of a biological rhythm. Biol. Bull **143**, 513–524 (1972)
Enright, J.T.: Heavy water slows biological timing processes. Z. vergl. Physiol. **72**, 1–16 (1971)
Eskin, A.: The sparrow clock: Behavior of the freerunning rhythm and entrainment analysis. Ph.D. Thesis, University of Texas (Austin) 224 pp. (1969)
Eskin, A.: Some properties of the system controlling the circadian activity rhythms of sparrows. In: Biochronometry (M.Menaker, Ed), pp. 55–80. Washington: Nat. Acad. Sci. 1971
Jahn, T.L., Crescitelli, F.: Diurnal changes in the electrical response of the compound eye. Biol. Bull. **78**, 42–52 (1940)
Johnsson, A., Karlsson, H.G.: The *Drosophila* eclosion rhythm, the transformation method and the fixed point theorem. Report, Lund Institute Techn. 51 pp. (1972)
Katz, J.J., Crespi, H.L., Czajka, D.M., Finkel, A.J.: Course of deuteration and some physiological effects of deuterium in mice. Amer. J. Physiol. **203**, 907–913 (1962)
Kramm, K.R.: Circadian activity in the antelope ground squirrel, *Ammospermophilus leucurus*. Ph.D. Thesis, U.C. Irvine, i-xviii, 267 pp. (1971)
Lohmann, M.: Ranges of circadian period length. Experientia (Basel) **23**, 788–790 (1967)
Maurer, A., Engelmann, W.: Effect of D_2O on the circadian rhythm of petal movement of *Kalanchoe*. Z. Naturforsch. **29c**, (1974)
McDaniel, M., Sulzman, F.M., Hastings, J.W.: Heavy water slows the *Gonyaulax* clock: A test of the hypothesis that D_2O affects circadian oscillations by diminishing the apparent temperature. Proc. nat. Acad. Sci. (Wash.) **71**, 4389–4391 (1974)
Page, T.L., Larimer, J.L.: Neural control of circadian rhythmicity in the crayfish. II. The ERG-Amplitude Rhythm. J. comp. Physiol. **97**, 81–96 (1975)
Palmer, J.D.: Biological clocks in marine organisms: the control of physiological and behavioral tidal rhythms. New York: Wiley, 1974
Palmer, J.D., Dowse, H.B.: Preliminary findings on the effect of D_2O on the period of circadian activity rhythms. Biol. Bull **137**, 388 (1966)
Pittendrigh, C.S.: Circadian rhythms and the circadian organization of living systems. Cold Spr. Harb. Symp. quant. Biol. **25**, 159–184 (1960)

Pittendrigh, C.S., Caldarola, P.C.: General homeostasis of the frequency of circadian oscillations. Proc. nat. Acad. Sci. (Wash.) **70**, 2697–2701 (1973)

Pittendrigh, C.S., Caldarola, P.C., Cosbey, E.S.: A differential effect of heavy water on temperature-dependent and temperature compensated aspects of the *Drosophila pseudoobscura* circadian system. Proc. nat. Acad. Sci. (Wash.) **70**, 2037–2041 (1973)

Pittendrigh, C.S., Daan, S.: Circadian oscillations in rodents: a systematic increase of their frequency with age. Science **186**, 548–550 (1974)

Pittendrigh, C.S., Daan, S.: A functional analysis of circadian pacemakers in nocturnal rodents. I. The stability and lability of spontaneous frequency. J. comp. Physiol. **106**, 223–252 (1976a)

Pittendrigh, C.S., Daan, S.: A functional analysis of circadian pacemakers in nocturnal rodents. IV. Entrainment: pacemaker as clock. J. comp. Physiol. **106**, 291–331 (1976b)

Pittendrigh, C.S., Daan, S.: A functional analysis of circadian pacemakers in nocturnal rodents. V. Pacemaker structure: a clock for all seasons. J. comp. Physiol. **106**, 333–355 (1976c)

Pohl, H.: Die Aktivitätsperiodik von zwei tagaktiven Nagern, *Funambulus palmarum* und *Eutamias sibiricus*, unter Dauerlichtbedingungen. J. comp. Physiol. **78**, 60–74 (1972)

Rusak, B., Zucker, I.: Biological rhythms and animal behavior. Ann. Rev. Psychol. **26**, 137–171 (1975)

Snyder, L.R.G.: Circadian rhythms and sun navigation in homing pigeons. Undergr. Thesis, Harvard Univ. (1969)

Suter, R.B., Rawson, K.S.: Circadian activity rhythm of the deermouse, *Peromyscus*: effect of deuterium oxide. Science **160**, 1011–1014 (1968)

Swade, R.H.: Circadian rhythms in fluctuating light cycles: toward a new model of entrainment. J. theor. Biol. **24**, 227–239 (1969)

Wada, S., Schneider, G.: Circadianer Rhythmus der Pupillenweite im Ommatidium von *Tenebrio molitor*. Z. vergl. Physiol. **58**, 395–397 (1968)

Wever, R.: A mathematical model for circadian rhythms. In: Circadian clocks (ed. J. Aschoff) pp. 49–63. Amsterdam: North Holland, 1965