## STUDIORUM PROGRESSUS

# Ranges of Circadian Period Length[1]

It seems certain that constant conditions of light and temperature did not play a major role in the evolution of circadian rhythms. Constant environments are uncommon, except in the deep sea; furthermore it seems unlikely that under such conditions a circadian rhythm would be of significant adaptive value. Diurnal periodicities as we find them today in a great variety of biological functions must be regarded in all cases rather as adaptations to cyclic environmental conditions. The recognition of properties of circadian systems as observed under artificial constant conditions of the laboratory are nevertheless of importance for the understanding of circadian mechanisms. Two major properties of circadian rhythms, observable only in experiments under constant conditions of light and temperature certainly are of broad significance. (1) The long term persistence of the periodicities and (2) the deviation of period length from the exact 24-h cycle of the natural day. Both have been interpreted as indications of a self-sustained basis for circadian rhythms.

The early finding (JOHNSON[2]), that the intensity of constant light influences the period length of circadian rhythms, has often been regarded as an interesting but minor side effect with no significance for the fundamental process of entrainment to environmental cycles. However, since ASCHOFF[3] summarized the effects of light intensity upon the changes in free-running period length, it has become increasingly evident that the behavior summarized in 'ASCHOFF's rule' or the 'circadian rule' is so general that it must be regarded as possible, if not probable, that these effects are involved in the entrainment to a Zeitgeber.

Though the influence of light intensity upon period length, as described by ASCHOFF's rule, has been demonstrated qualitatively in many cases, little is known about quantitative relationships between light intensity and period length on a supraindividual basis. If there are, as postulated above, any relationships between ASCHOFF's rule and the process of entrainment, it seems worthwhile to seek more quantitative information about the range of possible influences of light intensity upon circadian period length. In this article it will be shown that with sufficient experimental material it is possible to derive a quantitative 'endogenous range' of free-running periods; it is hoped that this information will have a direct bearing on the properties of the circadian system responsible for synchronization. (See discussion).

The effect of light intensity upon circadian frequency has been described by ASCHOFF[3] as follows: 'When the intensity of illumination...is increased light active animals will increase their spontaneous frequency while dark active animals decrease it.' For a quantitative investigation of this rule the dark active insect *Leucophaea maderae* (Blattaria) is a suitable object for several reasons: under all investigated light intensities the average circadian period ($\tau$) of running activity can easily be determined with an accuracy of about ± 5 min, based on observations over 2–4 weeks. (Other organisms often become aperiodic with light intensities that may still have a – non-detectable – effect on $\tau$.) Also the range of light intensities which influences period length is very small in *Leucophaea* with an upper limit of $< 5$ lux (or even $< 0.5$ lux, see also ROBERTS[4]) which eliminates experimental problems associated with heat production by the lighting. In preliminary experiments it was found that out of 16 animals only 1 showed a slight increase in period length (about 4 min) after the light intensity was increased from 0.5 to 5 lux. In the following paragraphs, all data with light intensities above 0.5 lux were pooled (LL) and compared with those derived under conditions of total darkness (DD).

The running activity of 32 adult *Leucophaea* males was recorded simultaneously. Two kinds of running wheels (8 of each) were utilized and 16 mechanically stable devices in which the activity was transformed into electrical impulses by means of changes in capacity. No significant differences with respect to period ($\tau$) or period changes ($\Delta\tau$) could be observed with the 2 registration methods. 141 $\tau$ values have been evaluated. These data, grouped in classes of 6 min (0.1 h) are presented in the diagram of Figure 1. The DD experiments are represented by 77 $\tau$ values and 64 $\tau$ values come from LL experiments. The ordinate in this Figure represents the % frequency of $\tau$ values/6 min class, for LL and DD values treated separately. The shape of the curves more or less approximates a normal distribution. The mean values of the 2 distributions with their standard deviations are: $\tau_{DD} = 23.84 \pm 0.24$ h, $\tau_{LL} = 24.32 \pm 0.27$ h. The difference between the 2 mean values is, of course, statistically significant ($P < 0.01$).

It is evident that *Leucophaea* has a very narrow circadian range, compared, for example, with finches, where $\Delta\tau$ values of 2–3 h are not uncommon. Even lumping all values in Figure 1, the standard deviation about the general mean of 24.08 h is only ± 0.35 h, i.e. about ± 1.5% deviation/period. It is interesting in this connection to note that human mechanical methods of time measuring allowed only a precision of ± 1%/day, until the pendulum was discovered in the 17th century (see BONANOMI[5]).

In the literature of circadian rhythms one can find many observations on highest and lowest $\tau$ values under

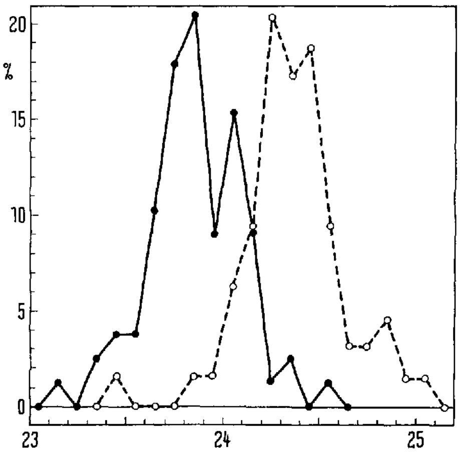

Fig. 1. Relative frequency distribution (% values as ordinate) of free-running periods of the cockroach *Leucophaea maderae* ●——● periods in constant darkness (N = 77); o-----o periods in constant light (N = 64).

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[1] Supported by a NATO fellowship.

[2] M. JOHNSON, J. exp. Zool. *82*, 315 (1939).

[3] J. ASCHOFF, Pflügers Archges. Physiol. *270*, 9 (1959).

[4] S. K. ROBERTS, J. cell. comp. Physiol. *55*, 99 (1960).

[5] J. BONANOMI, Naturwissenschaft und Medizin *3*, 22 (1966).

| Species | Illumination (lux) | Range of periods (h) | Author |
|---|---|---|---|
| *Homo sapiens* | | 24,5–25,9 | Aschoff and Wever 1962[6] |
| *Rattus norvegicus* | DD-LL | 24,0–27,0 | Folk, Brown et al.[7–8] |
| *Mus musculus* | 0,3–200 | 22,5–25,9 | Aschoff[9–11] |
| *Microtus ochrogaster* | DD-250 | 24,0–24,2 | Calhoun[12] |
| *Peromyscus leucopus* | DD-258 | 22,8–25,4 | Johnson[1], Rawson[13] |
| *Peromyscus Bardii* | DD-LL | 23,3–24,7 | Pittendrigh, unpublished |
| *Glaucomys volans* | DD-10 | 23,0–25,0 | DeCoursey[14,15] Aschoff[16] |
| *Mesocricetus auratus* | DD-20 | 23,9–24,8 | Aschoff[17], Pittendrigh, unpublished |
| *Sigmodon hispidus* | DD-250 | 23,7–24,5 | Calhoun[12] |
| *Tamias striatus* | DD-5 | 23,0–26,3 | Rawson[13] |
| *Fringilla coelebs* | 0,2–120 | 21,7–24,9 | Aschoff[3], Aschoff et al[18] |
| *Pyrrhula pyrrhula* | DD-LL | 22,0–26,0 | Aschoff[19] |
| *Sturnus vulgaris* | 0,6–320 | 22,5–24,3 | Aschoff[17], Hoffmann[20] |
| *Tyto alba* | 0,002–0,9 | 23,6–25,4 | Aschoff[16], Hoffmann[21] |
| *Lacerta sicula* | DD-420 | 22,5–24,8 | Hoffmann[22,20] |
| *Lacerta agilis* | DD-80 | 22,2–24,5 | Hoffmann[22] |
| *Leucophaea maderae* | DD-5 | 23,1–25,1 | Lohmann |
| *Byrsotria fumigata* | DD-250 | 23,9–25,5 | Roberts[4] |
| *Periplaneta americana* | DD-11 | 23,8–24,5 | Roberts[4] |
| *Velia currens* | DD-700 | 24,2–27,5 | Rensing[23] |
| *Tenebrio molitor* | 0,01–100 | 23,3–27,3 | Lohmann[24] |
| *Geotrupes silvaticus* | DD-15 | 24,0–24,7 | Geisler[25] |

various light intensities. However, in most cases it is not stated whether the intensities used can be regarded as limiting (i.e. beyond saturation value, as in *Leucophaea*), nor is it evident whether the cited example was an exception compared with other individuals of the same species. Nevertheless I have tried to compile the data from several species for comparative purposes, making no pretense of completeness.

The data are summarized in Figure 2 which gives the circadian ranges of period length for a variety of animals in which the circadian period has been determined by means of actograms of their running activity. The solid bars in the upper part of the diagram connect only extreme values, which in some cases were taken from reports by different authors. In the Table the species are tabulated in the sequence of their graphical presentation. In addition to the $\tau$ values, the light intensities and the authors are cited in this Table. The lower intensity as well as the lower period value has been generally mentioned first although in light active animals the smaller $\tau$ value usually is associated with the higher intensity of illumination.

It must be emphasized that most data on which Figure 2 is based were not derived from studies comparable in extent to those of Figure 1, so that the given range as well as its relative position (e.g. asymmetrical to 24 h) cannot always be regarded as reliable. For this reason, individual values are less interesting than the cumulative curve in the lower part of the diagram, which was derived by adding the species whose circadian range fully or partly covered a certain period length (the hours being divided into classes of 20 min). The sum of every class is given in % of all species. It is not surprising that the curve has its maximum at 24 h although the circadian range of many species seems to be asymmetrical to 24 h. The extremes extend from 21.8–27.5 h which can be expressed in % as a deviation of − 9.2% and + 14.6%. The total range of circadian periods for the cited animals therefore can be stated as less than 25%.

Summarizing these results it may be said that the interspecific range of circadian periods of locomotor activity approximates 24 h ± 12% whereas the interindividual variation (within one species) lies between 4% and 15%. Compared with other biological parameters the circadian period length can be regarded as relatively precise.

*Discussion.* Preliminary experiments with *Leucophaea* (Lohmann, unpublished) suggest that in this species the range of entrainment is not much larger than the range of free-running period. In one case a Zeitgeber period of 23 h only produced relative coordination (Aschoff[26]); in other cases LD periods of 26 h did not entrain the activity rhythm. These investigations, however, should be extended and confirmed, since it has been shown in other organisms that the range of entrainment can be appreciably larger than the range of free-running periods (Tribukait[27]), suggesting that there are additional influences upon $\tau$ of either the light to dark (and/or the dark to light) transition or additive effects of the light

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[12] J. B. Calhoun, Ecology *26*, 250 (1945).

[13] K. S. Rawson, Photop. Rel. Phen. (Ed. Withrow; Wash., AAAS 1959), p. 791.

[14] P. J. DeCoursey, Cold Spring Harb. Symp. quant. Biol. *25*, 49 (1960).

[15] P. J. DeCoursey, Z. vergl. Physiol. *44*, 331 (1961).

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[17] J. Aschoff, Cold Spring Harb. Symp. quant. Biol. *25*, 11 (1960).

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[19] J. Aschoff, Z. vergl. Physiol. *35*, 159 (1953).

[20] K. Hoffmann, Z. vergl. Physiol. *43*, 544 (1960).

[21] K. Hoffmann, *Circadian Clocks* (Ed. J. Aschoff; North Holland Publ. Co. 1965), p. 87.

[22] K. Hoffmann, Z. vergl. Physiol. *37*, 253 (1955).

[23] L. Rensing, Z. vergl. Physiol. 44, 292 (1961).

[24] M. Lohmann, Z. vergl. Physiol. *49*, 341 (1964).

[25] M. Geisler, Z. Tierpsychol. *18*, 389 (1961).

[26] J. Aschoff, *Circadian Clocks* (Ed. J. Aschoff; North Holland Publ. Co. 1965), p. 262.

[27] B. Tribukait, Z. vergl. Physiol. *38*, 479 (1956).

fraction and the dark fraction. The latter would then suggest a response curve to constant light similar in principle to the well studied response curves to light flashes.

It is obvious that further investigations of both ranges of entrainment and of ranges of free-running periods are necessary before any firm generalizations can be made. Very few experimental studies of ranges of entrainment have been published (Bruce[28]) and in most of those cases, data are not available on the range of free-running periods for the same organisms[29].

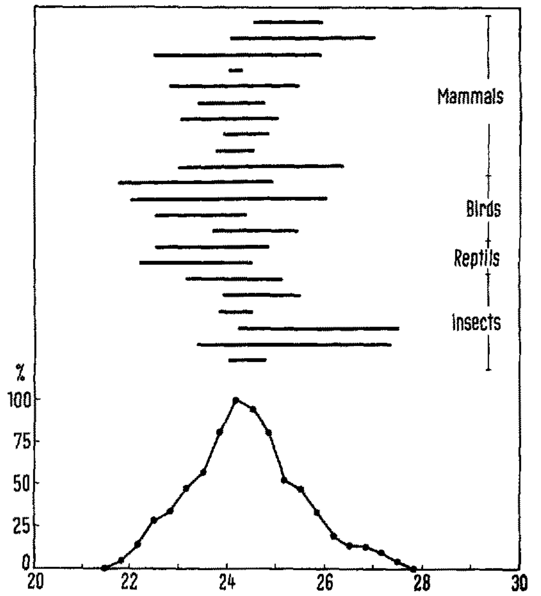

Fig. 2. The ranges of free-running periods (abscissa) of 22 species as compiled from the literature (ordered according to sequence in Table). The curve at the bottom summarizes the data (as % of all species) with which each portion of the period regime (20 min classes) is included within the several ranges of free-running periods. For example, the period ranges of 11 species overlap 23.3 h; 11 species 50%.

*Zusammenfassung*. Quantitative Untersuchungen zur Häufigkeitsverteilung circadianer Periodenlängen der Schabe *Leucophaea maderae* zeigen, dass diese Art ein sehr enges Periodenspektrum besitzt. Die lichtbedingten Abweichungen von 24 h betragen im Mittel nur ± 0,35 h oder 1,5%. Die Befunde werden mit Angaben der Literatur verglichen.

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[29] The author thanks Dr. C. S. Pittendrigh for the possibility to work in his laboratory and to use his excellent facilities.

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## PRO EXPERIMENTIS

### Use of Mixture of Ethanol–*n* Butanol–Xylene in Paraffin Method

During the study of cytology and development of some Indian Rusts Fungi infecting the leaves of their host plants, it was noted that during the monsoon season with high humidity, the transfers of dehydrated tissues in 100% ethanol to xylene, caused turbidity, perhaps due to the absorption of atmospheric humidity by absolute ethanol. While attempting to overcome this difficulty, the author noticed that the ethanol, which normally causes turbidity with xylene in high humid atmospheres, is easily miscible with it only in presence of *n* butanol.

Based on these observations, the author incorporated the following changes using the mixture of ethanol–*n* butanol–xylene, in conventional paraffin method. This method proved good for transferring the tissues of rust-infected leaves, without any turbidity or any other damage to the tissue, neither was the staining quality of the tissue affected. Moreover, the tissue remained soft during the process and the brittleness which is usually associated with xylene was avoided. Furthermore, the author also noticed that xylene is easily miscible with even much lower grades of ethanol, as much as 70% ethanol, in the presence of *n* butanol, without any fogginess being imparted to the mixture.

The use of the following working schedule is suggested to overcome the fogginess usually associated with transfers from 100% ethanol to xylene in humid atmosphere, and to avoid the brittleness of the tissue usually associated with xylene.

*Working schedule*. The material dehydrated through ethanol grades to 100% ethanol and ready to be transferred to xylene is to be processed as follows: (1) a mixture of ethanol–*n* butanol-xylene (Ebxol) (1:1:1) 6 h; (2) *n* butanol–xylene (1:1) 1–4 h; (3) xylene pure 1 h; (4) xylene pure.

The material is now ready for the further process of paraffin infiltration. The material may be left in the second step of mixture of *n* butanol and xylene but not in xylene pure[1].

*Zusammenfassung*. Es wird eine für die histologische Technik nützliche Methode beschrieben, um bei der Überführung von entwässertem Gewebe aus absolutem Alkohol in Xylol bei hoher Luftfeuchtigkeit Trübungen zu vermeiden.

A. V. Sathe

*Maharashtra Association for the Cultivation of Science, Poona 4 (India), 23rd March 1967.*

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