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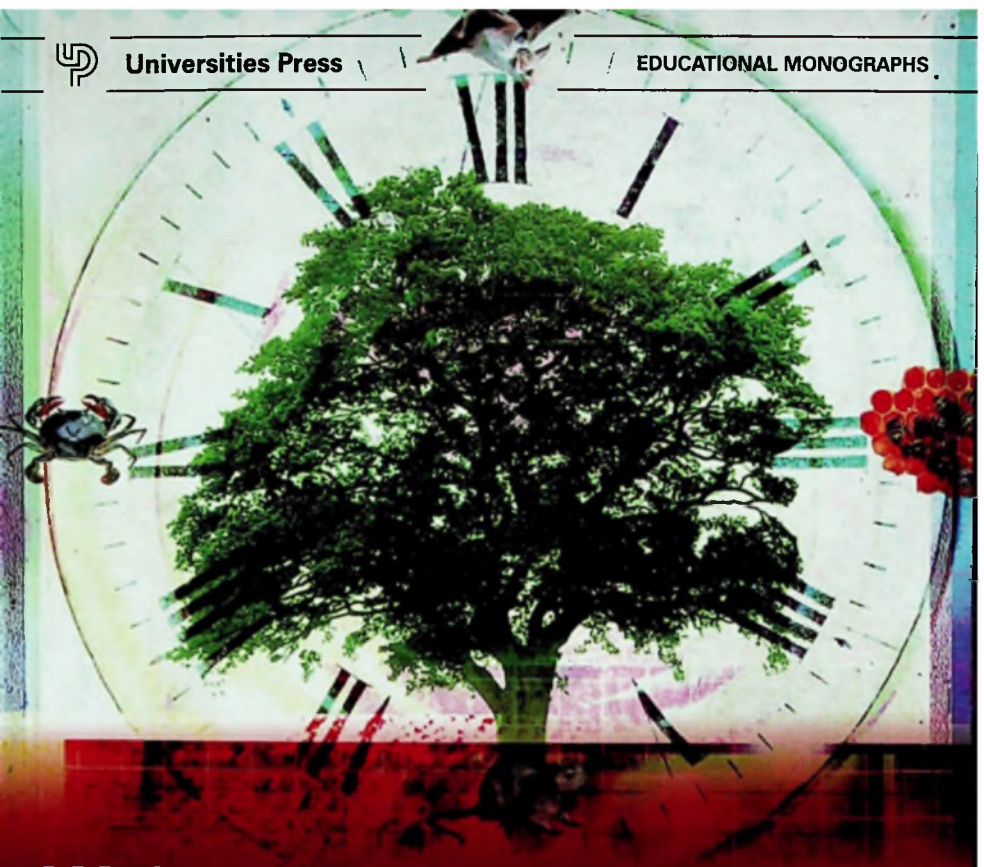

M K Chandrashekaran

Time in the Living World

Jawaharlal Nehru Centre for
Advanced Scientific Research

Time in the Living World

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Educational Monographs

Time in the Living World

M K Chandrashekaran
Evolutionary and Organismal Biology Unit
Jawaharlal Nehru Centre for Advanced Scientific Research
Bangalore 560 064
India

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Universities Press

Universities Press (India) Private Limited

Registered Office
3-5-819 Hyderguda, Hyderabad 500 029 (A.P.), India
e-mail: *hyd2_upilco@sancharnet.in*

Distributed by
Orient Longman Private Limited

Registered Office
3-6-752 Himayatnagar, Hyderabad 500 029 (A.P.), India

Other Offices
Bangalore / Bhopal / Bhubaneshwar / Chennai
Ernakulam / Guwahati / Hyderabad / Jaipur / Kolkata
Lucknow / Mumbai / New Delhi / Patna

First published 2005

Cover and book design

ISBN 81 7371 546 7

Set in Caxton Bk BT 10 on 12 by
OSDATA, Hyderabad 500 029

Printed in India at
Orion Printers Private Limited
Hyderabad 500 004

Published by
Universities Press (India) Private Limited
3-5-819, Hyderguda, Hyderabad 500 029

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FOREWORD

The Jawaharlal Nehru Centre for Advanced Scientific Research established by the Government of India in 1989 as part of the centenary celebrations of Pandit Jawaharlal Nehru, has completed fifteen years of its existence. The Centre is an autonomous institution devoted to advanced scientific research. It promotes programmes in chosen frontier areas of science and engineering and supports workshops and symposia in these areas. It also has programmes to encourage young talent. The Centre has now been recognised as a Deemed University by the University Grants Commission.

In addition to the above activities, the Centre has a programme of publishing high quality Educational Monographs written by leading scientists and engineers in the country addressed to students at the graduate and postgraduate levels, and the general research community. These are short accounts introducing the reader to interesting areas in science and engineering in an easy-to-read manner so that further study in greater depth and detail are facilitated.

This monograph is one of the series being brought out as part of the publication activities of the Centre. The Centre pays due attention to the choice of authors and subjects and style of presentation, to make these monographs attractive, interesting and useful to students as well as teachers. It is our hope that these publications will be received well both within and outside India.

M.R.S. Rao

PREFACE

This book is not a conventional monograph on biological clocks, since it describes only my own work beginning with my researches for the PhD degree in 1960 until 1996, carried out in three continents specifically on intertidal crabs, fruitflies, bats, mice and humans. It will be immediately clear to the reader that the choice of 'case studies' is not entirely fortuitous. The only red thread holding them together is the circadian organisation of their biology and behaviour.

All through my career I have worked on the single problem of how plants, animals and humans measured time. This is certainly an oddity in the fast changing world of biology. A monograph like the present one could not be stimulating reading if it is just a compilation of publications. So I have written a somewhat detailed introduction on biological clocks, which owes much to Erwin Bünning's 3rd English edition of the first monograph on the subject, *The Physiological Clock*, the first German edition of which appeared in 1958.

I stumbled into the field of biological rhythms when the study of rhythms did not even have a name tag and was treated worse than palmistry and astrology today. Now the scientific study of biological rhythms is called chronobiology and is presently the subject of countless papers in journals such as *Nature*, *Science* and *Cell* in addition to those that are featured in speciality journals such as the *Journal of Biological Rhythms, Chronobiology International, Journal of Circadian Rhythms* and *Biological Rhythm Research*. The 'clock' genes have been identified in a fungus, a plant, *Drosophila* and mammals including humans. I have not sought to give the latest developments in the field, which is largely in molecular genetics of circadian rhythms, for which the reader is advised to read frontline scientific journals or turn to the internet. There is a flourishing society, the Society for Research on Biological Rhythms (SRBR) in the USA, a European Society for Chronobiology and several national societies. The Gordon Research Conferences and the SRBR deliberate on the subject on alternate years. The journal *Science* in a December 1998 issue ranked some findings in the field of biological clocks as the first runner-up-breakthrough of the year. Paul Shaw (2003) wrote, 'The successes achieved by chronobiologists over the past decade have been the envy of the scientific community. Indeed it is not uncommon for scientists from a veriety of disciplines to cite the advances

in circadian rhythms as a proof of concept that they too will be able to unravel the mechanisms underlying a particular trait of interest".

This book has been a long time in the writing, mainly because I was collating all the older work by myself. Vidyanand Nanjundiah and R Gadagkar felt very strongly that the details of my career, especially after my return to India and setting up a laboratory, the only one of its kind anywhere, was bound to be of interest to younger scientists in India. Gadagkar had even set an example by writing a whole book on his work on the primitively eusocial paper wasp (Gadagkar, R. 2001. *The Social Biology of ropalidia marginata: Toward Understanding the Evolution of Eusociality.* Massachusetts, USA: Harvard University Press, Cambridge). In deference to Nanjundiah's and Gadagkar's suggestions, I had first included in the various chapters, details of my life and the circumstances and people who influenced me at the University of Madras, where I did my PhD and the University of Tübingen and Berkeley, University of California, where I had done postdoctoral work. The result was that the first version read like a monograph embedded in a memoir, and in retrospect it would have been very distracting to a reader who wanted to get at the science.

The dedicated copy editors at the Universities Press Pvt. Ltd. convinced me that I first narrate all I had to say about *Time in the Living World* and then write a last chapter 'Looking Back' of how it all came about. I am grateful to them for this practical advice. Therefore, the circumstances, ambience and my reminiscences of men and matters are all knit together in the last chapter from memory, not always the most accurate source of information. I hope at least some of the young readers who read this book will find it exciting to work on the problem of biological time-keeping, if it is 6in the laboratory, out in nature, within caves or even, inside a human isolation facility.

ACKNOWLEDGEMENTS

I am grateful to Erwin Bünning and Jürgen Aschof for the crucial role they played in my career; Hubert Markl for much early encouragement (and invitations to his laboratory at the University of Konstanz). John Barnabas and O Siddiqi reassured me early, that my own work on *Drosophila*, entirely carried out in the West, and the line of study I had initiated in Madurai were unique and important. Adventures and sustained achievement in science are possible only if one can find students who can go with one all the way. In this respect I was fortunate to have, among others, R Subbaraj, G Marimuthu, Dilip Joshi, N Viswanathan, L Geetha and Vijay Kumar Sharma as students. They cheerfully helped me to perform fascinating experiments in the Department of Animal Behaviour and Physiology, School of Biological Sciences, MKU (1975–1996). S Krishnaswamy made everything I did at MKU possible. Most of the post-1996 papers cited in the chapters on the circadian rhythms in *Drosophila* and *Mus booduga* are a testimony to the tremendous enthusiasm, enterprise and energy of V K Sharma, V Sheeba and A Joshi who have helped me lay the foundations for the Evolutionary and Organismal Biology Unit in this Centre, the best of its kind in India. The DAAD, DFG, Alexander von Humboldt-Stiftung, the Miller Institute for Basic Research in Science at UC, Berkeley, UGC, CSIR and the DST supported my work. Vidyanand Nanjundiah and Renee Borges read earlier drafts of this book and suggested many changes and corrections. A V Nagarathnamma gave me much advice on the preparation of the manuscript. N Mukunda suggested the title of the book, critically read some portions of the manuscript, and patiently waited. I am thankful to C N R Rao, for spontaneous help and encouragement at various stages of my career in India, for bringing me to this Centre and encouraging me to write this monograph.

July 2005 M K Chandrashekaran

1. INTRODUCTION

❄

Go wondrous creature, mount where science Guides,
Go measure earth, weigh air, and state the tides.
Instruct the planets in which orbs to run,
Correct Old Time, and regulate the sun.

ALEXANDER POPE

❄

Life on earth evolved in an environment which is spectacularly periodic. The periodicity is a consequence of planetary revolutions. The revolution of the earth on its own axis causes the 24-hour (h) day with its alternating day and night cycles. The revolution of the moon around the earth once every 29 days, causes the lunar month, the diurnal and semidiurnal ocean-tides. The earth itself revolves around the sun once in 365 days accounting for the seasons and the calendar year. Plants, animals and humans have adapted themselves in the course of millions of years of evolution to the geophysical and planetary cycles. Such adaptations express themselves in various biological rhythms such as (i) the ubiquitous approximately 24 h cycle, (ii) tidal and lunar rhythms and (iii) circannual rhythms. The study of how organisms are physiologically adapted to the *temporal* order of their environment is called chronobiology just as the study of organic adaptations to the *spatial* order of the environment is the central concern of ecology. But modern ecology incorporates both space and time in its coordinates.

The most frequently noticed biological rhythms are the so-called sleep movements of the leaves of plants. The leaves droop at night and assume a horizontal position during the day (Fig. 1.1) presumably for more efficient photosynthesis.

Several more adaptive physiological and seasonal functions are linked to these sleep movements (see Sections 1.7 and 1.8). Such sleep movements may also be seen in our common cotton plant, *Gossypium hirsutum*. Individual plants of the same species, and even different leaves of the same plant, can have varied periods of leaf movements. A period length in sleep movement rhythms is measured as the number of hours from one midnight position of the leaf until the next midnight position, the midnight position being indicated by the maximal drooping position of the leaf. Periods vary between 23 and 26 h. Bünning (1932) took two such plants of *Phaseolus multiflorus*, plant "*a*"

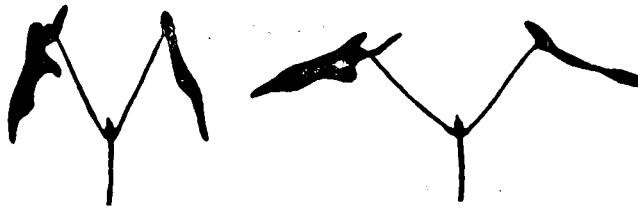

Fig. 1.1 *Phaseolus coccineus* (bean) leaves in (a) night and (b) day positions. There is a pulvinus between the leaf-blade and the stem. The antagonistic swelling of the two halves of the pulvinus causes the 'sleep' movements of the leaves. (After Bünning, 1958).

with a period of 23 h and plant "*b*" with a period of 26 h. He then crossed *a* and *b*, and the leaves of the F1 had an intermediate period of 25 h. The results indicated that the leaf movement rhythm had a Mendelian genetic basis. This was the first experimental demonstration of the genetic basis for endodiurnal rhythms, as circadian rhythms were then referred to in German literature. Several decades later, Konopka and Benzer (1971) isolated mutants in *Drosophila melanogaster* with significantly altered circadian periods. In each case, the period length was altered both for the rhythms in eclosion (emergence of adult insect from the puparium) and the locomotor activity. These mutant phenotypes suggest the presence of at least two and possibly three different genes, all of which map to the X chromosome (Jackson, 1993).

Halberg (1959) coined the expressive term *circadian* to describe endogenous daily rhythms, which were until then variously called daily, diurnal, endodiurnal and diel rhythms. The term derives from the Latin *circa:* about; *dies:* day. The experimental era of modern chronobiology may be said to have started in 1960 when the prestigious Cold Spring Harbour Symposium on Quantitative Biology deliberated on the subject of "Biological Clocks" with E.Bünning as Chairman (Chovnick, 1960). For the first time biological rhythm researchers from all corners of the world came together and attempted a collation of information and analysed the significance of rhythmic phenomena in the adaptation of organisms to their environment, and arrived at a broad agreement about nomenclature and terminology. Practically all the future leaders in the field of chronobiology were present at this landmark symposium. This was also the last time that the unfortunate controversy about the endogenous versus exogenous origin of circadian rhythms was seriously and scientifically discussed. Brown, however, continued to claim that exogenous factors such as cosmic ray showers, electro- and magnetostatic fluctuations incident upon the rotation of the earth on its axis, entrained circadian rhythms until his last breath (see Brown, 1974; Bünning and Chandrashekaran, 1975; 1998b).

Today several laboratories all over the world are working on the molecular biology of circadian rhythms. A leading molecular biologist wrote, "Some of us have been attracted to the study of biological rhythms because they are uniquely quantifiable. For example, it is hard to imagine that the relationship between specificity of environmental stimulus and behavioural response could be more readily demonstrated, and quantified, in any other science of behaviour. All this implies a discrete and sensitive mechanism" (Young, 1993). Circadian rhythms occur at all levels of physiological organisation and complexity, in the physiology and behaviour of practically all animals and plants – in fungi, algae, single-celled animals, worms, insects, amphibians, reptiles, birds and mammals (including humans).

1.1 Terminology and abbreviations

In the interests of easy readability I have decided to leave out as much of the jargon of chronobiology as possible. But some essential abbreviations and symbols, standardised by Aschoff (1965), and since then much in use, are listed below.

LD: Light/dark cycle

LL: Continuous light

DD: Continuous darkness

Free-running period (τ): Period of circadian rhythm in LL or DD

Zeitgeber(s): Entraining or synchronising agents. A German word now used in English frequently. Universal zeitgebers are LD cycles caused by sunrise and sunset.

T: Period of the zeitgeber (24 h in the case of a natural LD).

h: Hour(s).

Entrainment When a zeitgeber modifies, or synchronises endogenous rhythms such that it is equal to T.

Phase Any point along the circadian rhythm. It is often expressed as CT or "circadian time", denoting that it is not local time.

Phase angle difference The difference between a fixed phase of the circadian rhythm and a fixed phase of the zeitgeber. Examples are sunset and the onset of activity in a nocturnal animal, or sunrise and the onset of activity in a diurnal organism.

Phase shift Circadian rhythms, while in a state of free-run, respond to brief exposures to stimuli such as light, darkness, elevated or lowered temperature, or chemicals, with the displacement of the rhythm or shift of phase. Phase

shifts can be *advances* or *delays*. In computing phase shifts, discrete events, such as the onset of activity of an animal, median value in eclosion rhythms, or the lowest point in body temperature cycles, are taken as reference points.

PRC Phase response curve. A plot of the responses of a circadian rhythm in terms of phase shifts to perturbations as a function of phase.

There is a pseudoscience called *"biorhythm"*, which claims the existence of the 23-day male cycle, 28-day female cycle, 33-day intellectual cycle, a 38-day compassion cycle, a 43-day aesthetic cycle, a 48-day self-awareness cycle and a 53-day spiritual cycle (a National Institute of Mental Health (USA) paper classifies biorhythms rightly as mythology). This book will have not have anything to say about such biorhythms.

The defining characteristics of circadian rhythms are

1. They entrain to LD cycles in nature and in the laboratory.
2. Circadian rhythms free-run in LL and/or DD with periods deviating slightly from 24 h.
3. Circadian rhythms respond with phase shifts to light, temperature and chemical perturbations in a time dependent manner.
4. The period of circadian rhythms are compensated for temperature changes i.e., if a leaf movement rhythm has a period of 27 h in LL, it would show the same value regardless of whether the recording is made at 16° or 26°C. As a consequence, the $Q10$ (temperature quotient) values remain close to 1.
5. Circadian rhythms have a genetic basis.

It is good to remember that while all circadian rhythms are daily rhythms not all daily rhythms need be circadian rhythms. In literature all daily rhythms are loosely described as circadian rhythms. Fortunately most daily rhythms, on proper experimental investigation, indeed turn out to be circadian rhythms.

1.2 Historical background

Circadian rhythms were first discovered in the sleep movements of the leaves of plants. Sleep movements in plants are of widespread occurrence, especially in plants belonging to the family Papilionaceae. The first recorded daily rhythm was of the closing movements of the leaves of the tamarind tree (*Tamarindus indicus)* and was made by the Greek philosopher Androsthenes when he joined Alexander of Macedon in his march to India in the fourth century BC (Bünning, 1960). Carl von Linné (1707–1778) constructed a *floral clock* based on his knowledge of the opening of flowers at different hours of the day. The French astronomer De Mairan demonstrated in 1729 that the sleep movements of potted *Mimosa pudica*, the touch-me-not plant, persisted in the perpetual darkness of a cave. J.G.Zinn (1759) showed that the vertical and drooping 24 h

rhythms of the bean plant persisted even in the absence of LD cycles and alternating low- and high-temperatures. About a hundred years ago Wilhelm Pfeffer (1845–1920) recorded the sleep movements of the been plant on kymograph drums in his laboratory in the University of Leipzig. His laboratory was state-of-the-art even by modern standards with constant temperature rooms, automatic time switches and facilities for simulation of dawn and dusk conditions (Bünning and Chandrashekaran, 1975). The Indian physicist Jagadish Chandra Bose (1858–1937) wrote important papers in English on his findings on diurnal rhythms of plants (Fig. 1.2).

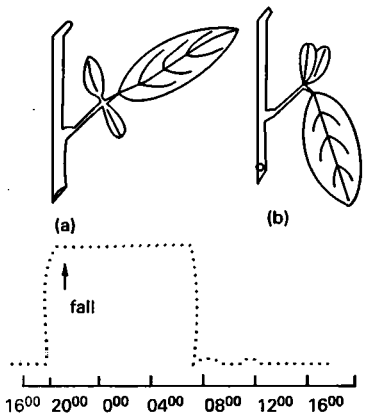

Fig. 1.2 Leaf of *Desmodium gyrans* in (a) open (day) and (b) closed (night) positions. (After Bose, 1927).

In 1919 Bose reported entrainment of leaf movements to light/dark cycles and free-running rhythms in continuous light and continuous darkness (Bose, 1919; Chandrashekaran, 1998a). R. Semon (1905; 1908) held that the daily rhythms of the leaves were endogenous cycles and heritable in nature. Darwin, in his book *On the Power of Movements in Plants* published in 1880, expressed the view that these movements were heritable and must be of utmost importance to the plants (Darwin and Darwin, 1880).

Research on circadian rhythms in animals began much later, the most thorough and elegant experiments being those of Karl von Frisch and his students on honey bees, published 1929 onwards. They trained honeybees to search for food at certain hours of the day by offering them sugar water at a particular spot. Such "time training" had to be repeated day after day at the same hour for a week or more. On the first experimental day, the bees began to arrive on the spot at the appointed hour(s) and searched for food even though it had been removed by the experimenter (Fig. 1.3).

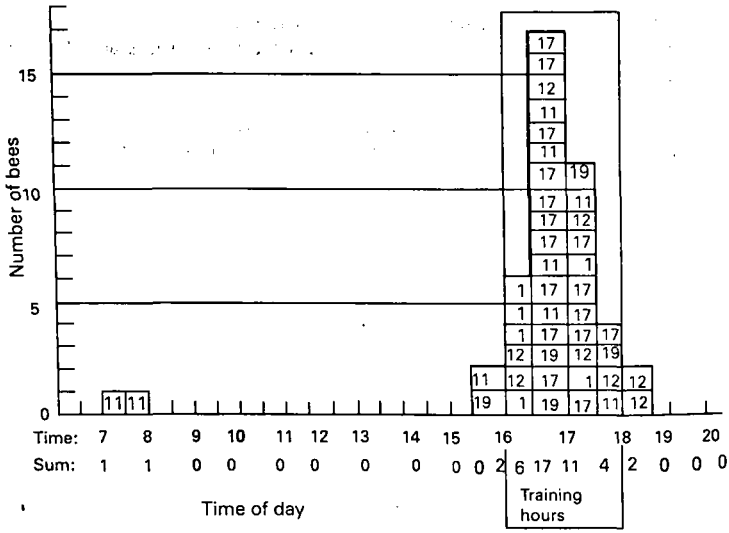

Fig. 1.3 Results of an experiment on 'time training' in honey bees. Bees were offered food over several days between 16-18 h and on the day of counting they were not offered any food; yet the bees swarmed to the empty dishes around the same time. The bees were numbered and the histogram illustrates 'bee visits' for ever ½ h from 06 to 20 h. (Modified after Beling, 1929).

Beling (1929), a student of Karl von Frisch, had strong evidence that the bees were thus orienting in time with the help of endogenous clocks. Richter (1922), working in the USA, published, in what was his Ph.D. thesis, data indicating entrainment of the locomotor activity rhythm in rats to light/dark cycles and restricted time of feeding and demonstrated that the rhythm free-ran under constant conditions in the laboratory. Aschoff and Wever (1962) experimentally confirmed the endogenous nature of circadian rhythms in humans when they studied a group of humans in a sealed cellar of a Munich hospital for 8–18 days.

Another important meeting was the one convened by Jürgen Aschoff at Feldafing in Bavaria on "Circadian Clocks" in 1963 (Aschoff, 1965b) This meeting, supported by NATO, was a watershed event, and along with the 1960 Cold Spring Harbour meeting, marked the advent of the modern era of research in chronobiology. The conceptual foundations of the field of biological rhythm research in this century were largely laid by J Aschoff, E Bünning and C S Pittendrigh. When in 1971, Ron Konopka and Seymour Benzer reported the discovery of the clock gene in *Drosophila melanogaster*, research in chronobiology entered the age of molecular genetic analyses of

circadian rhythms. Clock genes have now been reported in the bread mould *Neurospora crassa* (Feldman and Hoyle, 1973), the plant *Arabidopsis* (Millar et al., 1995) and cyanobacteria (Kondo et al., 1994). There was much rejoicing at the identification and cloning of the first clock gene in mammals – the mouse – by researchers led by Joseph Takahashi in the Centre for Biological Timing at the Northwestern University, Evanston, Illinois (Antoch et al., 1997). The feat has been hailed as a nugget of circadian gold.

1.3 Examples

Rhythms in plants

It is now well-known that all organisms, from fungi to humans have circadian clocks. Cyanobacteria exhibit circadian rhythms in their photosynthetic rate. Circadian rhythms express themselves in the sporulation of the bread mould *Neurospora crassa*. The molecular genetics and mechanisms of light entrainment of the *Neurospora* rhythm have now been worked out in some detail. The giant green alga *Acetabularia* (Schweiger et al., 1964) shows circadian rhythms in CO_2 output, photosynthesis, cytoplasmic movement, and cell wall electric potential. The green alga *Euglena gracilis* (Brinkmann, 1966) show phototactic (attraction to light) circadian rhythms in cultures maintained in dim light. The marine alga *Oedogonium* releases its spores in a circadian pattern. The dinoflagellate *Gonyaulax polyedra,* made famous by the researches on it by Hastings and Sweeney, expresses rhythms in spontaneous luminescence, photosynthetic ability and cell division (Fig. 1.4) (Hastings and Sweeney, 1958; 1964).

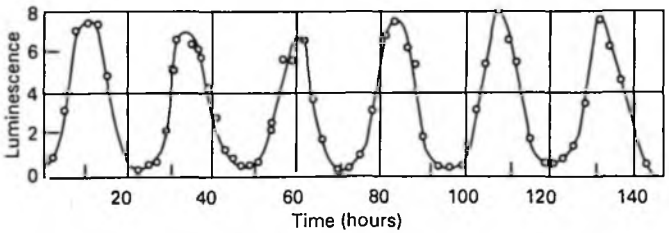

Fig. 1.4 Daily luminescence in the alga *Gonyaulax* (After Hastings and Sweeney, 1959).

The most common biological rhythm seen in India is in the "sleep" movements of the leaves of the avenue tree *Enterolobium saman,* appropriately called the sleepy-faced tree in Tamil, which anticipate sunset and fold together and open a little in advance of sunrise. The leaves of the tamarind tree, gooseberry, the touch-me-not plant and the Indian telegraph tree *Desmodium gyrans* also show

such rhythms in leaf movements. Certain flowers open their petals to coincide with the activity of the insects that pollinate them. The time of petal opening is species-specific. The lily *Nymphaea* opens its petals at night and closes them during the day. Daily rhythms, have also been reported in nuclear volume, turgor pressure in leaf pulvini, starch deposition, chlorophyll synthesis, heat resistance, activity of enzymes, stomatal openings, rate of transpiration, rate of exudation, sensitivity to toxins, shape of chloroplasts, protoplasmic viscosity, cytoplasmic streaming, sensitivity to alcohol, heavy water sensitivity and so on in various plants (Bünning, 1973).

Rhythms in animals

Insects show rhythms in the rate of oviposition, pupation, egg hatching, and eclosion. Many aspects of development happen only once in the life cycle of an insect. Their precise timing may be under the control of a circadian clock, and the occurrence of these events may therefore synchronise with a particular time of day or night. In such cases, a clear rhythm of a particular developmental event becomes apparent only in a population of mixed developmental stages. Haddow and Gillett (1957) showed that egg laying in the caged yellow-fever mosquito *Aëdes aegypti* occurred in well-defined peaks at the end of the light period in a normal tropical day of *ca.* LD 12:12 h, both in field and laboratory conditions. Populations of the mosquito raised in DD showed a weak periodicity but exposing the populations to just 5 min of light every 24 h made the rhythm distinct. In populations exposed to LL, the egg laying was completely arrhythmic (Gillett et al., 1959).

An interesting circadian rhythm has been reported (Loher and Chandrashekaran, 1970) in the oviposition of the Californian grasshopper *Chorthippus curtipennis*. These grasshoppers lay their eggs in a single pod. Populations of 50 females at a time were kept in a cage with moist compact sand at the bottom. The grasshoppers laid most of their eggs in the middle of the L-phase in LD 12:12 h cycles. In LL and DD, the oviposition rhythm showed persistence and was free running. There was entrainment even after the optic lobes were severed and the three ocelli destroyed by micro-incineration. The authors postulated that there was extracephalic photoreception and this accounted for the LD entrainment even after light was blocked from the compound eyes and ocelli. This is the first experimental demonstration of 'extra-optic and extracephalic' photic entrainment of an insect circadian rhythm. Dumortier (1972) demonstrated that two photoreceptive systems operate in entraining the stridulatory activity rhythm in the orthopteran insect *Ephippiger*, one system being extracephalic. In the pink bollworm *Pectinophora gossypiella*, oviposition occurred soon after the onset of darkness in LD cycles. The oviposition rhythm free-ran in DD with a period of 22.7 h (Danilevskii, 1965).

One of the most intensively investigated circadian rhythms is that of eclosion in *Drosophila pseudoobscura* and *D. melanogaster* (Fig.1.5).

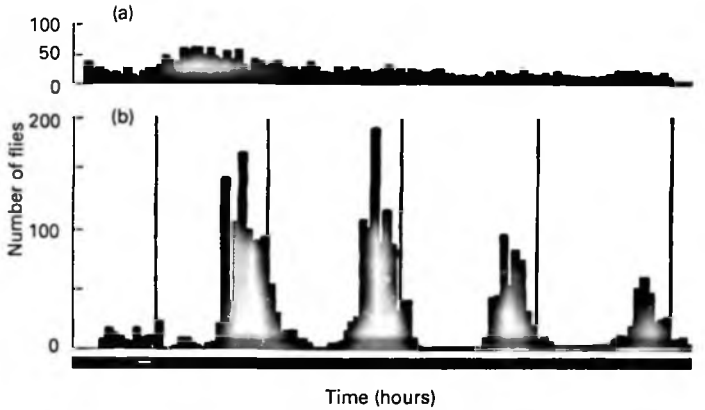

Fig. 1.5 Eclosion rhythm in *Drosophila*. (a) Control in DD; (b) Induction of a rhythm in eclosion by a 4 h light break given to a population of pupae raised in DD since the egg stage . The vertical lines coincide with 4, 24, 48, 96 and 120 h after beginning of the light break. (After Pittendrigh and Bruce,1957).

It was with the eclosion rhythm in *Drosophila* that Pittendrigh and Bruce (1954) first elegantly demonstrated the phenomenon of temperature compensation in biological clocks (Fig.1. 6) (see also Zimmerman et al., 1968).

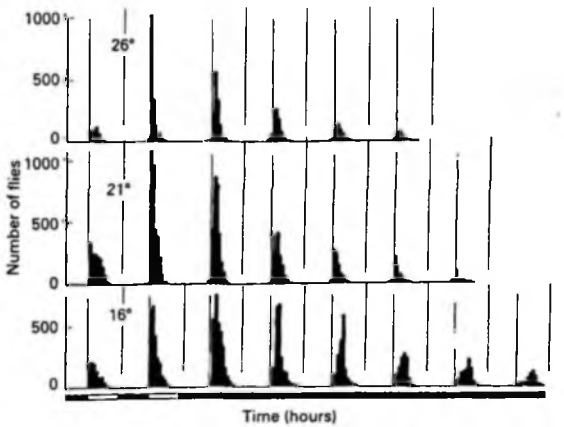

Fig. 1.6 Eclosion rhythm in *Drosophila* at 26°, 21° and 16° C. The vertical lines are 24 h apart. Temperature has only a small influence on period length. Light conditions are contained in the bar at the bottom. (After Pittendrigh and Bruce, 1954).

A less thoroughly investigated rhythm is cuticle deposition in the cockroach, grasshoppers and locusts. One can test this using the leg (the femur) of grasshoppers or the common cockroach and a new razor blade. Figure 1.7 shows how one can investigate the daily growth rhythms in the leg of a cockroach with the unaided eye or with a simple magnifying lens.

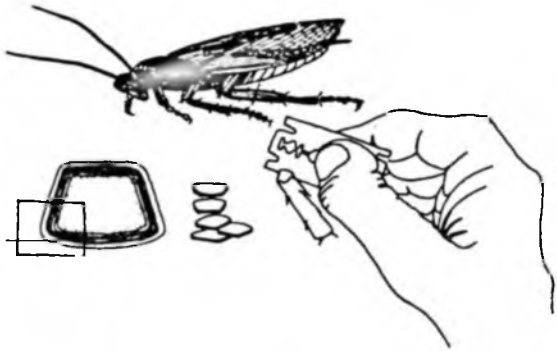

Fig. 1.7 Chitin growth rings in the leg of the cockroach *Periplaneta americana*. The fine slice may be made from the femur. (After Engelmann and Klemke, 1983).

Cockroaches such as *Periplaneta americana, Leucophaea,* and *Blatta orientalis* show excellent circadian rhythms in their locomotor activity. The biting cycles in mosquitoes are also under circadian control (Jones, 1976; Jones and Naylor, 1970; Pandian and Chandrashekaran, 1980).

Among the earliest experiments on circadian rhythms in animals were the elegant studies performed on honeybees by Beling (1929), Wahl (1932) and Kalmus (1934), in the laboratory of Karl von Frisch in the University of Munich. Beling time trained bees by offering them sugar water for 2 h every 24 h for several days. The bees came looking for sugar water at expected times on subsequent days even if no food was offered. Training was successful only when food was offered once in every 24 h but not when offered every 19 or 27 h. Clearly 19 and 27 h were outside the *limits of entrainment*. It was demonstrated that this "time sense" (Zeitsinn) was inborn, not learnt and that honeybees that had never experienced LD cycles could also be entrained to 24 h restricted feeding in DD. Kalmus (1934) performed the earliest experiments with cold and warm pulses and wrote of a 24 h *Eigenfrequenz* (period) in honey bees which was phase shifted by 2 h low and 2 h high temperature pulses. The body of early work on circadian rhythms on honey bees remained comparatively unknown to chronobiologists working outside Germany, since the authors called the phenomenon *Zeitsinn* or *Zeitgedächtnis* (time memory, Forel, 1910). Having called the phenomenon by this name, the researchers began looking for external events like the position and angle of

the sun, light and temperature fluctuations, polarisation of light, landmarks and other such exogenous factors which could effect the phenomenon to the total exclusion of the internal circadian organisation.

Feeding rhythms have still remained the main circadian behaviour studied in bees (Moore and Rankin, 1985; Moore et al., 1989). Frisch and Aschoff (1987) performed laboratory experiments on a colony (hive) of bees and showed that the locomotor activity rhythms of all members of the hive free-ran with a period slightly longer than 24 h and that the hive entrained to feeding cycles. Several authors have reported distinct times of activity and rest in isolated bees kept in LL or DD. The period of these activity rhythms deviate from 24 h (21.8 h: Spangler, 1972; 23.5 h: Kaiser and Steiner-Kaiser, 1983; 23.0 h: Moore and Rankin, 1985, in DD). Differences between the periods of worker bees and drones have been found, as well as changes in activity patterns with age. Under identical conditions in DD, the periods of the activity rhythms of the worker bees are 21.8 h and those of the drones 23.7 h. In contrast, no circadian pattern of activity was found in freshly emerged bees (Spangler, 1972). There are only a few reports of activity rhythms of entire colonies (Kefuss and Nye, 1970; Frisch and Aschoff, 1987). There is an obvious *social* synchronisation of the circadian clocks of individual bees in nature as well as in experiments in the laboratory. Medugorac and Lindauer (1967) first reported evidence for social synchronisation in bees. They found that honey collecting bees introduced to a foreign colony visited a food source at their own feeding time as well as the feeding time of the host colony. Southwick and Moritz (1987) reported that two groups of bees that were out of phase with respect to their metabolic rhythms became synchronised within 2 days when physical contact among them was allowed. Similarly groups of bees show synchronisation of their circadian locomotor activity pattern after contact (Sasaki, 1992).

Colonies and isolated bees of the Cape honeybee, *Apis mellifera capensis* Esch, were investigated for evidence of circadian rhythmicity under constant conditions (Frisch and Koeniger, 1994). Colonies developed free-running activity rhythms in self-selected LD cycles, which had a slightly shorter period than 24 h. (A self-selected LD regime is one in which the members of a bee colony come out of the darkness of the hive into daylight and later return to the darkness of the hive at hours determined by themselves). The periods of the activity rhythms of individual isolated bees were longer than 24 h in self-selected LD cycles and LL, while they were shorter than 24 h in DD. The authors conclude that the periods of common activity and common rest of bees within a colony, in an entrained state or under free-running conditions result from mutual *social* synchronisation rhythms of the individual bees. The honeybee joins the select ranks of animals in which genuine social synchronisation has been experimentally demonstrated, others among them are fish, birds (Gwinner, 1966; Menaker and Eskin, 1966), the mouse *Mus musculus* (Halberg et al., 1954), the field mouse *Mus booduga* (Vishwanathan and Chandrashekaran, 1985; 1988), beavers (Bovet and

Dertli, 1974), and the insect bat *Hipposideros speoris* (Marimuthu et al., 1978; 1981; Chandrashekaran 1982). All reptiles, birds and mammals that were investigated show circadian rhythms in activity and rest and other physiological processes. Based on information and facts available at present, it appears that all animals and all green plants including unicellular organisms possess a circadian organisation. Indeed circadian rhythms are so frequently found in mammals that Davis-Walton and Sherman (1994) reporting sleep arrhythmia in the eusocial naked mole-rat, claimed "This naked mole-rat *Heterocephalus gaber* is the only wild mammal that does *not* regularly exhibit circadian sleep–wake cycles."

1.4 Effects of LL and DD on circadian clocks

While LD cycles entrain (synchronise) circadian rhythms, LL tends to lengthen or shorten the period of the rhythm. The period-modifying effect of LL was first reported in the spontaneous locomotor activity rhythm of the white-footed mouse by Johnson (1939). In mice the period length is 25–26 h in LL and decreases to 23–23.5 h in DD. In rats the period length in LL could increase by nearly 2 h compared to the period in DD. Bullfinches kept in DD show a period of 24 h. Experiments on the flight activity of two microchiropteran bats, *Taphozous melanopogon* (Subbaraj and Chandrashekaran, 1981) and *Hipposideros speoris* (Marimuthu and Chandrashekaran, 1983) in LL and DD indicate that the periods of both bats were longer in LL than in DD. Aschoff formulated a general rule (Aschoff's Rule) which states that the period length of light-active animals decreases with increasing intensities of LL whereas the period length of dark-active animals increases with increasing intensities of LL (Aschoff, 1959). According to Aschoff (1960), the frequency changes linearly with the logarithm of the light intensity (Fig. 1.8).

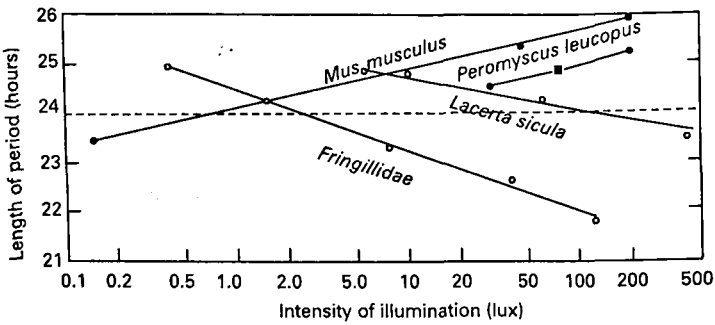

Fig. 1.8 Spontaneous activity period of animals in constant environment, depending on the intensity of LL. (After Aschoff 1959).

A few exceptions to this rule have been reported. Aschoff's Rule might also apply to situations in nature and therefore have a deeper meaning in the context of mechanisms. The phenomena described above about the effects of light on period length relate to white light and only to animals. Bünning has described instances where monochromatic light modulates period length of plants as a function of colour (wavelength). In plants an antagonistic effect of red and far-red light was found. The leaf movements of beans (*Phaseolus coccineus*) show different periods, depending on the quality of light (Lörcher, 1958). Similar results were also found in the leaf movements of *Coleus*. These factors vary from case to case. Plants etiolate and do not grow properly in DD since they need some light for photosynthesis.

1.5 Phase angle differences

To recapitulate Section 1.1: phase angle differences are the difference between the phase of the rhythm and a fixed phase of the zeitgeber. In nature, the maxima and minima of circadian rhythms do not have a fixed phase angle relationship with light intensities during LD entrainment. Phase angles are caused by a variety of environmental factors, which are often complex; they may be caused by how gradual light intensity changes are during twilight. Duration of twilight, both at dawn as well as dusk, are relatively brief closer to the equator and become longer and longer with increasing latitude towards the Arctic as well as the Antarctic. In Madurai, India (9°58' N lat; 78°6' E long) dawn and dusk last just over 12 min. Phase angle properties also change in the course of the seasons. At 49°20' N lat impressive changes have been demonstrated in the timing of the onset and termination of the flight activity in the jackdaw *Coleus monedula* L., which closely paralleled changes in sunrise and sunset times over a one- year period (Aschoff and von Holst, 1960). Erkert (1974) in his studies on the effect of moonlight on nocturnal mammals, carried out under natural conditions near the equator in Colombia (4°35' N lat; 74°27' W long), concluded that the activity of the fruit bat *Rousettus aegyptiacus*, living in the open, started 30–60 min after sunset when the light intensity varied from starlight to variable moonlight intensities, that depended on the phases of the moon. Evidently in this fruit bat, a *fixed* lower threshold of light intensity triggers onset of flight. In the cave-dwelling Indian tomb bat *Taphozous melanopogon* there is no such *fixed* lower threshold (Subbaraj and Chandrashekaran, 1977). Unlike in the jackdaw, the onset and end of activity were also not strictly parallel to the changes in the time of sunrise and sunset over the seasons. Consequently, the onset, the midpoint and the end of activity undergo seasonally varying phase angle features. As a result, the bats began their foraging flight when it was pitch-dark during the shorter days of December at light intensities typically of 0.1–0.3 lux, and flew out even as the sun was setting and its tip visible at the horizon during relatively longer summer days at light intensities as high as 50 lux (Subbaraj

and Chandrashekaran, 1977). In contrast, the onset of emergence activity in the insectivorous bat *Hipposideros speoris,* at the same latitude, occurred within a narrow range of environmental light intensity and varied over the seasons (Marimuthu, 1984). These variations in the lowest light intensities that trigger exodus flight in bats indicate that absolute light intensity is not *the* cue for the onset of colony activity in both species, but rather *the rate of change* of light intensity which play a role in regulation of the onset and end of activity.

1.6 Biologically important light intensities

Since circadian rhythms are used for daily and seasonal time measurement, a high degree of precision in the functioning of these clocks is important. Therefore, reliable entrainment to exactly 24 h cycles and a stable phase angle relationship with the zeitgeber become necessary preconditions for accurate biochronometry. Organisms can arbitrarily choose light intensities following sunrise and sunset for the purpose of entrainment. This is not a reliable device for the fluctuations of light intensities during these phases of the solar sky are much too great from day to day and depend especially on cloudiness, particularly in temperate countries within high latitudes.

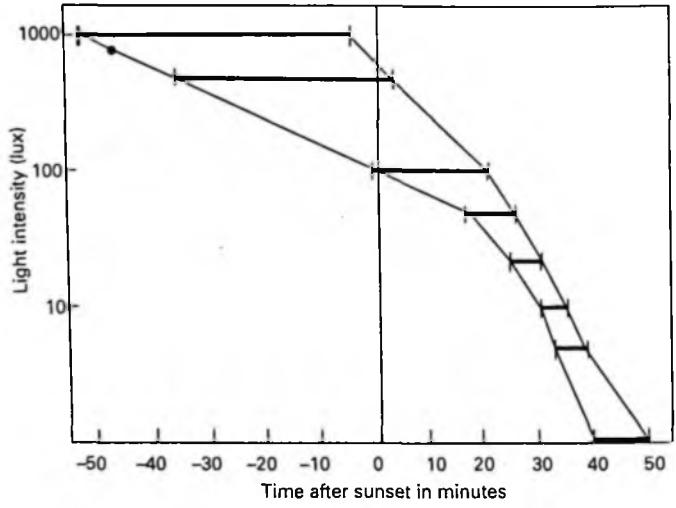

Fig. 1.9 Daylight intensities reaching a flat surface measured in Tübingen 1-2 March 1969. It is obvious that the rate of change of light intensity is most rapid when the light intensity at dusk is close to 10 lux. Even dally variations incident upon cloud cover did not matter much in the 10 lux region. (After Bünning, 1969).

The most suitable and noise-free reference points are values of light intensities between 1 and 10 lux that occur *before* sunrise and *after* sunset. In this range of light intensity, the rates of intensity changes are the greatest, and variations in time due to weather conditions usually do not exceed a few minutes (Fig. 1.9).

Actually plants and animals make use of these dim light regions as reference. Within intensities of 10 lux or a little more, the phase angle difference between the solar cycle and the circadian rhythm remains nearly independent of light intensity (Bünning, 1969; Chandrashekaran and Loher, 1969a) (Figs. 1.10 and 1.11).

Needless to say the threshold intensities of 1 to 10 lux would themselves vary in time depending on the seasons of the year. In northerly climes and high latitudes as in Spitzbergen in Norway, the day length in summer could be 16 h or more and in winter months 8 h or less – hence, the sobriquet "lands of the midnight sun", for countries around the polar cap. On the equator, the day length over the seasons is invariant and it may be expected that the period length of circadian rhythms in this region may be indistinguishably close to 24 h.

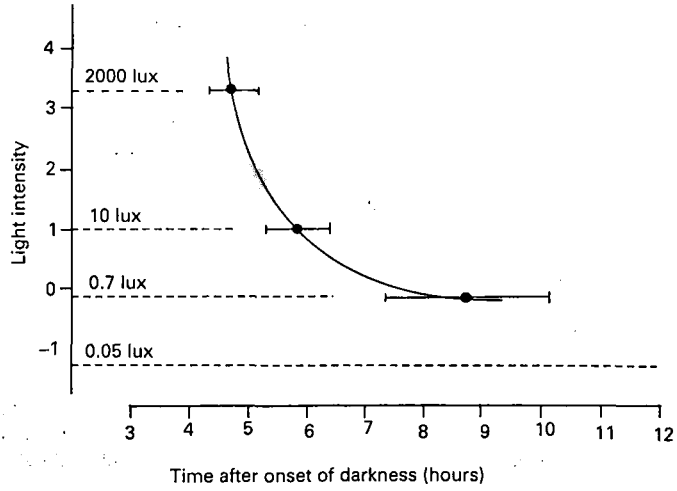

Fig. 1.10 Leaf movement rhythm in *Glycine max* (soyabean) with LD 12:12 h. On the abscissa is the hour of midnight position (in hours after onset of D). There is no entrainment with light intensity of 0.05 lux. (The rhythm free-ran with a period of 26 h; not shown in the figure). There was entrainment to LD cycles in which light intensity was 0.7 lux and above. The figure illustrates that the phase angle difference between LD cycles are invariant above 10 lux. (After Bünning, 1969).

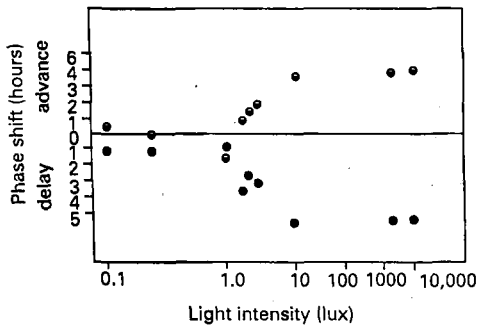

Fig. 1.11 *Drosophila pseudoobscura* eclosion rhythm. Phase shifts effected by 15-min light pulses of different intensities. The 0 line indicates values obtained in controls which did not experience light pulses. (After Chandrashekaran and Loher, 1969a).

1.7 Use of the circadian clocks for measurement of day length

The process by which organisms differentiate short days or long nights rom long days and short nights is known as photoperiodism. The most spectacular photoperiodic phenomenon and annual rhythmicity we get to see is in the flowering of trees and are so common that we fail to take particular note of it. Nor do textbooks on botany in the market in India treat the subject of photoperiodism with the sense of wonder that it richly deserves. The wonder lies in the fact that in tropical and sub-tropical climes day length and temperature do not really vary so much and yet plants do have an annual flowering season. Earlier, it was believed that annual changes such as those in temperature, light intensity and rainfall, were responsible for the adjustment made by organisms of their development to seasonal changes. This may be true if organisms can rely on changes in these factors as an absolute indicator of the time of the year. Temperature changes in some oceans are indeed good indicators of seasons and many algae and marine organisms programme their development by temperature. In cases where temperature is not a reliable factor to indicate the course of seasons, organisms submit themselves to photoperiodic control. In photoperiodic responses, organisms measure the real day length (or night), approximately from dawn.

The first evidence of photoperiodic control of physiological processes were obtained from the studies of Henfrey (1852) on flower formation in plants. Klebs (1913) expressed his results on flowering in the plant *Sempervivum funkii* more precisely: "In nature, the time of flowering is very probably determined by the fact that day length increases after the spring equinox (21 March). After the day reaches a certain length, the formation of flowers is initiated. Light probably does not function as a nutrient factor, but more

in a catalytic way." The real physiological causes underlying photoperiodic phenomena were not appreciated until the 1930s. The involvement of day length in causing annual events such as trees shedding leaves in autumn and later vernalisation with the advent of spring was not properly understood. Photoperiodism was vaguely associated in the minds of people as something connected with flowering. This was especially the case after the work of Garner and Allard (1920; 1923) on photoperiodism in plants. The term *photoperiodism* was coined by the United States Department of Agriculture (USDA) scientist O. F. Cook and was introduced in literature by Garner (Goldman, 2001). It is not surprising that photoperiodic control is found most frequently in organisms living between the latitudes of 35° and 40°. In this region temperatures are the least reliable as indicators of seasonal changes and changes in day length.

1.8 Critical day length

Botanical examples

One prerequisite for photoperiodic control of flowering is that an interaction must exist between leaves that perceive the day length and the apex that actually changes into the flower. Bünning (1969) has given an excellent example of this "correlative interaction" on the basis of data derived from elegant experiments. Soybean plants (*Glycine max* var. Tübingen) showed leaf movements, which were in direct synchrony with flower induction/ inhibition rhythms (Fig.1.12).

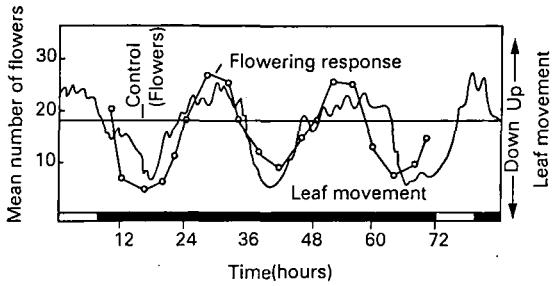

Fig. 1.12 Synchronous circadian modulation of the leaf movements and the photoperiodic sensitivity in flower induction in *Glycine max* in 8 : 64 h LD. The plants were initially placed in a cubicle with a bright light of 10,000 lux and then brought into the experimental room. They were then given at different phases 30-min light pulses for flower induction. The plant whose leaf movements were being traced were not given any light breaks to avoid phase shifting. (After Bünning, 1969).

Bünning pointed out that the leaf movement rhythm can be looked upon as a good "hand" of the clock and that it is also reflective of the time course of the endogenous circadian clock (Bünning, 1971b). The leaves exert influences on the apex, which promote or inhibit flowering. Phenomena such as the role of the so-called flowering hormone or the action of growth substances will not be discussed here for they do not relate directly to the circadian clock. We are interested here only *in the time-measuring ability* that finds its expression in *photoperiodic responses.* Plant species are known in which at least one of the following processes depends on day length – tuber formation, seed germination, vegetative development, succulence, cambium activity, tissue differentiation and induction or termination of dormancy of buds and bulbs.

Long-day plants form flower primordia after being exposed to light periods of more than 12 h for a few days. If the light period is shorter than this critical day length of 12 h, no flower primordia will be formed at all. In a few long-day plants even one long day is sufficient for induction. The so-called *short-day* plants can grow *vegetatively* from several months to an unlimited time, if the light period is longer than a critical day length of say, 12 h. Yet if the light period is shorter than the critical day length for just a few days (in some species a single short day is sufficient) then flowering ensues rapidly (Bünning, 1973). Figure 1.13 illustrates the underlying physiological principle succinctly. There are also *day-neutral* plant species that flower independently of day length.

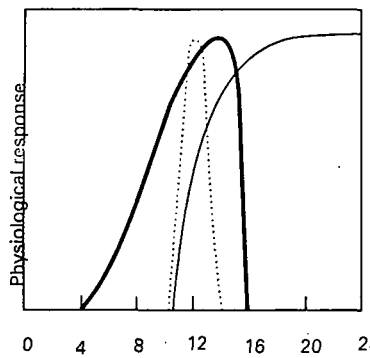

Fig. 1.13 The important interrelations between day length and physiological and photoperiodic responses in plants and animals. Thick curve : The physiological response is possible only in short days. The upper limit of the example chosen here is 16 h. Thin curve : The physiological response will set in only when day length exceeds a certain number of hours (11 h in the example chosen). The dotted line : The physiological responses occur around midway (eg., LD 12:12 h) (After Bünning, 1977).

Zoological examples

Kogure (1933) discovered photoperiodic control of diapause in the commercial silkworm *Bombyx mori.* Diapause is a rest period in insects comparable to the dormancy of buds or bulbs in plants and characterised by severe reduction of many metabolic processes. Marcovitch (1924) reported the photoperiodic control of the annual cycle in the behaviour of the plant lice *Aphis forbesi.* Schäfer (1907) first suggested that day length might be the controlling factor in the migration of birds as well as in the annual gonadal cycle. Rowan (1926) reported that increasing the daily light period in autumn can induce the enlargement of gonads and the impulse to migrate northward in *Junco hyemalis, Corvus brachyrhynchos* and *Serinus canarius.* These phenomena are otherwise restricted to spring.

The decisive factor in photoperiodic reactions normally consists of exceeding a critical day length, regardless of whether the examined process of development is being promoted or inhibited. The length of this critical light period varies usually being between 10 and 16 h, the limits indicating durations of shortest and longest days in temperate regions within 35° and 40°. The oscillator model by Bünning (1936), a model which explains the mechanism of photoperiodism, is gaining increased credence. It was proposed for photoperiodism in plants and insects and was christened the *Bünning hypothesis* by Pittendrigh (1960). The Bünning hypothesis states that photoperiodic time measurement is accomplished by means of an endogenous

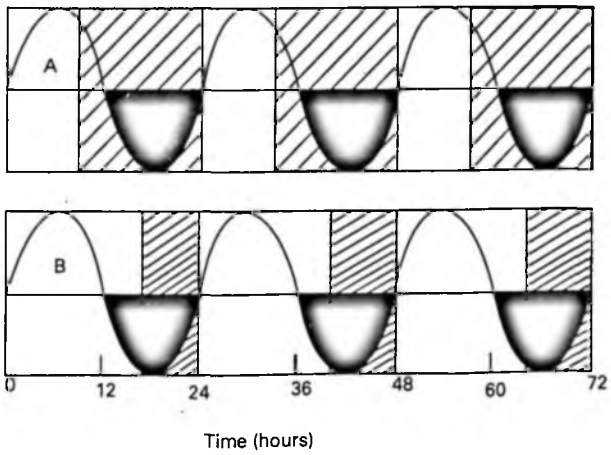

Fig. 1.14 Bünning's (1960) model showing the phase relationships of the hypothetical rhythm of light sensitivity under (a) short and (b) long day conditions (After Lees, 1971).

24 h rhythm of light sensitivity consisting of two half cycles of 12 h of photophilic (light loving) and scotophilic (darkness loving) affinities (Fig.1.14).

The details are not important. Bünning's entirely original model postulates that the circadian rhythm in light sensitivity acts much like a *yardstick* in seasonal events such as photoperiodic phenomena. Bünning found an adaptive function for circadian rhythms which had eluded Charles Darwin and August Weismann (1834–1914). For a detailed treatment of the subject of photoperiodism the general reader is directed to consult the classic *Insect Clocks* by Saunders (2002).

1.9 The clock - bird migration and direction finding

Birds are the most mobile group of vertebrates. Roughly half the known species perform some kind of migration. The ability to fly, homeothermy and relatively long life-spans enable birds to migrate to distant areas, mostly to escape the severity of environmental conditions. Alerstam (1990) believes that migration has existed for as long as birds have been present on earth (*ca.* 10 million years). In most cases migration is simply the trip from the breeding grounds to the winter quarters and back. Almost all areas between the Arctic and the Antarctic are used as breeding grounds. There are no geographical barriers such as deserts, mountains and oceans that cannot be regularly crossed by migrant birds. The most spectacular migration in terms of distance is performed by the Arctic tern, *Sterna paradiaea*, which annually migrates from Arctic breeding grounds to winter quarters in the Antarctic continent. Individuals may cover a distance of up to 50,000 km per year and account for a "life-time" distance of 1 million km – about three times our distance to the moon. Maximum non-stop flights range between 5000 and 7000 km in warblers, which undertake long trans-oceanic flights from Alaska to the Pacific Islands, or from Siberia to New Zealand. A single spell of non-stop flight may last 80–100 h. Passerines can fly non-stop for over 2000 km and humming birds up to 800–1000 km. The ruby-throated humming bird *Archilochus colubris*, weighing just 4.8 g, regularly crosses the Gulf of Mexico in a non-stop flight of about 18 h requiring 3,200,000 wing-beats (Nachtigall, 1993).

Migratory restlessness

Many purely diurnal species of birds become more or less nocturnal during the migratory season. Evidence for this nocturnality comes from the analysis of calls of nocturnal migrants, nocturnal lighthouse casualties, use of moonwatch methods and the absence of many migratory species in diurnal migratory studies. The nocturnal bouts of activities show up in perch-hopping (see Section 1.13) laboratory studies and are attributed to migratory

restlessness or "Zugunruhe." In 1949 Kramer observed that starlings (*Sturnus vulgaris*) kept in an outdoor aviary showed "Zugunruhe" and oriented in the normal migratory direction in most cases. The birds also exhibited this type of oriented behaviour in a smaller cage where the sight of landmarks was excluded. When the sky was clear the birds oriented, but not when it was heavily overcast. By experiments, using mirrors, Kramer (1950) conclusively demonstrated that the sun was the orienting stimulus. Since the same direction was followed at different times of the day, Kramer (1950; 1952; 1953) concluded that the birds were able to compensate for the sun's movement. It has been demonstrated in more than 25 bird species and populations that migratory restlessness is closely associated with the specific migratory seasons, the duration of the migratory period, the distance being covered and the ecological characteristics of the migratory journey (Berthold, 1993).

Genetic basis of bird migration

Until recently, when discussing the intricacies of natural phenomena, it was fashionable to talk about "the mystery of bird migration." How do the birds know in what direction to take off? How far to fly? And how did they know where to alight in the over-wintering landscape? Now, owing largely to the research of German ornithologists and ethologists we have the answers to most of these questions. Eberhard Gwinner and Peter Berthold carried out most of the work in the Ornithological Station in Radolfzell close to Lake Constance in Germany. Even though migratory restlessness was reported and precisely described by Johann Andreas Naumann in the nineteenth century, it was only in the 1960s that it was discovered that it was an innate property. It has now been conclusively demonstrated that there is a genetic basis for bird migration. If starlings were kept captive inside activity cages, they would fly around for as long as their migratory conspecifics kept flying to their wintering grounds. Johann Andreas Naumann remarked that the golden oriole *Oriolus* "migrate far away presumably as far as Africa" after noticing their "Zugunruhe" in cages. On the basis of their findings from three crossbreeding studies, two carried out with blackcaps and one with redstarts, Berthold and Querner (1981) reported the details of the genetics of bird migration. The parental stock of the blackcaps was central European birds which migrate regularly to the Mediterranean and display in laboratory studies large amounts of migratory restlessness, consistent with their long migratory routes. They were crossed with African conspecifics that exhibit small amounts of migratory restlessness, and with those from Cape Verde Islands where blackcaps are resident and do not display migratory restlessness. In both crossbreeding studies, the F1 hybrids expressed intermediate patterns of migratory restlessness (Fig.1.15).

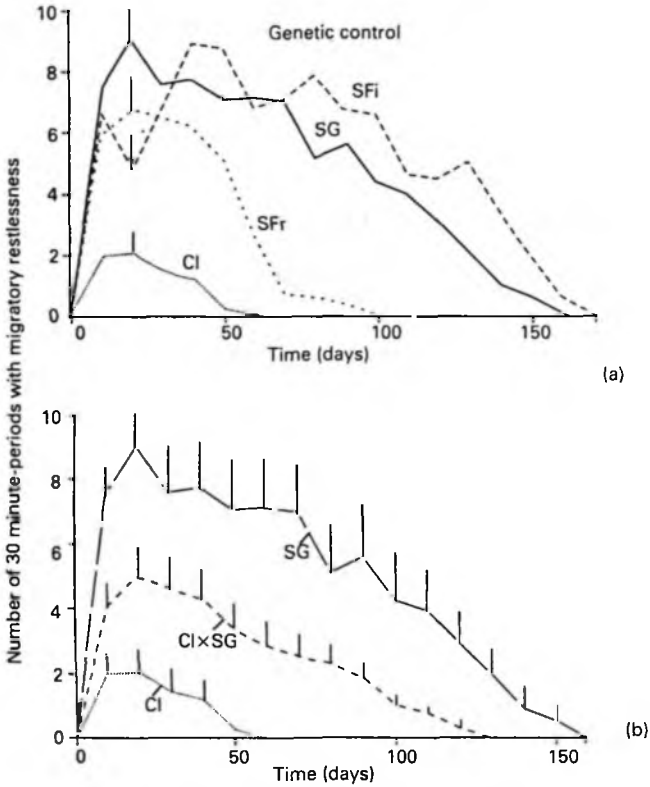

Fig. 1.15 Time course of migratory activity in groups of hand-raised blackcaps from (a) four populations and (b) of hybrids.
SFi: Southern Finland; SG: Southern Germany; SFr: Southern France; CI: Canary islands, Africa; Vertical lines : standard error. (After Berthold and Querner, 1981).

These results show that in the populations examined, migratory activity, expressed as migratory restlessness, is an inherited population-specific characteristic. Readers interested in details about bird migration should refer to the books of Alerstam (1990) and Berthold (1993).

1.10 Circannual rhythms

In spite of the fact that only 20 bird species have been experimentally studied for persistent circadian and circannual rhythms, there is no doubt

that migratory restlessness is closely related to circadian rhythms. Kumar et al. (1991) have shown that photoperiodic effects on body fattening and metabolic events in the migratory buntings, are mediated by circadian rhythms. Hoffmann (1960) demonstrated with a set of elegant experiments that the clock underlying sun compass orientation even free-runs in LL. Pengelley and Asmundson (1970) were among the earliest to report free-running circannual rhythms in the golden-mantled ground squirrel *Citellus lateralis*. Gwinner (1967) and Berthold (1971) obtained the first fully convincing proofs for genuine circannual rhythms in birds, i.e., in Old World warblers. The circannual rhythms in migratory restlessness and of body mass changes (fattening) are under endogenous control and free-run with periods of 10–12 months if the birds are kept under constant conditions (mostly in LL). Where do circadian rhythms come into the picture of bird migration? Migratory birds must prepare themselves well in advance to fly southwards, even before the first snow falls. They moult and acquire new feathers, fatten themselves and store energy to be expended in the course of the arduous journey. This means having to anticipate and programme their activities well in time. The circadian sensitivity of the birds to daylight enables them to sense the shortening of the days in winter. During the homeward spring migration, the birds do just the opposite – sense the lengthening of days. The photoperiodic programming of bird migration has been known for over 70 years.

1.11 Clocks in celestial orientation

Celestial orientation in animals was discovered in 1911, by a few simple but ingenious experiments, Santschi showed that ants could use the sun to find their way back to the nest. When he shielded them from the direct light of the sun, and reflected its rays with a mirror, the animals predictably altered their course. In the following years a similar mode of orientation has been found in many other insects as well as in other groups of animals. Since the sun, which is the light and orientational source, provided a reference direction as does the needle of a compass, this type of orientation was termed "light compass orientation." If an ant uses the sun to find the direction back to its nest, it must be assumed that it does so by remembering the angle of the sun on the way out, and following the angle corresponding to the opposite direction on return. Since the sun moves in the sky in the course of time, the ants must be able to make allowance for the time elapsed thus compensating for the sun's motion. A continually consulted clock mechanism would suggest itself. In fact such a possibility was already mentioned by Santschi (1911) but seemed implausible at that time. The hypothesis had to await the work on the orientation of birds and the work of von Frisch (1950) on the orientation of bees with the help of the sun, to be revived 35 years later. Brun (1958) recognised this kind of orientation in ants. By placing them in a light-tight box he prevented

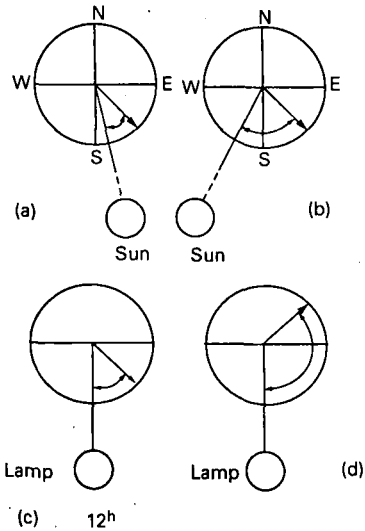

Fig. 1.16 The circadian rhythm and sun compass orientation. An animal is searching for the SE direction, home or the direction to a feeding place. (a) and (b) the animal chooses different angles to the sun depending on the time of day. The double-headed arrow shows this angle, the single arrow the choice of direction. (c) and (d) show corresponding behaviour without the sun. The animal changes the angle with the static artificial light source during the day. (After Bünning, 1973).

the animals from continuing their way to foraging for several hours. Individuals of the ant *Lasius niger* continued on their way after being released without accounting or compensating for the changed position of the sun. Therefore they headed off in the wrong direction. Given this information it is not clear if the *Lasius niger* does have a circadian clock at all. If it does, it is not putting it to use for direction-finding. In contrast, *Formica rufa*, maintained its original direction of movement, thereby showing that it was able to take into account the movement of the sun that had occurred while the animal was inside the box. Animals use the sun azimuth compass in almost all cases that have been closely investigated, i.e., only the horizontal position of the sun's direction is considered. The altitude of the sun does not seem to offer additional information. The basic principles of orientation by the sun and the reactions to "artificial suns" are shown in Fig.1.16 (Hoffmann 1953).

Orientation by means of the internal clock has been found in several arthropods. The investigations from von Frisch's laboratory demonstrated that bees follow this principle. Very interesting in this context are the experiments of Papi (1960) with the wolf spider *Arctosa perita*. This animal,

which inhabits banks of rivers and lakes, if placed on the water hurries to the bank in a direction perpendicular to the shoreline. But if it is taken to the opposite bank and placed on the water there, then the animal runs across the water, or at least it tries to do so. It runs in the direction to which it was adjusted by the position of the sun. The sand hopper *Talitrus saltator* is another example. The escape direction of this animal also depends on a sun compass and on a time sense. These animals live on the wet sand of beaches. If they are transferred to dry sand, they will escape back to the sea at right angles to the shoreline. This angle is determined on the basis of the position of the sun. Some specimens of *Talitrus* were taken from Italy to Argentina. Their escape direction was oriented to the sun in accordance with their inner clock, which had been set in Italy. The possession of the clock does not mean that it is always used or even that it can always be used. In physiological terms, it means that certain processes are coupled to the clock tightly, or not at all, depending on internal and external conditions. Hoffmann (1960) trained starlings to take off at a certain angle of the compass by appropriately positioning feeding plates (Fig. 1.17). He then exposed the animals to a LD cycle, which was shifted by 6 h, compared to

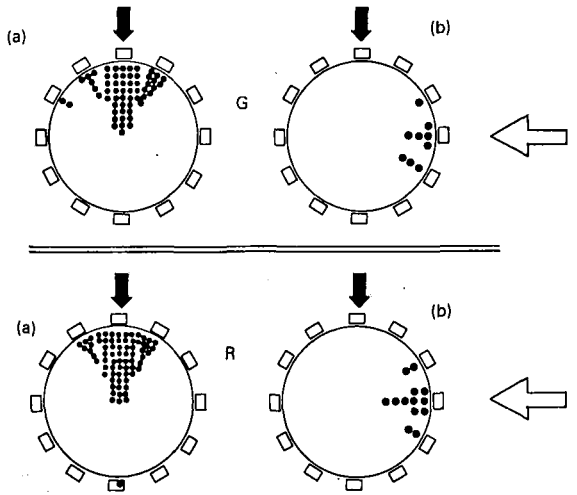

Fig. 1.17 Compensation for sun movement while direction finding of two starlings. The black arrow indicates the direction of food training before the test. The rectangles represent the (now empty) food containers. Each dot represents one choice. (a) shows the starling G and R direction finding after the training period without resetting the clock. (b) shows the choices after the internal clock of the birds have been advanced by 6 h. The clock had been advanced by offering LD cycles with a 6 h phase shift for 12-8 days. Unfilled arrows indicate expected choice of direction. (After Hoffmann, 1953).

the original day/night cycle. Thus, he could shift the clock within a few days so that the birds now took off to search for food in a direction deviating by 90° from the training direction.

The deviation occurred to the right or to the left, depending upon whether the clock has been advanced or delayed by 6 h. The orientation for the return to home base must be much more difficult and would require a time sense with an estimated accuracy of 2 min per day. The free- running periods of most circadian rhythms do display this kind of accuracy. Experiments by Schmidt-Koenig (1975) with an experimentally phase shifted clock (as by Hoffmann) indicate that the same kind of orientation as in the directed take-off does in fact participate in the return of carrier pigeons. Experimental data on the ability of other vertebrates to use time compensated sun compass orientation are now available for mammals, fishes, lizards, turtles and frogs.

1.12 Loss of overt rhythmicity

Arctic and Antarctic regions

Some beetles of the arctic region showed only a faint rhythm that did not even entrain to LD cycles. A circadian activity pattern may be altogether missing in the penguin *Pygoscelis adeliae.* Some arctic rodents showed only nocturnal activity during the arctic winter (DD) and diffuse activity throughout the 24 h during arctic summer (LL). Eskimos are reported *not* to show the normal 24 h periodicity of urine production and potassium secretion, even when they followed a regular daily routine (Lobban, 1967). In contrast, people from temperate regions, even after living in the arctic for several years, continue to show the 24 h periodicity of excretion. Several species of plants growing in extreme arctic conditions (Spitsbergen, latitude 76°– 80°) still have circadian leaf movements (Bünning, 1973).

Caves

A loss of circadian rhythms in activity and physiology can be expected in organisms living in caves. Park et al. (1941) could not find any circadian rhythm in the activity of the cave crayfish *Oronectes pellucidus.* Similarly, no circadian rhythm could be detected in the eyeless cave crayfish *Niphargus puteanus.* In Madurai we investigated the locomotor activity of a millipede living inside a natural cave in pockets 40 m deep in perpetual darkness, constant temperature and high humidity to see if it shows circadian organisation. The millipede might have lived in its present habitat for thousands of years as evidenced by morphological features which hinted at adaptation to the cave environment – no pigmented eye-spots, severe reduction in the number of ommatidia and a total lack of pigments giving

them a milk-white appearance. The monitoring of activity was made in the cave with an actograph fitted with far-red light emitting diodes and photoelements. When the millipedes intercepted the red beam during locomotion in the aktograph the writing stylets of an Esterline Angus A 620 X event recorder were activated. Careful inspection of activity revealed a wobbly circadian rhythm in the activity of millipedes which was capable of entrainment by LD cycles artificially offered inside the cave or in the laboratory. The circadian rhythm had survived the constancy of the environment and absence of zeitgebers for thousands of generations (Koilraj et al., 2000). The instances cited have all been investigated with reference to locomotor activity. If carefully investigated, other rhythms besides the one in locomotor activity, (as in oxygen consumption, for example) may be present. In all these cases of arrhythmia, we may even be dealing with an uncoupling of the locomotor activity from the circadian clock , i.e, uncoupling of the activity being measured from the underlying clock.

1.13 Methods of recording rhythms

The methods of recording rhythms described here can be adopted even in schools and junior colleges. The two most important criteria in studying biological rhythms are: ready availability of the biological material and a function that is easily recorded, without disturbing the rhythm itself. For over a hundred years now these criteria have certainly played a role in making leaf movement rhythms the clear favorite of biologists studying daily rhythms. Figure 1.18 shows a simple procedure for recording leaf movements.

We adopted this method for recording the sleep movements of the leaves of the Indian cotton plant (*Gossypium hirsutum*). The cotton plant, *Gossypium hirsutum* L. exhibits circadian sleep movements of its leaves. Topical application of fusaric acid (5-*n*-butyl-pyridine 2-carboxylic acid), an *in vivo* toxin produced during pathogenesis of the wilt disease, to the lamina caused phase shifts advances and delays that varied in degree and magnitude as a function of the treated phases (Sundararajan et al., 1978). The trifoliate leaves of *Oxalis* show closing and opening 24 h rhythms. They close together in a clasping fashion for the night and spread out for the day. The arrangement shown in Fig. 1.19 may be used to measure this rhythm.

A photoelement is placed underneath the plexiglass cuvette holding the leaves. The photoelement is connected to a device that measures the amount of light falling on it and the fluctuations of light cause parallel changes in the electrical resistance measured in millivolts (mV). The course of the size of the shadow falling on the photoelement during the folding and opening of the leaflets is traced on an X-Y recorder. The chart of the recorder moves at a known and controllable speed.

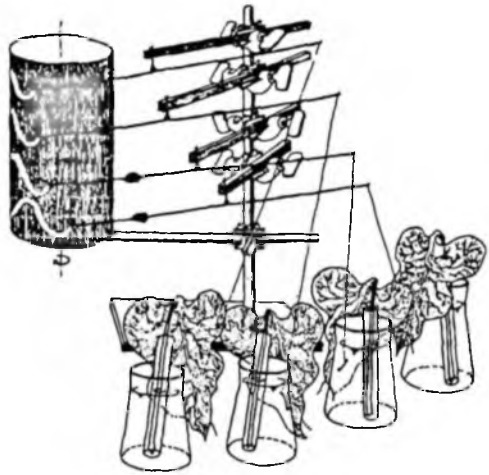

Fig. 1.18 A set up to register sleep movements of leaves (in this case of a bean plant) widely in use ever since the days of Wilhelm Pfeffer. The thermohygrograph drum wrapped in paper and coated with soot, completes one revolution in 7 days.(After Engelmann and Klemke, 1983).

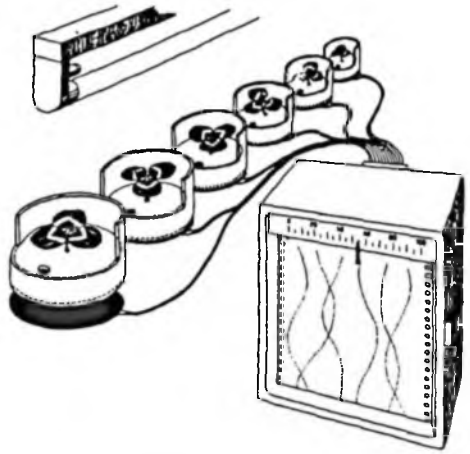

Fig. 1.19 A photoelectrical method of recording leaf movements in *Oxalis*. The shadows of the leaves fall on the circular photoelements placed below. The changes in the electrical resistance caused by the folding and unfolding leaves are measured on a six channel X-Y recorder. (After Engelmann and Klemke, 1983).

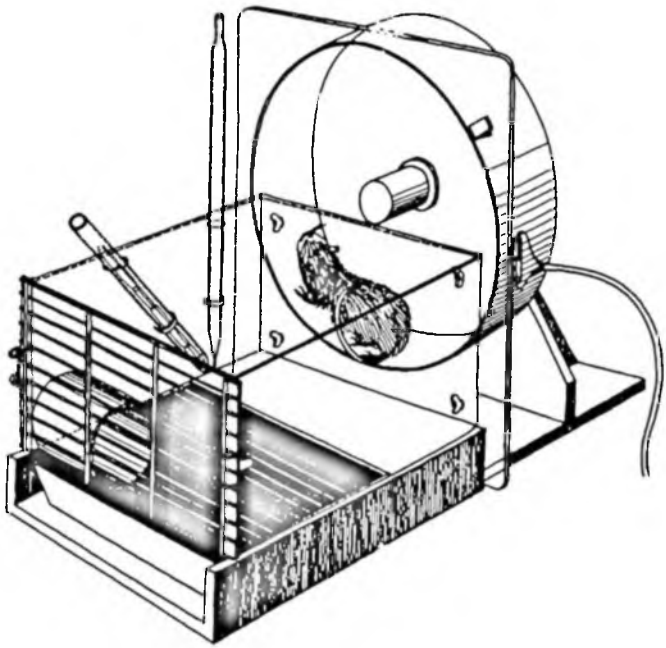

Fig. 1.20 Activity running wheel with which the locomotor, feeding and drinking activity of small mammals can be recorded. The wheel itself can be made from a suitable slice of PVC tube of desired diameter. The magnet attached to the wheel activates when in rotation caused by the running of the animal, a reeds relay and activity stylet inscribes each revolution as the deflection of the stylet on an event (X–Y) recorder (not shown). The wheel is covered in the front by plexiglass with a hole to permit the animal to get into and out of the wheel. The smaller tube in the larger cage is for the animal to sleep in. (After Engelmann and Klemke, 1983).

Another favourite object of study in biological rhythm research has been the locomotor activity of cockroaches. They are highly recommended in view of their sturdiness and easy availability. Food and water through a cotton wick can be thrust into the sides of one flank of the activity wheel. Birds and mammals are also excellent subjects in the study of circadian rhythms. The experimental arrangements for recording the activity of small mammals such as hamsters, guinea pigs, squirrels and mice can be a running wheel set-up similar to the one shown in Fig. 1.20.

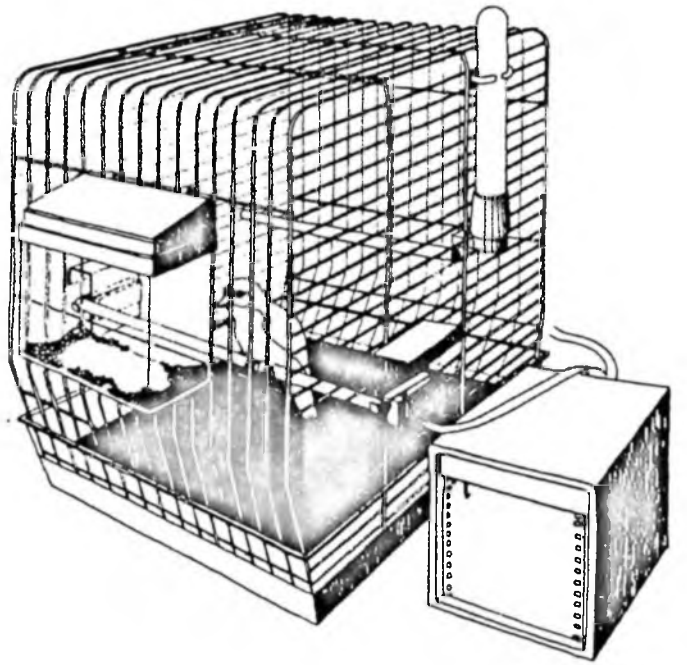

Fig. 1.21 Activity rhythms in birds. One of the perches is mounted on two microswitches which activate writing stylets when the birds hop on and off, and record activity on the X-Y recorder (Modified after Engelmann and Klemke, 1983).

Figure 1.21 shows the standard perch-hopping activity cages used to measure flight activity in birds.

If birds like finches or sparrows are caged and therefore cannot fly around freely, they begin hopping about or cling to the sides of cages if they find a perch. If offered crossbars they then hop from one to the other. The two perches in a cage are mounted such that they activate microswitches connected to writing stylets of X-Y or event recorders every time the bird alights on them or leaves (Engelmann and Klemke, 1983).

The choice of the organism obviously would depend on the kind of questions a researcher has. If it is bird migration, or migratory restlessness that is being investigated, then the researcher must find a migratory bird. If the effects of pharmacological and other rhythm affecting drugs are to be checked, it makes sense to settle for leaf movement rhythms, for the reagents can be measured carefully and mixed with the water in which the plant leaves are

held in hydroponics experiments. For our experiments with bats, we had to design our own flight activity cages made of aluminum frames (50 × 50 × 50) cm and covered in mosquito netting material with a sleeve to feed the bats. The cages were suspended from a sturdy bar by springs. When the bats flew inside the cage it jiggled and such movements activated the stylets of an Esterline Angus event recorder. Often, the elegance of recording methods would depend on the ingenuity of the experimenter. This brief introduction to the subject of chronobiology is to help the reader to better understand the rest of this book.

2. Tidal and Lunar Rhythms

The true worth of an experimenter consists in pursuing not only what he seeks, but also what he did not seek.

Claude Bernard

Tides are a response of the waters of seas and oceans to the gravitational pull of the moon and to a lesser extent the sun. The moon rises 50 min later every day, therefore, the time of the high and low tide will also be correspondingly later every day. Twice each month when it is the *new* moon and again when it is the *full* moon, the strongest tidal movements are evoked accounting for the highest flood tides and the lowest ebb tides of the 29-day lunar month. These are called the spring tides.

2.1 Re-discovery of tidal and circadian rhythms in a crab

Many authors had reported the occurrence and persistence of tidal rhythms in crustaceans and other inter-tidal organisms, the earliest reports coming in over a hundred years ago (Gamble and Keeble, 1903). Unfortunately, the journals in which the reports of JT Enright and D Neumann appeared were not to be found in the library of the University of Madras, the only scientific library that I had access to in the early 1960s and I was personally not aware of many of the reports, until much later. The reader must forgive me for getting personal in one or two places – but sometimes the wonder of science and discovery can only be narrated in first person.

Textbooks stated that the anomuran crab *Emerita asiatica* always remained under a few centimetres of water at the very edge of the sea. It was also known that to remain so the crab had to periodically migrate up the slope of the beach when the sea rose and reverse the direction and migrate down the slope of the beach when the sea ebbed (McGinitie and McGinitie, 1949). In the course of their four (two upward and two downward) daily migrations, the crabs always faced the sea, extended their plumose antennae with fine hair-like structures, and strained the protozoans, bacteria and other tiny planktonic organisms that

were being transported by the "wash" of waves rushing seawards. The crabs fed on such tiny organisms. The larger females of about 4–6 cm remained buried in the sand with just their stalked eyes sticking out. On sunny days, the thousands upon thousands of the slender stalked eyes gave to the shore surface the glistening appearance of creamy velvet. At the approach of a shadow or bird all the eyes would be retracted in amazing synchrony and it was only then that the wet brown of the sea sand could be seen.

I was attempting to determine, as my PhD thesis, the minimum amount of oxygen that would be needed for this crab to survive. The story reproduced here now, narrates how I accidentally discovered for myself, most unexpectedly, the tidal rhythms in the inter-tidal anomuran crab. It beautifully demonstrates the element of surprise and open-endedness of some discoveries in science (Chandrashekaran, 1996).

2.2 The experiments

I constructed respirometers in which fresh seawater could be made to flow continuously at desired rates (Fig. 2.1) (Chandrashekaran, 1965).

Oxygen content in the water flowing into the respirometer and the oxygen content in the water flowing out of the respirometer was estimated with the crab within it all the time. Since the rate of water flow (volume) was known, the actual oxygen consumed by the crab per unit time could be calculated, which was a good measure of the metabolism. But to obtain basal or standard values, the readings had to be taken when the crab remained still. In the case of humans, the measurements would be made typically on subjects lying on a bed and listening to music some 8 h after a light meal, hardly conditions I could impose on my crabs. The swimming activity and rest patterns appeared to me to be bizarre, for there was no saying when they would swim and when they would stay still. I first noticed that the crabs swam vigorously bottom up at unexpected hours or sat still at the bottom of the respirometers at other hours. Within the respiration chamber was the activity cage enclosing a crab connected to a Palmer's lever. The fulcrum is not shown in Fig. 2.1 but a moveable weight screwed on the free arm of the lever is shown. This weight could be so adjusted that the cage touched the bottom of the respiration chamber when the crab rested. When the crab swam up, the cage would also rise and these movements of the cage were traced by the writing tip of the Palmer's lever, as vertical markings on a slow revolving kymograph drum. The kymograph traces were preserved by passing them through appropriately thinned solutions of shellac.

One day – it was a pitch-dark Monday night – I came into the aquarium at 2100 h to check out 400 freshly captured crabs which I had placed in 20 glass troughs of ca. 30 cm diameter each containing 20 crabs. When I turned on the laboratory lights, I was struck by the lovely sight of 400 grey crabs swimming with their chalk white abdomen showing. They all swam synchronously – all

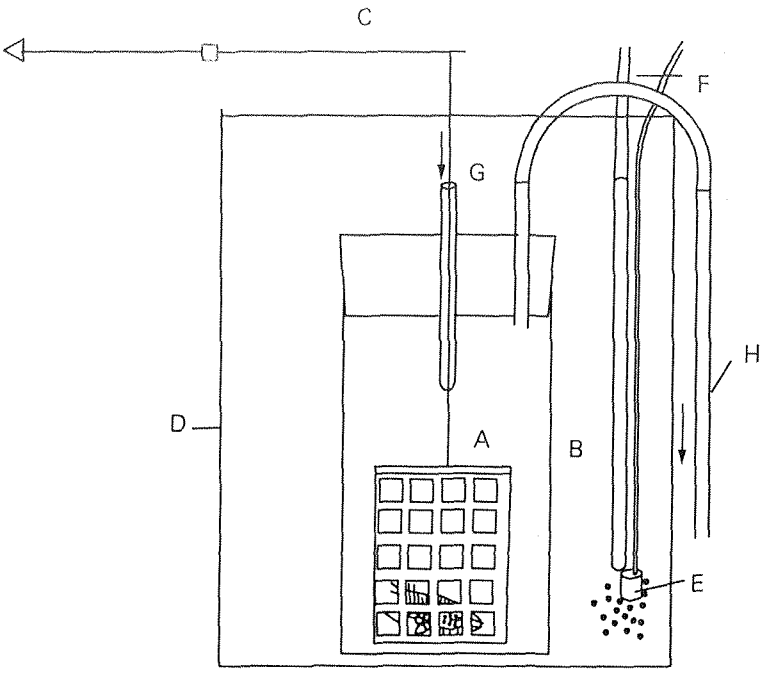

Fig. 2.1 The simple plastic cage device used to record the swimming activity of the crab *Emerita asiatica* with the continuous flow of seawater arrangement to enable oxygen consumption estimations. (A) activity cage; (B) animal chamber; (C) marking lever; (D) constant water level trough (not drawn to scale); (G) inlet; (H) outlet. When the crab swam up into the water away from the bottom of the activity cage, the cage rose inside the chamber. Such bouts of activity registered themselves through the writing lever on a kymograph drum. (After Chandrashekaran, 1965).

the crabs in all the troughs. I attributed the swimming and commotion to the lights coming on after a spell of darkness. In a while the swimming abated and a couple of hours later even subsided. I concluded that the crabs were getting used to the light and were therefore not reacting to it anymore (habituation).

For verification, I repeated the events the next Monday, but when I turned the lights on not a single crab stirred! It was as though a flashing contagion had killed them all. Each crab, on being prodded with a pencil or a stick, stirred and moved but would not swim. They all sat still – all the crabs in all the troughs. That ought to have been my moment of discovery, but my mind was not prepared to read the story that was literally being thrust upon me. "In the field of observation, chance only favours those minds which have been prepared", said Louis Pasteur.

Given the unpredictable patterns of the activities of the crab (as I had then erroneously believed), I decided to monitor the swimming activity of the crab with the aid of the simple actograph/respirometer set-up also shown in Fig. 2.1.

Using this I could simultaneously measure oxygen consumption and thus monitor swimming activity. Thus I made the crabs write their own story and the kymograph records were "fixed" and kept for future graph-plotting. Given the burden of physical work associated with these experiments, graph-plotting was undertaken roughly a year after the Monday to Monday experience. Late one night I began to transfer the activity data from kymograph records to millimeter paper when I literally saw beautiful activity peaks and rest troughs emerge at regular intervals. It was the wee hours of the morning. I had hit upon *tidal rhythms*! The data of one long experiment is reproduced in Fig. 2.2.

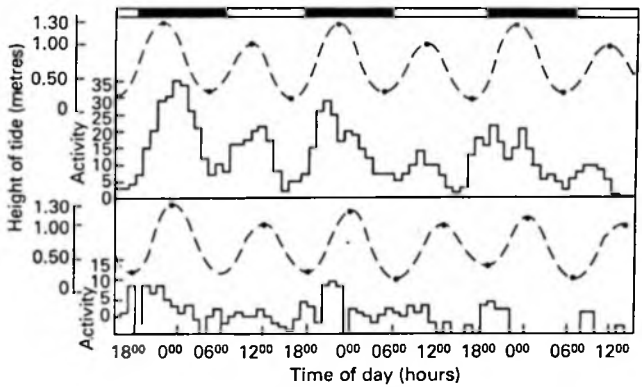

Fig. 2.2 Tidal rhythms in the spontaneous swimming activity of *Emerita asiatica* in the constant conditions of the laboratory and its waning in the course of six days. Dark bars denote hours of darkness outside. Other details in text. (After Chandrashekaran, 1965).

The laboratory did not have a copy of the Tide Tables published annually by the Geodetical Survey of India. For the reader's information, the Madras coast has a semi-diurnal tidal environment with high and low tides alternating at 6-hourly intervals. Thus, two high or two low tides of a 24.8 h lunar day would be 12.4 h apart. In the Madras coast the difference in height between the highest spring tide and the lowest neap tide is ca. 2 m although the daily amplitude of the twice-daily high and low tides can be a mere 30 – 40 cm.

The tidal curves were fitted in the course of the next few days. This is a truly unbiased re-discovery, which had to wait its moment of unfolding. Note the gradual waning of the tidal rhythm in the activity of the crab after the 4 or 5 days of the constant conditions of the laboratory (Fig. 2.2).

Perhaps now the Monday to Monday anomalous finding can be understood with hindsight. On the first Monday night it was obviously ***high tide*** in Madras at 2100 h. The crabs in the laboratory were re-enacting the activity of their

conspecifics outside along the shoreline. The time of high tide moves by 50 min per day in keeping with the time of moonrise. Therefore, in 7 days until the next Monday the tidal progression was 50 × 7=350 min which is close to 6 h. Since a high and succeeding low tide are 6 h apart, on the second Monday it would have been *low tide* in Madras at 2100 h. The crabs in the laboratory were again only enacting the drama of what was happening to their conspecifics in the beach 2 km away. These crabs swim vigorously in nature during high tide and remain still during low tide.

Further details of the data needed to be sorted out. For complex geophysical reasons, the night high tide in the Madras coast often tended to be higher than the day high tide. Given the near 12:12 h LD of Madras, one of the high tides always coincided with hours of darkness. From Fig. 2.2 it can be very clearly seen that the amount of the nightly high tide activity of the crabs were much higher than their day high tide activity. Was the amount of activity then a function of the height of the high tide? For higher the tide, the greater will be the area of the beach inundated, and therefore, more abundant the food. Another possibility was that the nightly exaggerated activity arose because of a super-position of a *circadian* component on a *tidal* component. Predation by birds would also be low at night time thus enabling the crabs to be very active safely. Experiments done on rare days when the day time and night time high tides were of equal height, as given in the Tide Tables, revealed that the nightly high tide activity was always higher than the activity coinciding with the high tide of day hours. Figure 2.3 illustrates data from such experiments.

It was concluded that the exaggerated nightly activity coinciding with the nighttime high tide arose because of a super-position of tidal and diurnal rhythms. This was at a time when even the existence of an endogenous diurnal rhythm was regarded, by most biologists, as subscribing to a mytical or metaphysical notion. If even the ubiquitous *circadian* rhythms were suspect in those days, *tidal* and *lunar* rhythms strained the very limits of scientific credulity!

2.3 Earlier work on tidal rhythms

Several organisms living in the inter-tidal zone of the sea show tidal rhythms. In the Welsh coast near Cardiff and Swansea and in the beaches in Brittany, in a narrow zone just below the high tide mark of neap tides, lives a small acoelous turbellarian worm, *'Convoluta roscoffensis*. Over the hours of darkness of the night and during the tides, these zoo-xanthellae-bearing worms live buried in the sand but emerge on to the surface during daytime low tides. Thus, exposed to the sun the symbiotic algae within the beautifully sinuous body of the planarians photosynthesise, giving the worms a grass-green sheen. Since these worms occur in great density of thousands per square metre, large expanses of the beaches at low tide appear spinach-green. When the sea rises and covers the *Convoluta* patches, the worms burrow into the

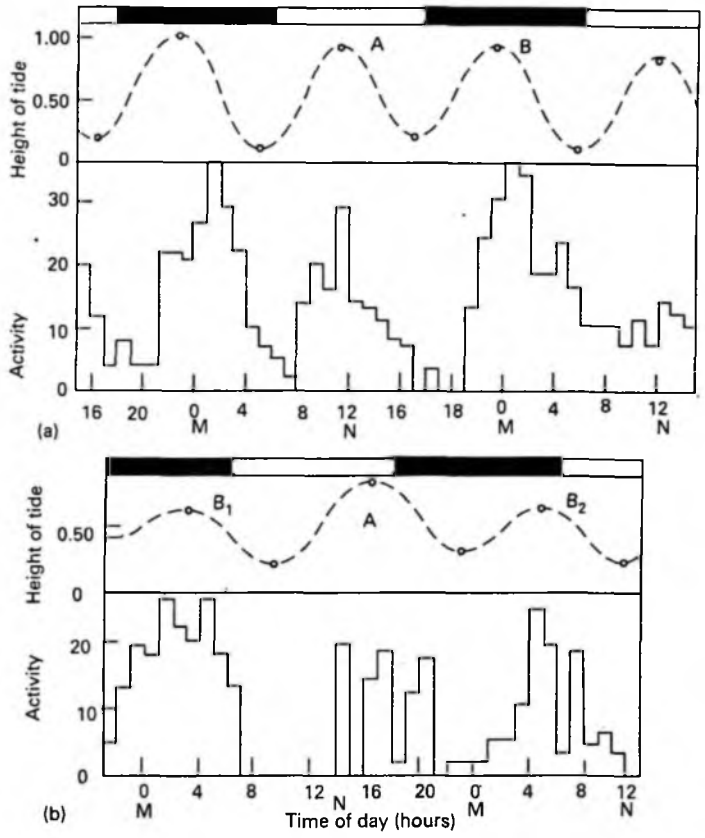

Fig. 2.3(a) illustrates the tidal rhythm in the swimming activity of a female *Emerita* over forty-eight hours. Note that (A) daytime and (B) nighttime high tides on these days were of equal height. Even so, the activity coinciding with the high tides occurring during hours of darkness on both nights was much higher than the activity coinciding with the daytime high tide.
(b) Illustration of the tidal rhythm in the swimming activity of a female *Emerita* recorded through thirty nine hours when (A) a daytime high tide was even higher than (B) the night high tide. Shaded bars indicate hours of darkness outside. (After Chandrashekaran, 1965).

sand to avoid being washed up on to the beach. They re-emerge with the advent of the low tide (Gamble and Keeble, 1903; Bohn, 1903). These movements of the turbellarians are a type of *vertical migration.*

If these worms are brought into the laboratory and placed in petri-dishes or small tanks with seawater and sand in continuous light, the vertical migrations persist in the laboratory for 4–7 days in approximate synchrony with the tides (Martin, 1907). That phototactic and photosynthetic components are involved becomes apparent in that the vertical circatidal migrations of *Convoluta* persist in LL but not DD. Diatoms and euglenoids are also known to undergo vertical tidal rhythms which reportedly persist in the natural LD conditions in the laboratory for a few days. Bohn (1906) reported that the sea anemone *Actinia equina* contracted and expanded with tidal frequency for over 8 days after being brought to the laboratory aquarium. It must be pointed out that results of later experiments performed in 1964 cast some doubt about the duration over which tidal tendencies persist in *Actinia equina*. However, I know from personal experience with inter-tidal invertebrates in Madras and *Emerita talpoida* in Wilmington, N. Carolina that the number of days tidal rhythms persist may depend on the conditions of the experiments as well as on the species and even the ambient temperature and oxygen partial pressure of the seawater. But enough evidence has been accumulated now to indicate that circatidal rhythms are endogenously programmed, much as circadian rhythms, and that they do persist in the constant and aperiodic conditions of the laboratory up to 7–14 days.

Buried in the upper reaches of the beaches of southern California, lives the amphipod *Synchelidium*. Its habitat is submerged during high tides when the amphipods come out, and swim about and feed in the wave wash. Two or three hours later when the sea ebbs, the amphipods burrow into the moist sand to safety. Enright (1963) investigated the persistent tidal rhythmicity of the swimming activity of the crustacean in the constant conditions of his laboratory in the Scripps Institution of Oceanography in La Jolla. He placed large populations of *Synchelidium* in cuvette-like glass troughs with seawater and sand, and followed with time-lapse photography the number of amphipods swimming. There was an impressive tidal rhythm in the swimming activity of these animals (Fig. 2.4) which, however, damped out after about 3 days in the laboratory.

The first two peaks even faithfully mirrored the phase, height and semi-diurnal inequalities of the tides of the Californian coast at La Jolla. Three other crustaceans occurring in the same beach, an anomuran *Emerita*, a mysid *Archaeomysis* and an isopod *Excirolana chiltoni* all possessed similar tidal rhythms as shown by *Synchelidium* (Enright, 1963).

The isopod *Excirolana chiltoni* which also occurs in the same beaches of California with its complex patterns of persistent tidal rhythms was the object of Enright (1972) and his student Klapow's (1972) study. They claimed that the patterns of swimming activity of this burrowing crustacean in the constant conditions of the laboratory mirror the amplitude and timing of the tides, which were in themselves quite complex. The tides undergo diurnal to semi-diurnal appearances and amplitudinal inequalities. There was,

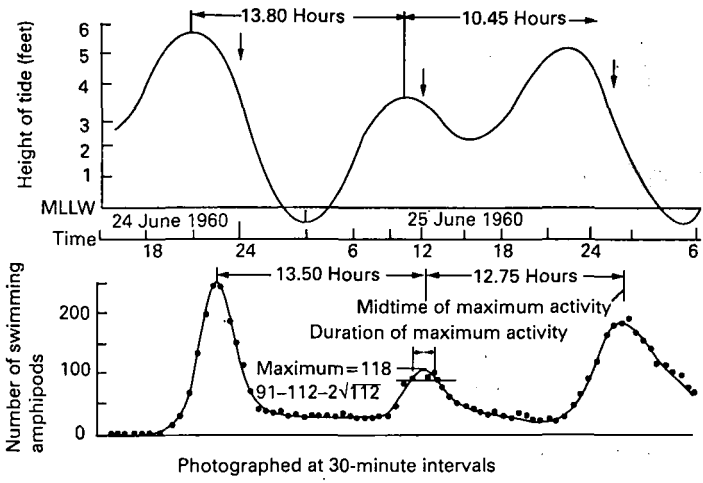

Fig. 2.4 Tidal rhythm of freshly collected *Synchelidium* sp. Upper graph : tidal profile from U.S. Coast and Geodetic Survey predictions for La Jolla, California; O: mean lower low water; Lower graph : counts of number of swimming amphipods during laboratory observation. Two photographs were made per hour.(After Enright, 1963).

however, quite a bit of variability in the persistent tidal rhythms of *Excirolana*. It was reported in a later paper (Enright, 1972) that there were especially tenacious specimens of the *Excirolana* (virtuoso isopods) whose tidal rhythms in the swimming activity persisted up to 65 days, all the while mimicking the changing features and inequalities of the tides. The unanswered question is how even without reinforcing zeitgeber inputs could there be such intricate and long-lasting synchronisation of activity patterns to the actual height of the high tides.

The inter-tidal fish *Blennius pholis* shows clear-cut tidal rhythms in its swimming activity when observed under laboratory conditions for at least 5 days (Gibson, 1967). This is a littoral fish which lives under loose stones in pools between the high and low tide zones and migrates between feeding and resting grounds with each flood tide. Gibson studied the locomotor activity of this fish in the laboratory in an actograph that was designed on the knowledge that the fish sank and rested at the bottom of the sea between bouts of activity coinciding with the high tide. Recordings made in LL and DD at constant temperature and away from the tides in the laboratory revealed the impressive (Fig. 2.5) and overt tidal rhythmicity in swimming activity, which, however, waned and damped out after 4–5 days.

Honegger (1973), who worked on California fiddler crabs, has suggested that tidal rhythms may only be modified circadian rhythms, which in the

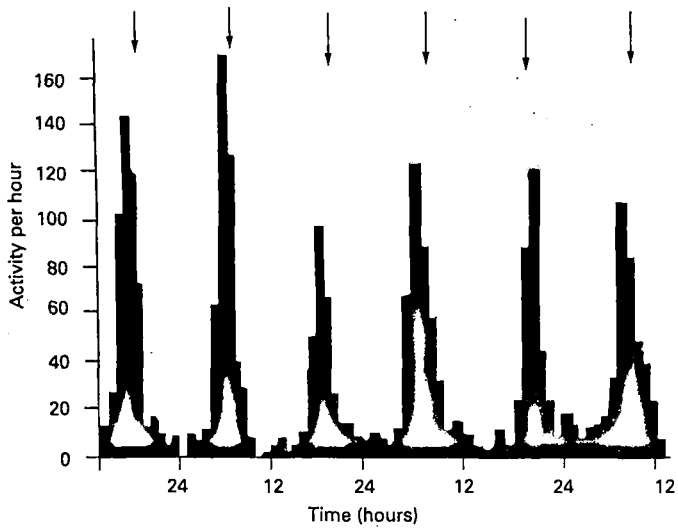

Fig. 2.5 The persistent tidal activity of the shanny *Blennius pholis* in DD. Arrows predict time of high tide (modified from Gibson, 1965).

course of evolution had become refractory to LD cycles and gradually entrained to the tidal cycles of the marine environment. In India one may spot fiddler crab males wave their claws (chelicerae) on the banks of estuaries during low tide – I have witnessed this on the banks of the Cooum and Adyar estuary in Madras and on the banks of the Mandovi river in Goa. The waving of the enlarged chelicera is very marked and in great synchrony when low tide coincides with sunrise and is reminiscent of dhobies (washermen) beating clothes on stones at the banks of rivers – a common sight before washing machines came into vogue. So the older literature of Indian origin refers to fiddler crabs as "dhobi-crabs." When the water rises at high tide the crabs disappear into their burrows, plug them and stay virtually under water.

2.4 Zeitgeber(s)

The zeitgebers (entraining cues) of tidal rhythms are really not known. Hydrostatic pressure, turbulence of the waves and resultant mechanical agitation have all been claimed to be entraining cues. I have noticed that two species of burrowing anomuran crabs, *Emerita asiatica* and *Albunea* sp. and a clam *Donax cuneatus* showed twice-daily tidal migrations in the

Madras coast. These animals are washed up the shore during high tide and 6 h later down the shore during low tide, by so-called breakers. Breakers are big waves which occur once every 10–15 waves and end with a big swishing noise and a lot of foam. The mystery to me was, how these animals "knew" a breaker was rolling towards them – especially the clam, which had white and sturdy shells. The clams literally popped up from the sand when the breaker was near to be washed away by as much as 15–20 cm each time, where they promptly reburied themselves in the sand and disappeared from sight. Turner and Belding (1957) suggested that the tidal migratory limits of the bivalve *Donax variabilis* occurring in the east coast of the USA were probably set by the "acoustic shocks" generated by the breakers. To prove this, they went to the seashore, stomped on the wet beach and saw that the bivalves just popped out. I performed an experiment in the laboratory to investigate if *Donax cuneatus* responded to these "acoustic shocks". I used a Philip's audio-frequency generator (GM 2308/01) with a 20 watt amplifier and a 10 watt diaphragm loudspeaker for the purpose. I discovered that *Donax cuneatus* responded to acoustic bombardments of frequencies 50, 100 and 150 c/s with increasingly rapid shell movements and protrusion of the foot and siphons (Chandrashekaran, 1963). This interestingly is the range of acoustic/mechanical vibrations the breakers generate in the beach. If one lay on the beach with an ear to the ground, one could hear the breakers rumbling with these typical frequencies. The clams did not respond to higher frequencies of 500, 1000, 1500, 2000 and 2500 c/s, which obviously do not have any environmental relevance to them. The results of these experiments confirm that the acoustic bombardment generated by the breakers trigger the popping up of organisms like *Emerita, Albunea* and *Donax cuneatus* from underneath the sand, to be washed up the beach at high tide, and down the beach during the ebbing sea, but do not shed any light whatsoever on the zeitgebers regulating tidal rhythms. Tidal rhythms wane rapidly, they are impervious to LD entrainment and the zeitgebers are unknown. To this extent they are ephemeral phenomena to the rhythm researcher. The precision of tidal rhythms are also in no way comparable to that of circadian rhythms and lunar/tidal rhythm researchers, for practical reasons, can work only in marine biological institutions situated close to the sea.

Textbooks inform us that a lunar day is 24 h 50 min long. Two high tides must therefore occur 12 h 25 min apart and so on. But a careful study of the tide table will make it clear that the situation never really happens in the real world outside owing to the topography of the tidal strip and metereological conditions. Compared to the very predictable sunrise/sunset timings, the tidal timings may be said to be "noisy." The tidal timings and height of tides, on the other hand, are often dictated by the topography of the coast, wind velocity and moon-related phases, in contrast to the noise-free state of LD zeitgebers. Nature obviously cannot programme to accommodate for noise, which may be why tidal rhythms lack the precision

of circadian rhythms. It is also not known if tidal/lunar rhythms have a separate genetic basis, unrelated to the 'clock' genes. Lability of the period may be an asset in a constantly changing environment like the inter-tidal environment.

2.5 Lunar and semi-lunar rhythms

Several marine organisms are known to have lunar breeding cycles with their physiological activities coinciding with neap or spring tides. They either have the lunar monthly period of 29 days or semilunar periods of 14–15 days. I describe here only some of the better known cases. The palolo worm of the Pacific and Atlantic oceans has probably the most famous lunar rhythm of all. This animal reproduces only twice a year, namely, during the neap tides of the last quarter moon in October and November. The grunion fish *Leuresthes tenuis* of the California coast takes advantage of the highest tides. Riding on the crest of the waves, it arrives on the beach, where it deposits its eggs and sperm. The fertilised eggs develop in the warm, moist sand and are safe for the water does not reach them for the next two weeks. Only by the next spring tide would the fish have developed sufficiently to be freed from their eggs and washed out into the open sea. Activities with such a lunar periodicity have also been described in mussels, sea urchins, and discharge of gametes in algae. The brown alga *Dictyota dichotoma* discharges its gametes twice in a lunar cycle. The remarkable feature of these rhythms is that some of them persist in the laboratory without tidal or moonlight exposure making it likely that they are also endogenous.

The moon affects life on earth in two ways: (1) through the direct influence of light (especially during full moon) and (2) through the tidal changes it brings about. Moonlight is sunlight reflected from the lunar surface with nearly the same spectral composition but with a slight shift towards red. Moonlight has an intensity of about 0.20–0.25 lux depending on latitude. Hauenschild (1960) showed that a weak light of moonlight intensity phased the lunar cycles of the marine worm *Platynereis*. This is also true for the brown alga *Dictyota*, which exhibits this relationship even more clearly. Bünning and Müller (1962) showed that the maximum discharge of eggs in *Dictyota dichotama* takes place 9 days after exposure to moonlight. The next maximum then follows after an interval of 15–16 days. Midges of the genus *Clunio* are found in the inter-tidal zones of the Atlantic and Pacific shores from the temperate areas to the Arctic. They have very curious and complicated life cycles. The *Clunio marinus* of the Atlantic coast of western Europe, for example, lives in the *lowest* parts of the inter-tidal zone exposed only during spring low water. It is only during these times that the insects are able to emerge (Neumann, 1963). Both sexes are extremely short-lived (ca. 2 h) and copulation and oviposition must occur before the tide rises and covers the larval site. On the Normandy coast, low tide occurs twice a

day at intervals of 12.4 h; consequently, low and high tides are about 50 min later each day and it takes a period of about 15 days before the times of low and high tides come full circle. Superimposed on this semi-diurnal tidal cycle is a semi-lunar tidal range. Eclosion of *Clunio marinus* in the Normandy coast is restricted to evening low tide (circadian component) and semi-lunar (lunar component) rhythms.

In a series of elegant experiments Neumann (1963; 1966a and b) analysed eclosion rhythms in *C. marinus* and showed it to be governed by the superposition of the circadian rhythm governing eclosion and the semi-lunar rhythm governing initiation of pupation. In populations of *C. marinus* reared in the laboratory in LD cycles of 16:8 h, eclosion occurred towards the end of the photoperiod. LL cultures showed arrhythmic eclosion but transfer to LD or exposure of the LL cultures to a single dark period re-initiated a circadian rhythm with periods <24 h. Neumann's results show that, the *C. marinus* eclosion rhythm is controlled by a circadian clock. The semi-lunar rhythm of pupation which is superimposed on the circadian cycle was shown to be entrained by natural or artificial moonlight (Neumann, 1966b). Cultures of *C. marinus* from Normandy were raised in LD 12:12 h or LD 16:8 h and then exposed to pulses of weak light (0.4 lux) during the dark period for 4–6 days at intervals of 30 days. This treatment initiated and entrained a semi-lunar rhythmicity, which was, absent from control populations without moonlight.

Considerable differences are known to occur between populations of these midges in different localities and different species of *Clunio*. These differences have been shown to be genotypic (Neumann, 1966a and b) and polygenic. Lunar rhythms of activity or eclosion (with a period of ca. 28 days) have been described under field conditions for a number of insects (Saunders, 1976). In at least two examples, in the mayfly *Povilla adusta* and ant lion *Myrmeleon obscurus* (Hartland-Rowe, 1958; Youthed and Moran, 1969a), these rhythms have been shown to be endogenous. Hartland-Rowe (1955; 1958) showed that the mayfly *Povilla adusta* emerged from the waters of Lake Victoria in its greatest numbers just after the full moon. This rhythm was maintained even after the nymphs had been kept in the dark for 10 days and in two individuals for 6 weeks. The second case concerns the rhythm of pit-building activity by larvae of the ant lion *Myrmeleon obscurus*. Using mean pit volume as a measure of activity, Youthed and Moran (1969b) showed that the maximum activity occurred at the time of the full moon. There was also a clear lunar day (24.8 h) rhythm with a peak in activity about 4 h after moonrise. The authors demonstrated that the observed lunar rhythm (period about 28 days) was a combination of this lunar day and the solar day (circadian) rhythm described earlier (Youthed and Moran, 1969a), and which produces a peak in activity shortly after dark. The functional significance of these rhythms was not clear.

Gwinner (1973) established that the nocturnal restlessness of robins and redstars increased during the migratory season in conjunction with the

nocturnal illumination from the moon. In the night monkey *Aotus trivigatus*, activity increased with moonlight but in the phyllostomatid bat *Artibeus literatus*, activity decreased with moonlight (Erkert, 1974). Lunar monthly rhythms have been reported from light-trap studies in the abundance of the number of insect species, most of them from tropical latitudes. In a few species of microchiropteran bats in Madurai we found that there was very little overt foraging activity during full moon nights and enhanced activity during new moon nights. Morrison (1978) reported that the fruit bat *Artibeus jamaicensis*, returned to their roosts between 01.00 and 07.00 h on full moon nights even though the sky was overcast. On new moon nights they continued to forage outside throughout the night. Morrison postulated that the activity cycle in the fruit bats was in some manner locked to the phases of the lunar cycle and called the phenomenon *lunar phobia*.

My students at the Madurai Kamaraj University were out making bat counts of a few species of insectivorous bats on the night of 13 March 1979 when unknown to them a lunar eclipse occurred from 01.15 to 04.30 a.m. The bat counts made were mainly on *Pipistrellus* spp., *Rhinopoma hardwickei* and *Hipposideros speoris* or *H. bicolor*. Our observations were limited to these species. The observers worked in two groups in two sites A and B at eastern and western extremes of the university campus. They found that the bat foraging activity (bat counts) suddenly increased during the eclipse (Usman et al., 1980). Figure 2.6 sets out the data on bat counts and insect abundance at the two sites for the night of 13 March 1979.

The time and duration of the lunar eclipse during this full moon night are indicated by the black areas of the horizontal bars on top. The graphs at the bottom shows control measurement at the same sites on a full moon night a lunar month later (After Usman et al., 1980).

We interpret our bat activity data to mean that full moon light is far too bright and increases the risk of predatory attacks on these echo-locating bats by owls that rely on eyesight. Therefore the bats start to fly under canopy cover. The only environmental factors we had noticed that can suppress the activity of these bats and drive them to their daytime roosts are gusty winds and torrential rains. In spite of the early reports that moonlight acts as a zeitgeber to lunar cycles, more careful experiments still need to be done to clinch the issue.

2.6 Interpretations of the interaction of the two rhythms

One of the most striking features of tidal rhythms is that they do *not* entrain to LD cycles in nature or in the laboratory. On the contrary, one of the defining characteristics of circadian rhythms is that they entrain to LD cycles. The interesting question then is the relationship of tidal and circadian rhythms when they occur in the same organism, as for example in the mole crab *Emerita asiatica*. If we plot imaginary curves with a peak and a trough,

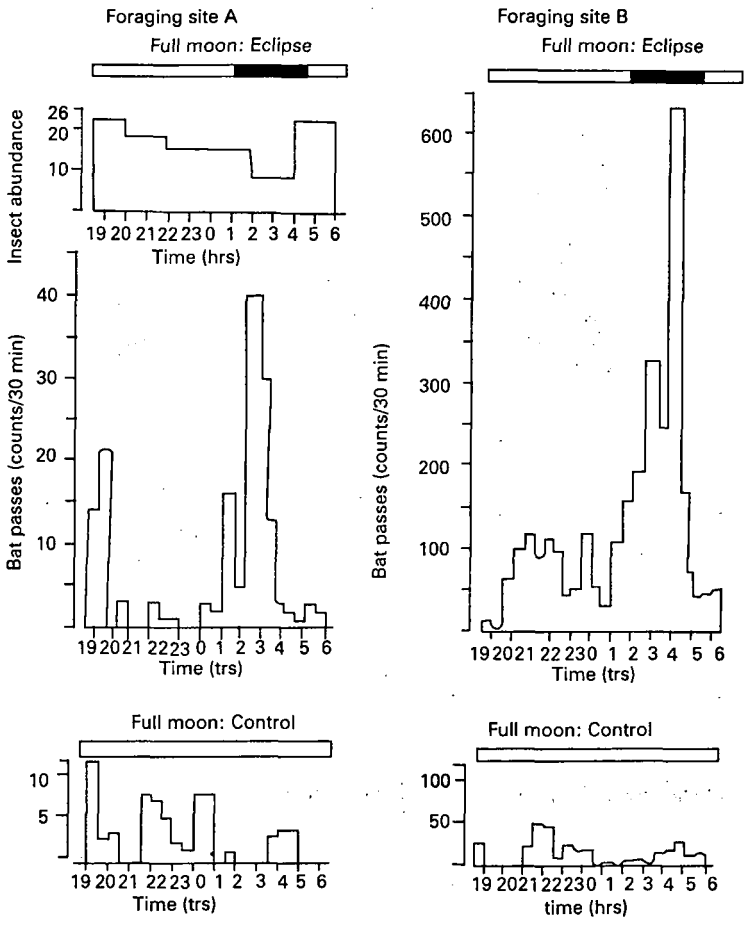

Fig. 2.6 Flight activity patterns of microchiropteran bats on the night of 13 March 1979 recorded simultaneously by two groups of researchers at two foraging sites A and B.

the LD12:12 h entrained 24 h circadian rhythms on a graph paper and again separately the 24.8 h lunar day rhythm on the same X-axis, it would be noticed that the lunar rhythm will progress to the right by 50 min everyday relative to the circadian rhythm, and would have drifted in 29 days 29 × 50 = 1450 min. which is very close to 24 h (1440 min). The lunar day peaks

would thus "beat" or coincide with the entrained circadian peaks once in 29 days, which is the duration of the lunar month. Bünning and Müller (1962) postulated that this periodic coincidence of the tidal (lunar) and circadian component also occurred in nature, and accounted for fortnightly and lunar monthly breeding cycles of marine invertebrates and other organisms. Naylor (1961) observed in the locomotor activity of the shore crab *Carcinus maenas*, tidal and circadian components with the tidal component drifting at tidal frequency in the laboratory and literally coinciding with the "diurnal" component at intervals of about 15 days. Any final conclusions on the "beat hypothesis" of lunar cycles would require detailed studies on the responses of the *circadian* and *tidal* rhythms under the experimental conditions of Bünning and Müller. Results of experiments on the lunar rhythms of the marine annelid *Platynereis* and the green alga *Halicystis parvula* and the isopod *Excirolana chiltoni* do *not* support the beat hypothesis of lunar and semi-lunar cycles.

2.7 Ecological importance

In most cases the ecological importance of the lunar cyclic phenomena is quite obvious. Reproduction could be attuned to the environmental conditions of spring and neap tide. In some cases, the release of male and female gametes is synchronised and the chances of fertilisation considerably enhanced. In addition reproduction being restricted to a few days in the lunar cycle, it is often also restricted to a certain time of year and even to a certain time of the day. This confines the reproductive processes in marine organisms, both plants and animals, sometimes to even a few hours in an entire year. This holds, for example, for the alga *Dictyota* and perhaps even more strictly for some marine animals. By such "gating" of gamete release, the chances of male and female gametes uniting are increased a thousand-fold over the chances when liberation is at random. The endogenous nature of the rhythmicity enables the organism to find the *appropriate* time in nature, even when the moon is hidden for 2 or 3 months because of a perpetually overcast sky.

3. THE *DROSOPHILA* CIRCADIAN CLOCK

❄

"Why this absurd concern with clocks, my friend?"

WALTER DE LA MARE

❄

3.1 Light and the eclosion rhythm in *Drosophila pseudoobscura*

The effects of light and temperature on the circadian rhythm of eclosion in *Drosophila* spp. have been the subject of early investigations in chronobiology and first attracted the attention of Kalmus and Bünning in 1935. Formal genetic studies of circadian behaviour began after the discovery of the *period* (*per*) gene of *Drosophila melanogaster* by Konopka and Benzer (1971). They found that one *per* mutant, was arrhythmic, another was rhythmic with a short period of 19 h and a third mutant was rhythmic with a long period of 29 h. The effects of the mutation were seen at the population level in eclosion and the level of locomotor activity of single flies (Kyriacou and Hall, 1980). Further developments in genetic, molecular, cellular, and behavioural studies of the *per* locus and its products in *Drosophila melanogaster* have been excellently reviewed by many workers (Dunlap, 1999; Hardin et al.,1995; Rosato and Kyriacou, 2001; Foster and Helfrich-Förster, 2001; Ashmore and Sehgal, 2003).

It is relatively easy to raise populations of *Drosophila* in the laboratory. A standardised fly medium of cornflour and molasses can be cooked and poured while hot into a wide-mouthed milk bottle and stoppered with cotton plugs. After the medium has sufficiently cooled, 40 to 60 adult flies, both male and female, from an earlier culture can be introduced and the cotton plugs replaced.

Adult females prefer to lay their eggs on the medium where it is not glassy smooth. If shallow furrows are made with the aid of a scalpel, the flies lay better. The eggs hatch, the first instar larva begin feeding, after which it undergoes two moults, crawling away from the moistness of the medium to become pupae which attach themselves to the walls of the rearing bottlle

Lights "on" and "off" rhythms

Laboratory populations of flies and pupae raised in LL or DD for a few generations were arrhythmic with flies eclosing at all hours of day and night. A single non-recurring transfer of the potentially arrhythmic populations from LL to DD initiated the so-called "off"-rhythm in eclosion. The off-rhythms were so designated because the act of switching off the light synchronises the oscillations of the arrhythmic pupae, which were obviously out-of-phase relative to one another and hence 'arrhythmic', and sets the eclosion rhythms in motion. The time of LL/DD transfer was arbitrarily called 0 h. The "on"-rhythms were similarly set in motion by the act of switching on the light at 0 h.

The transfers were made a couple of days before the first flies in a pupal population of mixed ages began to emerge, which was about 20–21 days after oviposition at 20°C in this strain of *D. pseudoobscura* (Princeton University 301, culture gifted to W. Engelmann by C.S.Pittendrigh). The off-rhythm was circadian and persisted with a period of 24.5 h for as long as there were flies to eclose in a culture. Figure 3.1 illustrates the time course of on- and off-rhythms.

The eclosion peaks on day one after transfers at the arbitrary hour "0" were poorly synchronised and therefore not shown in the figure (Chandrashekaran and Loher, 1969b). This was probably due to the fact that pupae which were on the verge of eclosing in the next few hours were developmentally too advanced to respond to the light changes.

The interesting feature was the fact that all off-rhythms were stable regardless of the intensity of the preceding LL. The peaks occurred ca. 15, 39, 63, 87, 111, 135, 159, etc., hours after LL/DD and eclosion lasted until there were no pupae left. In contrast, the on-rhythms were poorly synchronised and eventually dampened and disappeared. The eclosion rhythm waned with time in LL of intensities as low as starlight. The time course of on- and off-rhythms are 180° (12 h) out-of-phase relative to each other, which at one time tempted Pittendrigh to compare them to "morning" (M) and "evening" (E) oscillators. Pittendrigh had made a very crucial observation about the effect of light (LL) on the *Drosophila* oscillator. Pittendrigh (1966) postulated that the pacemaker oscillator was frozen by light at CT-12 phase, if LL lasted beyond 12 h. The phase at lights-on is 0 CT – a subjective sunrise time. On this scale, 12 CT would then be the subjective sunset time. Several experiments performed by Pittendrigh (1966) and Winfree (1971) proved that if populations of pupae are returned from LL which had lasted beyond 12 h, to DD, then the rhythm reappeared by the formula $n \times \tau + 15$ h, n = number of days. This was invariant. According to Pittendrigh, the clock "is released" from CT 12 position by the onset of DD. He dismissed whatever on-rhythms that persist as being *transients* (see Section 3.4). Pittendrigh (1981) had also investigated the effect of different light intensities (LL) in dampening the rhythms and causing

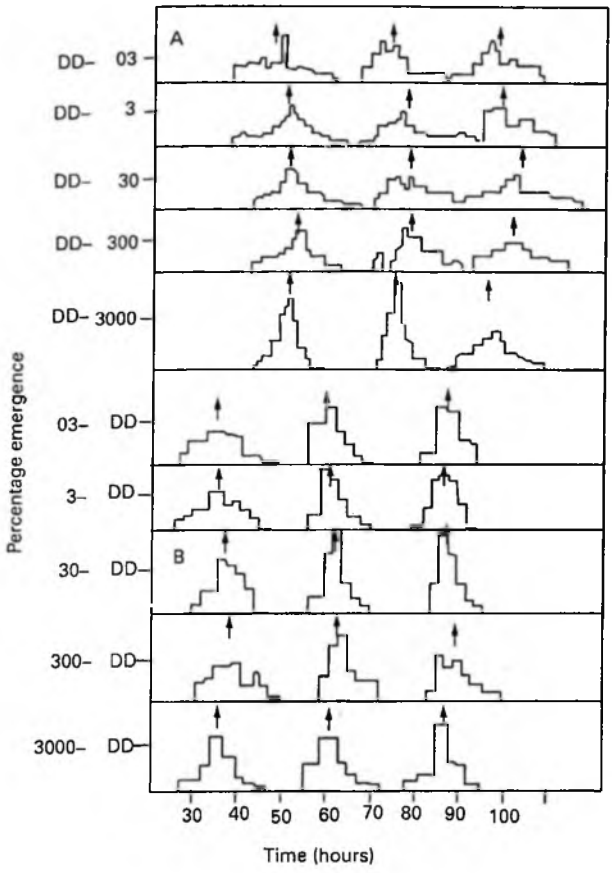

Fig. 3.1 On- and off-rhythms in the eclosion of adult *Drosophila pseudoobscura* flies. Populations were raised in either DD and then transferred to LL (upper panel), or were raised in LL of different intensities (lux) and then transferred to DD (lower panel). Tansfers were made on day 20 after oviposition and the time of transfer arbitrarily designated hour as 0. First eclosion peaks are not shown since synchronisation was generally poor in all cases. Arrows denote medians (After Chandrashekaran and Loher, 1969).

arrhythmicity. He showed that with increasing LL intensities the "width" of the eclosion peaks increased with successive cycles of a free-run (Fig. 3.2 and 3.3).

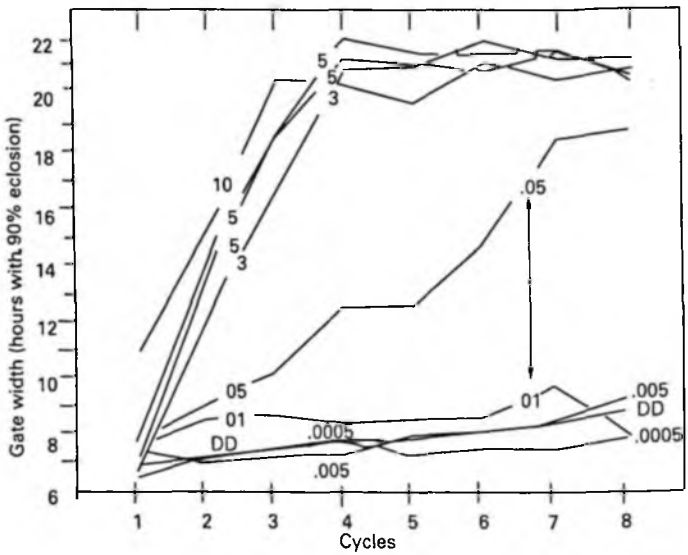

Fig. 3.2 The effect of low intensity LL on *D. pseudoobscura* eclosion rhythm. The approach to arrhythmicity at different light intensities is shown as the increase in the width of the gating of the daily eclosion rhythm in LL. At very low intensities (0.0005 to 0.005 lux), the width of the daily peak in LL is the same as that in DD. At higher intensities(>0.01 lux) arrhythmicity sets in rapidly. (Modified after Pittendrigh 1981).

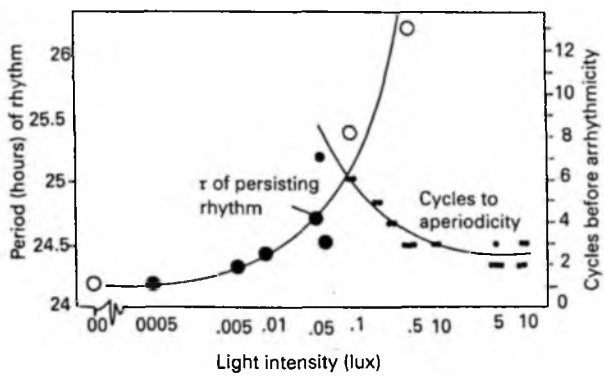

Fig. 3.3 The effects of low intensity LL on *D. pseudoobscura* eclosion rhythm. The curve to the right summarises the rate at which rhythmicity is lost as a function of light intensity; the curve to the left illustrates the dependence of the period on ambient LL intensity. (Modified after Pittendrigh,1981).

At very low intensities (0.0005 and 0.005 lux), the width of the gate of the daily peak is nearly the same as that in DD. At higher intensities (>0.01 lux) arrhythmicity is reached rapidly (Pittendrigh, 1981). The curves in Fig. 3.3 summarise the rate at which rhythmicity is lost as a function of light intensity and the dependence of the period (in whatever periodicity persists) as a function of light intensity.

The off-rhythms described here and those created by entrainment in LD and transfer to DD on day 20 are identical in their time course; so much so that all the rhythms Winfree induced in his pupal populations were done by exposing them to LL for several days and transferring them to DD.

3.2 The *Drosophila* PRC

The phase response curve (PRC) is a plot of the responses of a circadian rhythm in terms of phase shifts to perturbations (of light, temperature and chemicals) as a function of phase. A vast body of scientific literature has accumulated on this subject. A PRC indicates the state of sensitivity of the basic oscillation to zeitgeber stimuli at any given phase. Such information cannot be had, for instance, when we record discrete events such as locomotion and rest, which start and end abruptly. There is no clue to the state of the clock during hours of rest when nothing outwardly happens. Further all external events, both observable and measurable, may be in the nature of the "hands of the clock." Pittendrigh stated that the *Drosophila* PRC reflected *the time course and waveform* of the basic oscillator. This is true of all PRCs, but it was Pittendrigh who first used such a picturesque description.

It is not yet clear who first plotted the PRC. Hastings and Sweeney (1958) constructed a light pulse PRC as early as 1958 for the circadian rhythm in luminescence of the dinoflagellate *Gonyaulax polyedra.* While in 1929, Kleinhoonte (1929) published a paper on the effects of light on circadian cycles. Referring to this paper, Bünning wrote (1971a): "Kleinhoonte showed that even pulses of weak light, not more than a minute long, effectively cause delays or advances of circadian cycles and synchronise them. These were the first steps in studying what is now known as *phase response curves* of circadian rhythms."

The eclosion rhythm of *D. pseudoobscura* is very sensitive to perturbations of light. The responses of the rhythm appear as discrete displacements of eclosion peaks along the time axis and are designated as phase shifts. As would be expected in a genuine circadian rhythm, the magnitude and directions of phase shifts (advances or delays) are functions of the phases perturbed. Figure 3.4 illustrates the raw data and the methodology of evoking systematic phase shifts based on which PRC are constructed. Figure 3.5 illustrates a PRC constructed from data obtained from the kind of experiments shown in Fig. 3.4.

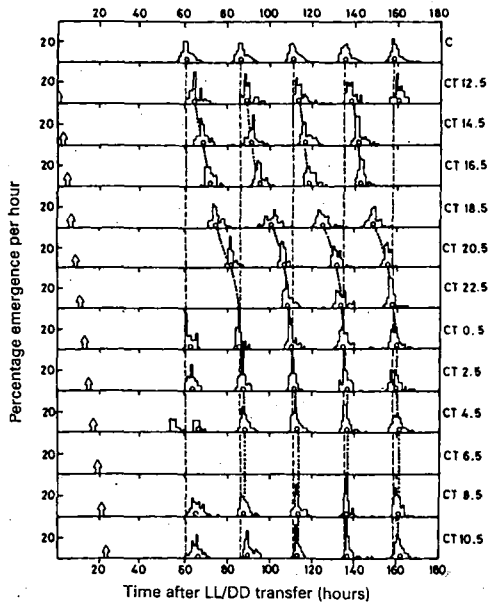

Fig. 3.4 Circadian rhythm of eclosion in populations of *Drosophila pseudoobscura* which were transferred from light to darkness at hour '0'. The uppermost row shows eclosion of flies in a control group 'C'. The other groups received 100 s blue light pulses of 442 + 17 nm of 100 μW cm^2 irradiance (arrows) at phases indicated by numbers alongside the groups. Eclosion within a peak have been normalised to 100%. The corresponding medians (white dots) are connected. (After Hamm et al.).

The light perturbations were 100 μW of 442 $\pm$ 17 nm of blue light given for 10 s, which was the most effective wavelength in shifting the phases of insect rhythms . The rhythm is refractory to light stimuli for the best part of the subjective day, but responds with increasingly dilatory phase shifts during the first half of the subjective night. At subjective midnight the system switches over, as it were, from massive delays to massive advances. The advances progressively diminish as the night progresses towards dawn. However, it must be pointed out that the phase shifts can be plotted as delays all the way, for example, a 6 h-advance phase shift can be depicted as an 18 h delay phase shift. The resulting curve would then be "monotonic" but in this chapter the proposition will be advanced and supported with empirical data that early subjective night, late subjective night and the subjective midnight separating them, all have their own interesting and ecologically meaningful phase responses to brief light perturbations. The

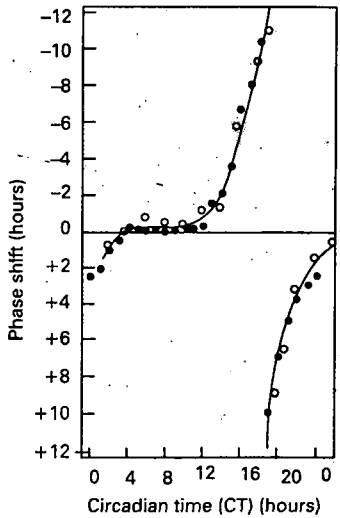

Fig. 3.5 A phase response curve (PRC) for *Drosophila pseudoobscura* obtained from experiments of the kind described in Fig. 3.4 using (saturating) blue light (442 + 17 nm) of 10–100 s duration and 100 μW cm^2 irradiance. The time course, wave-form and magnitude of phase shifts of this PRC are identical to those of Pittendrigh and Minis (1964) obtained in response to 15 min 100 ft. cd. (foot candle) white light pulses (After Hamm et al.).

Drosophila pseudoobscura PRC shown in Fig. 3.5 is constructed on the basis of steady-state phase shift data obtained from experiments which *preclude* transients (see Section 3.5).

3.3 Spectral sensitivity and intensity effects

The action spectrum describes the most effective wavelength in visible light of ca 350 to 700 nm for bringing about a physiological response, which may be induction of diapause in insects, breaking diapause or shifting the phases of circadian rhythms. A spectrograph, usually consisting of a quartz-iodine lamp or a Xenon high irradiance bulb, is used for light projection. The light beam is made to pass through monochromatic interference filters to obtain light of desired wavelengths. Monochromatic light intensities are expressed in μW cm^2 units and not in lux. Action spectra for the light-induced phase shifts of the *Drosophila pseudoobscura* circadian rhythm have been determined (Frank and Zimmerman, 1969). Pulses containing 15 min of monochromatic

light were then applied to the free-running rhythm at two phases – at 17 CT to cause delays and 20 CT to cause comparable advances. The results showed that the *direction* of the phase shift is not affected by wavelength, but the *magnitude* of the response increased with the intensity of the signal. Action spectra for both advances and delays were similar. The most effective wavelengths were between 420 and 480 nm and there was no response beyond 600 nm. This is the reason why I have used red light of >610 nm as "safe" light in all experiments. Safe light means that the period length of *Drosophila* rhythms, and the periods of circadian rhythms in the other animals investigated, did not alter in light with wavelengths beyond 610 nm.

3.4 Transients and the coupled oscillator model

The phase shifts that follow light pulses applied to the various phases of the subjective night (whether advances or delays) do not express themselves in full measure in the same, or even in the next cycle. It takes the rhythms 3–4 days for the steady state of the stable phase shifts to occur. These cycles occurring between the original and altered steady states have been called *transients*. The coupled oscillator model of Pittendrigh and Bruce (1957) first described transients in the context of circadian rhythms. Their model envisaged a coupled oscillator arrangement with one of the oscillators, called *A* oscillator, being the pacemaker. *A* oscillator was phase shifted instantaneously by light pulses and its period was compensted for changes in temperature. The *B* oscillator, on the other hand, was refractory (insensitive) to light but sensitive to temperature and *slave* to the *A* oscillator. *A* drove *B* but *B* did not feedback on *A*. Instantaneous phase shifts of the *A* oscillator were of the magnitude represented in the PRC; *B* gradually caught up with *A* in 3–4 circadian cycles, thus restoring the original steady-state phase angle. The efforts of the *B* oscillator to regain a stable phase angle with the *A* oscillator expressed itself in the form of overt *transients*. The coupled oscillator model thus elegantly explained the phenomenon of transients. Furthermore, the two main postulates of the model, that (1) the basic oscillator was reset instantaneously by light and that (2) the *transients* did not reflect the true phase of the basic oscillator, were stated without ambiguity and lent themselves to direct experimental verification.

Soon after the coupled oscillator model was proposed, Bünning and Zimmer (1962) gave a different interpretation to transients. They concluded from their studies on the petal movement rhythms of the crassulacean plant *Kalanchoe blossfeldiana* that the transient oscillation of this plant following light signals, which occured in the next 2 cycles, did reflect the behaviour of the basic oscillator. They found that the several phases of the transients respond to a light signal in a manner similar to the movement phases of the original (steady-state) rhythm. Thus, the interpretations of Bünning and Pittendrigh of the 'transients' hinged on whether or not they

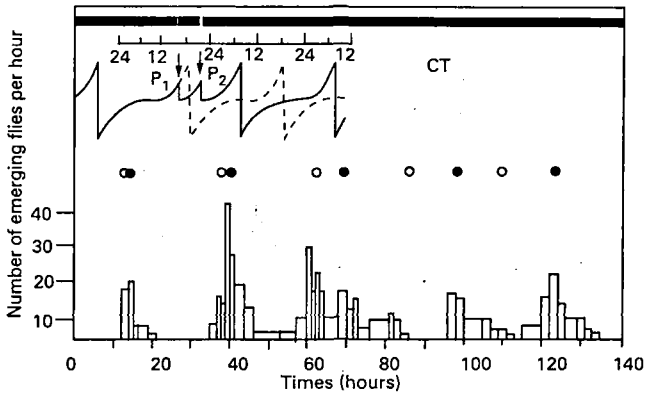

Fig. 3.6 The phase-shifting effect of two light pulses (P_1 and P_2) of 15 min and 300 lux on the eclosion rhythm of *D. pseudoobscura*. P_1 was given at 15.5 h CT and P_2 at 22.0 h CT. The solid line curve above the raw data of eclosion schematically depicts the phase shifts effected by the light pulses. The dotted line curve indicates the time course of unperturbed controls. Open circles denote calculated medians of peaks and solid circles indicate medians of populations experiencing light pulses. (After Chandrashekaran, 1967a).

described the time course of the basic pace-maker oscillator. Further Bünning and Zimmer had no need for a second (*B*) oscillator. In the course of 1966 I designed critical experiments with *Drosophila pseudoobscura* to further examine the two views about transients (Chandrashekaran, 1967a). In

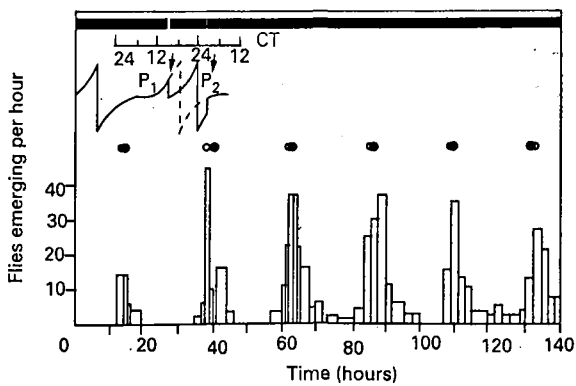

Fig. 3.7 The phase-shifting effect of two light pulses (P_1 and P_2) of 15 min and 300 lux on the eclosion rhythm of *D. pseudoobscura*. P_1 was given at 15.5 h CT and P_2 at 02.5 h CT. Other details as in Fig.3.6. (After Chandrashekaran, 1967 a).

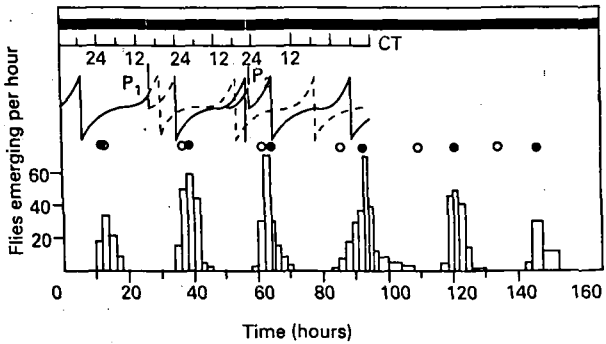

Fig. 3.8 The phase-shifting effect of two light pulses (P_1 and P_2) of 15 min and 300 lux each, on the eclosion rhythm of *D. pseudoobscura*. P_1 was given at 15.5 h CT of the *second* cycle and P_2 at 22.0 h CT of the *third* cycle. P_1 coincides with phase 27.5 after LL/DD transition of the experimental population and P_2 coincides with phase obtaining 58 h after LL/DD. The objective was to subject the *transients* to light pulses. Other details as in Fig.3.6. (After Chandrashekaran, 1967a).

planning experiments the PRC was assumed to truly reflect the *time course* and *waveform* of the basic oscillator. Two light pulses were employed, the first to shift phase and the second to determine how fast the phases did shift. The results and details do not belong in this book, but were unequivocally indicative that both the tenets of the coupled oscillator model were valid: (1) that phases of transients did not reflect phases of the basic oscillator and (2) that phase shifts affected by brief light pulses were *instantaneous* (Figs. 3.6, 3.7 & 3.8).

Pittendrigh (1967) and Pittendrigh and Skopik (1970) have also published results of experiments they had performed employing two light pulses spaced 12 h apart, calling one (M) morning pulse and the other (E) evening pulse, pointing out that the *instantaneous* nature of the phase shifts caused by these pulses enabled one to compute the entrained steady state. The last three decades have brought in more experimental findings supporting instantaneous phase-shifting of the basic oscillator for a fungus (Dharmananda 1980), a sparrow (Binkley and Mosher, 1987), and in our laboratory for the field mouse *Mus booduga* (Sharma and Chandrashekaran, 1997; 2000). (If the reader finds Section 3.6 very technical, it may be skipped without detriment to the story of biological clocks being told here.)

3.5 "Dawn" and "dusk" effects

In a more recent review, Foster and Helfrich-Förster (2001) conclude: 'Until recently, circadian biologists have tended to use light merely as a "hammer" to

shift the clock, but of course twilight detection is not a straightforward stimulus. It is highly dynamic and subject to considerable noise. Yet despite the high degree of environmental noise, entrained organisms show remarkable precision in their daily activities. Thus, the photosensory task of entrainment is likely to be very complex. The time is now right for circadian biologists to think about photoentrainment in a different way, to stop asking "what is the circadian photopigment?" and ask the more sophisticated question of "how do multiple photic channels interact to reduce the noise problem inherent in twilight detection?'

Earlier scientists like Darwin, Haberlandt and Bünning have indeed given thought to problems like twilight and entrainment. In the course of some of my early experiments (Chandrashekaran, 1967a), some data seemed to indicate that the first half of the subjective night of the circadian clock of *Drosophila* showed qualitatively different responses to the "on" and "off" components of even very brief light pulses, than the second half of the subjective night. More precisely, the first half of the subjective night seemed to respond only to the light "off" transition of a light pulse (probably simulating "dusk"); the second half appears to selectively respond only to the light "on" transition of a light pulse (probably simulating "dawn") (Chandrashekaran et al., 1973). Figure 3.9 illustrates the results of an extended series of experiments which indicate that the "dawn" and "dusk" effects of our light pulses indeed occur and such responses may mirror natural events. The logic of the experiments, the data of which are presented in Fig. 3.9 is as follows : If the light-off component alone has a triggering role in effecting *delay* phase shifts during the first half of the subjective night, then light pulses varying in duration from 15 min to 6 h (a 24-fold difference in duration) must *all* cause delay phase shifts of comparable magnitude as long as their light-*off* transitions are experimentally manipulated to occur at the same phase (CT 18 in the figure). This is illustrated for the seven experiments of populations bunched together in batch 'a' of Fig. 3.9. A 1 h light pulse and a 6 h light pulse both evoked ca. 8 h delay phase shifts, regardless of the *duration*. For the second half of the night the logic is: If indeed the light-on component alone has a triggering role in effecting *advance* phase shifts, then light pulses varying in duration from 15 min to 6 h must *all* cause advance phase shifts of comparable magnitude as long as their light-*on* transitions are experimentally manipulated to occur at the same phase (CT 19 in the figure). This is illustrated for the seven experiments of populations bunched together in batch 'c' of Fig. 3.9. In these experiments also a 1 h light pulse and a 6 h light pulse both evoked ca 9 h advance phase shifts, regardless of *duration.* The results of these experiments seem to confirm that brief light pulses can stand in for *dusk* until midnight and stand in for *dawn* after subjective midnight.

More experiments were performed in pursuit of the quest to discover further differences in response features and energy requirements of the two

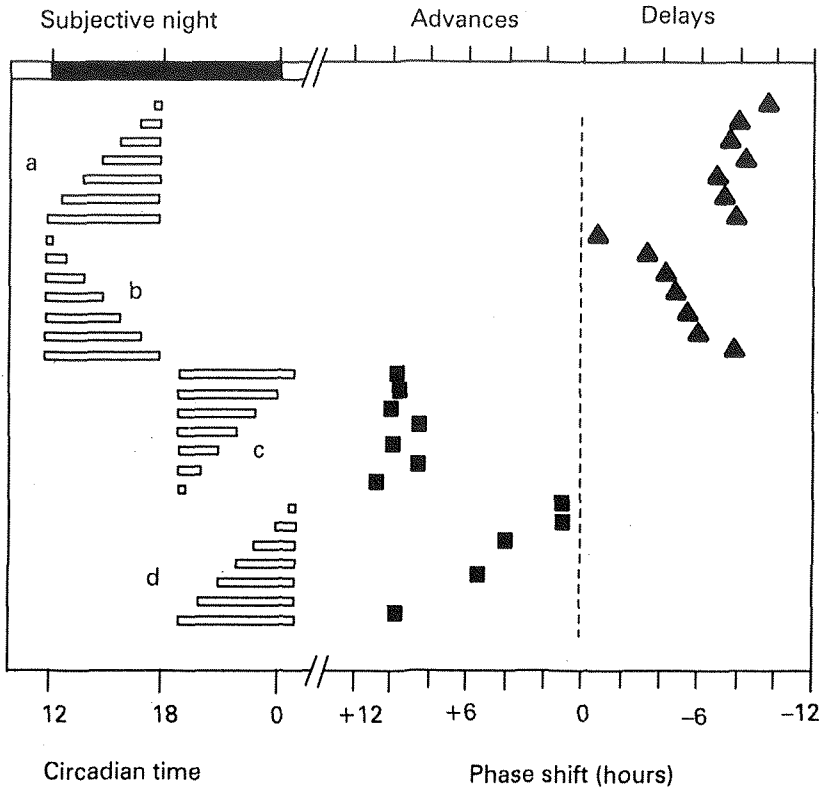

Fig. 3.9 Shifting the phases of the *D. pseudoobscura* eclosion rhythm with light pulses of 1000 lux and varying duration. The light pulses are represented by unfilled bars arranged in the figure in four batches. In batch 'a', different pupal populations experienced the 'on' transitions of the light pulses at 12 h, 13h, 14 h, 15 h, 16 h, 17 h and 17.75 h CT and the 'off transition at 18 h CT for all seven populations. In batch 'b', the experienced the 'on' transition at 12 h CT but the 'off' transition, staggered by 1 h for each population, at different hours. Batches 'a' and 'b' scan the first half of the subjective night, batches, whereas 'c' and'd' scan the second half of the subjective night on the pattern depicted in the figure. Filled triangles represent the averaged median *delay* phase shift values on days 4 and 5 after light treatment. Filled squares represent averaged *advance* phase shift values on days 4 and 5 after light treatment. (After Chandrashekaran et al., 1973).

halves of the subjective night of the *Drosophila* circadian clock. A series of experiments using blue light of 442 ± 17 nm at 18 CT (end of the first half of subjective night) and 19 CT (beginning of the second half of subjective night) interestingly revealed that the 18 CT phase was ten-fold *less* sensitive than the 19 CT phase (Fig. 3.10) (Chandrashekaran and Engelmann, 1973).

There is further evidence that these differences in energy requirements between 18 and 19 CT phases are also true of other phases of early and late subjective night. Very interestingly, in a series of more recent experiments

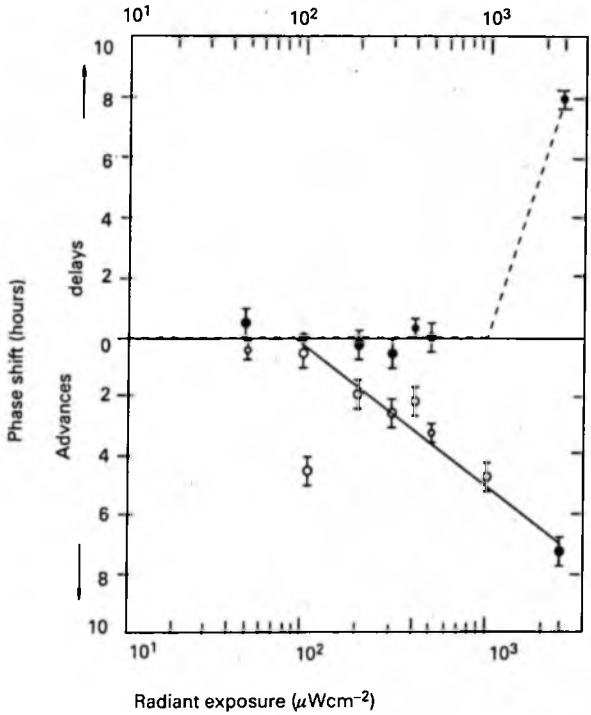

Fig. 3.10 The extent of phase shifts as a function of the monochromatic blue light pulses of 1000 ergs/cm and 442 + 17 nm and varying energies. Filled circles denote delay phase shifts in response to light pulses given at 18.0 h CT. Open circles denote advance phase shifts in response to light pulses given at 19.0 h CT. It is clear that the 18.0 h CT phase is ten-fold less sensitive than the 19.0 h CT.

performed with light stimuli on the circadian clock of the field mouse, we observed that there is a similar non-linearity of responses in these nocturnal mammals between the early and late subjective night phases, indicating that we are probably dealing with the basic characteristics of animal circadian systems (Sharma et al., 1999).

At this point it might be desirable to state the motivations for my carrying out these apparently esoteric experiments in the mid-1960s when rhythm research was still being looked upon as metaphysics. There were models and models to explain how circadian rhythms functioned, such as the coupled oscillator model, all of which did not take the organism nor the biological

and ecological aspects into consideration. Only the Bünning's hypothesis sought to reconcile endogenous processes such as the photophilic and scotophilic half cycles of organisms with exogenous events such as daylength and the seasons. I was deeply interested in the subjective night half of the *Drosophila* circadian clock, for this was where phase shift responses appeared to be dynamic and happening. The phenomenon of phase jump occurring at the subjective midnight phase and the differential transient kinetics associated with advance and delay phase shifts all had to have a role to play in the universal process of entrainment of circadian rhythms. In order to understand events that happen at the subjective midnight point it appeared important to work out the phase response kinetics and energy requirements of early and late night phases. This idea of looking for asymmetries between the two halves of the subjective night prompted the experiments described in Section 3.6.

3.6 Stopping the *Drosophila* clock?

Pavlidis (1967) predicted that the *Drosophila* clock must possess a "point of singularity" on theoretical considerations. Drawn in the form of a phase-plane limit cycle diagram the singular status (which is supposed to send the oscillator to a "phaseless" state and cause arrhythmicity) will be caused by light pulses of critical strength S^* administered at critical time T^*. Winfree (1970) defined the values of these two parameters for the *Drosophila* eclosion rhythm (the basis of his Ph.D. thesis submitted to Princeton University), which if given in combination, nearly abolish the eclosion rhythm in *Drosophila pseudoobscura*. In that state flies emerge at all hours of day and night without a trace of the "gating" of eclosion. Figure 3.11 shows the format of the singularity point experiments of Winfree. S^* was blue light of 460 ± 17 nm of an intensity of 100 ergs/cm² lasting 50 s.

T^* was 6.8 h after LL/DD transfer of pupae (18.8 CT). In a series of experiments we could severely attenuate (Chandrashekaran and Engelmann, 1976) the circadian rhythms with blue light pulses (442 ± 17 nm) whose *product of radiant exposure* i.e., intensity × time, was the same as the

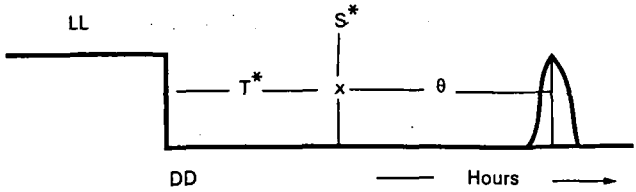

Fig. 3.11 Format of the rhythm-attenuating '*singularity point*' experiments. T^* and S^* are explained in the text. (Modified after Winfree, 1970).

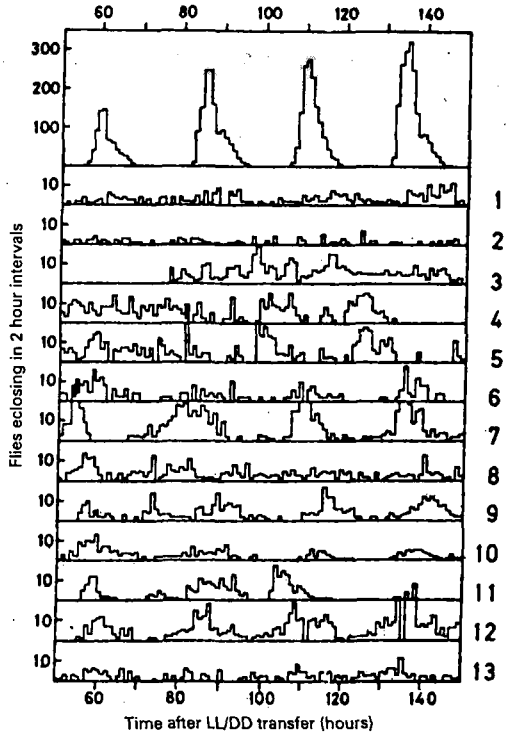

Fig. 3.12 Raw data of experiments showing the actual pattern of eclosion subjected to different T^* and S^* combinations whose product of radiant exposure were the same as the *singularity inducing stimulus* of Winfree (1970). The records illustrate the eclosion pattern in different populations of pupae between the 50th and 150th h after LL/DD transfer. The uppermost histogram was derived from pooled eclosion data from 11 untreated LL/DD populations. The perfectly rhythmic pattern eclosion has an R value of 15. Other details in text. (After Chandrashekaran and Engelmann, 1976).

singular stimulus of Winfree. In our experiments, we systematically varied the *irradiance* (I) and *duration* (t) components within the limits of reciprocity, which were surprisingly vast. In terms of irradiance it varied from very bright light of 12,5000 to 0.01 μW/cm and duration could be varied, from brief light flashes of 0.04 a to long pulses of 5000 s. Figure 3.12 sets forth the raw data illustrating arrhythmicity and attenuated rhythmic trends obtained in our experiments.

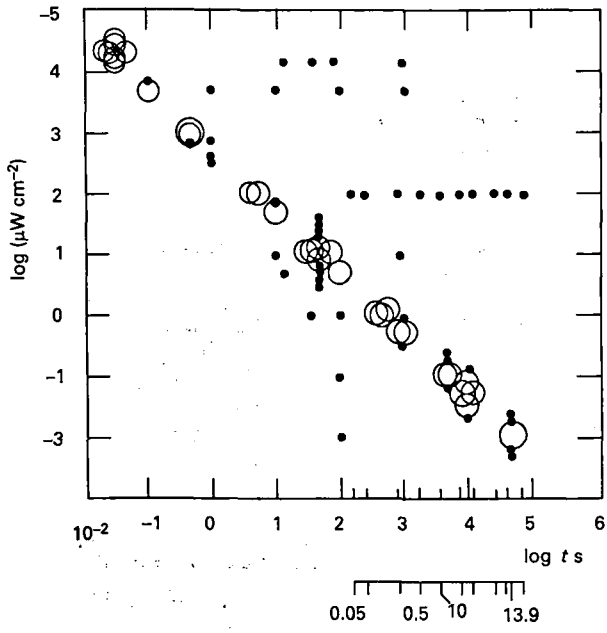

Fig. 3.13 The reciprocal relationship between irradiance (I) and duration (t) of rhythm attenuating light pulses. The larger circles represent populations in which the rhythm severely attenuated and hence had R values above 50, the diameter of the circle being a measure of the degree of arrhythmicity. Filled circles indicate populations in which the rhythm continued unaffected or experienced small phase shifts. (After Chandrashekaran and Engelmann, 1976).

These experiments were extremely difficult and often successful attenuation of the rhythm was possible only in 10% of the trials. The populations of pupae came from the same general stock and the LL/DD transfers were made at an arbitrary hour "0," each of the 20 populations of an experiment experiencing the transfer 10 min apart. This was undertaken so that the same monochromatic projection source of blue light, with the same interference filter and neutral density heat-absorption glass filters could be employed, thus narrowing down sources of variation. Figure 3.13 summarises the data of all the experiments performed. The larger open circles in Fig. 3.13 represent populations in which the rhythm was attenuated by light pulses whose radiant exposure ($I \times t$) was 500 μW/cm.

The diameter of open circles roughly represents the degree of arrhythmicity. On the contrary, filled circles denote populations that

remained (often perfectly) rhythmic in spite of light treatment, and also those populations that responded with variable amounts of phase shifts. The clear implication of our results is that the point of singularity is unstable relative to other phases, say CT 18 or CT 19 and the variations are a measure of the frequency of occasions on which the light stimuli 'hit' or 'missed' the T^*.

3.7 Temperature and the *Drosophila* clock

The independence of the periods of circadian rhythms while experiencing varying ambient temperatures is apparently achieved by means of active compensatory processes. Bünning (1974) considered that the temperature compensation of circadian rhythms is connected with the different influences of temperature on the various phases of the cycle especially on the energy requiring and non-energy requiring phases. He felt that the phenomenon of temperature compensation could be explained by Darwinian selection. In cases of absence of selection pressure as in plants and homeothermic animals, Q-10 values between 0.85 and 1.5 are known to occur. It is this temperature-compensated nature of the periods of circadian rhythms that enables them to participate in time measurement (chronometry). Although the free-running periods of circadian rhythms are stable over a vast range of "constant" temperatures, any temporary, non-recurring increase (step-up) or decrease (step-down) in temperature affects the rhythm and causes "advance" and "delay" phase shifts, respectively. The amount of phase shifts caused by the temperature steps, and whether only delay phase shifts are caused or only advance phase shifts are evoked, are functions of the phases of the rhythm experiencing the step (Bünning and Tazawa 1957; Moser 1962). Temperature pulses, which contain both steps, on the other hand, cause both advance and delay phase shifts again as a function of phase (Pittendrigh and Bruce 1959). Scientists working on the responses of the *Drosophila pseudoobscura* rhythm to temperature perturbations have obtained conflicting results. Zimmerman et al., (1968) reported persistent advance and delay phase shifts in response to high (20°/28°/20°C) and low (28°/20°/28°C) temperature pulses lasting 12 h and beginning at different phases. Winfree (1972), on the other hand, reported that for his strain of flies, the impact of a 12 h incubation at 28°C for pupae raised at 20°C was "severe" at all phases. Eclosion peaks occurs $12+24 \times n$ h after termination of the 12 h incubation regardless of phase, indicating that the rhythm was being *reset* each time. Maier (1973) also working with *Drosophila pseudoobscura* PU 301 strain, studied the effects of 4 min 40°C temperature pulses on the rhythm and obtained *delay* phase shifts at *all* phases, even though the amount of phase shifts showed phase dependence. In view of these widely divergent responses obtained on the same system, I carried

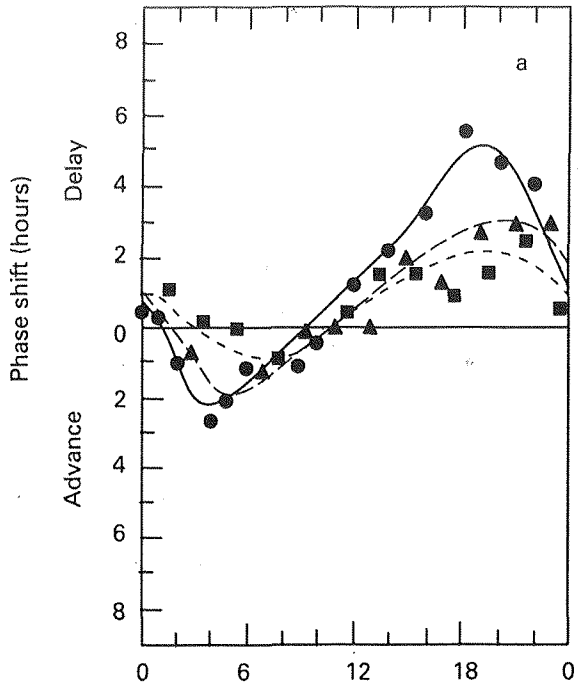

Fig. 3.14 Phase response curves obtained with high temperature pulses (HTP 20°/30°/20° C) of 3 h (■), 6 h (▲) and 12 h (●) duration with phase shift values plotted against the CT value at midpoint of perturbation. (After Chandrashekaran, 1974).

out a series of experiments with temperature pulses, varying in the process, a few parameters such as duration and the range of both the low and high temperature pulses. The results being summarised here represent as of now, the most thorough investigation of temperature pulse PRC made for any circadian system – plant, animal or human (Chandrashekaran, 1974).

Phase shift data for the responses of the rhythm to HTP (20° /30° /20° C) of 12, 6 and 3 h durations are presented in Fig. 3.14 and Fig.3.15 presents phase shift data to LTP (20°/10°/20°C) of 12, 6 and 3 h durations.

In both figures the phase shift data are shown against the h of "midpoint" of the pulses. The responses to LTP of all durations are *inverted* relative to the responses to HTP given at corresponding phases. As a consequence, phases responding with delays to HTP respond with advances to LTP and vice versa. The scatter of phase shift values is relatively higher for responses to LTP and is markedly so for responses to 12 h LTP. This scatter becomes exaggerated close to the region of the rhythm where responses switch from delay phase shifts to advance phase shifts. The 12 and 6 h LTP given in this region affect the *amplitude* of the rhythm in addition to shifting it and this resulted in broadened eclosion peaks and even *arrhythmicity*. The PRC for 12 h HTP appears to be of the weak type (type 1) in the terminology of Winfree (1971).

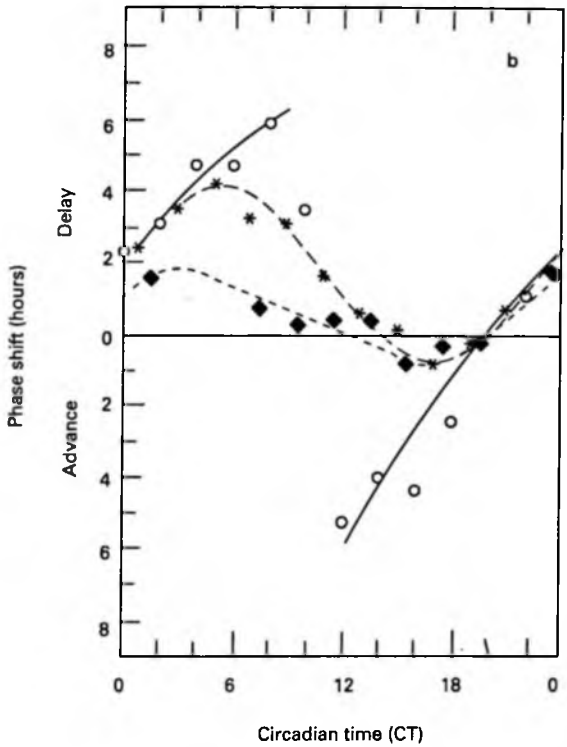

Fig. 3.15 Phase response curves obtained with low temperature pulses (LTP20°/10°/20°C) of 3 h (■), 6 h (✱) and 12 h (○) duration with phase shift values plotted against the CT value at midpoint of perturbation. (After Chandrashekaran, 1974).

Weak type PRC arise when phase shift responses switch smoothly from delays to advances and advances to delays. The PRC for 12 LTP, on the contrary, is of the strong type (type 0) where the system switches abruptly from pronounced delays to pronounced advances. The manner in which the PRC are plotted i.e., whether the "onset", "midpoint" or "end" is chosen as the point of reference appears to be important, especially where longer signals are involved. Thus a 12 h HTP/LTP which starts (has 'onset') at CT 12 has a 'midpoint' at CT 18 and the 'end' at CT 0 literally covering half the circadian cycle. The argument is not that any one portion of the temperature perturbation (onset/midpoint/end) as such effects the phase shift, as has been claimed for light pulses, but that diverse methods of plotting responses against phases may better reveal similarities in the action of signals of widely varying durations. And unlike

light pulses, temperature pulses may bring about phase shifts by some kind of continuous and additive (parametric) action.

3.8 Recent work on *Drosophila melanogaster* oviposition, eclosion and locomotor activity rhythms

The above summary of the classical experiments of Pittendrigh and colleagues carried out over three decades (1954–1984) on the formal properties of circadian rhythms in the eclosion of *Drosophila pseudoobscura* paved the way and set the stage for rapid progress in unravelling the genetic bases and molecular mechanisms of circadian rhythms. *Drosophila* continues to be one of the favourite model systems, besides cyanobacteria (Ouyang et al., 1998), *Neurospora*, *Arabidopsis*, *Gonyaulax* and rodents for researches on the molecular genetics of circadian rhythms. To round off the story of circadian rhythms in *Drosophila*, I summarise here the results of our more recent researches on circadian rhythms in oviposition, eclosion and locomotor activity, carried out on large populations of *Drosophila melanogaster* that had been maintained in an aperiodic environment (LL) for more than 600 generations in incubators under constant temperature (25°±1°C) and humidity (ca. 90%) with food and mates being available *ad libitum*.

In a first series of experiments, developmentally asynchronous eggs were collected from the adult flies in the population and introduced into LL, LD 12:12 h and DD at 25° ± 1°C. The number of eclosing flies was recorded every 2 h, yielding a time series comprising four consecutive 24 h days for each environmental treatment (Fig. 3.16).

Data were statistically treated for the removal of any linear component and periodograms were obtained using fast Fourier transforms. Eclosion data for LL treatment did not reveal any statistically significant periodicity, whereas data from the LD and DD treatments revealed significant periodicity of 24 and 26 h, respectively (Fig.3.16) (Sheeba et al., 2001), clearly indicating entrainment to an external LD cycle and an intrinsic, free-running circadian periodicity of eclosion rhythm under DD. Not only had the circadian rhythm in eclosion persisted in this population after 600 generations in an aperiodic environment, it had also maintained an impressive degree of precision (Sheeba et al., 1999). The duration in each cycle within which the bulk of the eclosion takes place under LD conditions ("gate") is approximately 8 h which is comparable in magnitude with gates seen in the eclosion rhythms of *Drosophila* spp. that have been reared under LD conditions (Pittendrigh and Skopik, 1970). The fact that the eclosion rhythm was not observed in LL suggests that individuals within the population were desynchronised relative to one another over the course of their 600-generation-long history in the laboratory. This is not surprising for the circadian rhythm of eclosion in *Drosophila pseudoobscura* is known to damp out quickly even in very low intensities

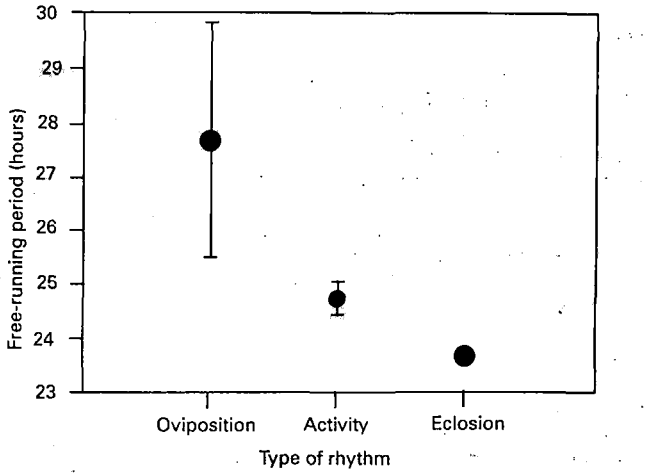

Fig. 3.16 Plot of free-running periods of eclosion, locomotor activity, and oviposition rhythms with error bars representing a 95% confidence interval around the mean for visual hypothesis testing. The values of the free-running period of the eclosion, locomotor activity and the oviposition rhythms were 23.64 h, 24.73 + 0.29 h (mean + 95% CI) and 27.66 + 2.16 h (mean + 95% CI), respectively. (After Sheeba et al., 2001).

of LL (Chandrashekaran and Loher, 1969a; Pittendrigh, 1981; Winfree, 1974). The arrhythmicity in eclosion in LL also suggests that it is unlikely that individuals within these populations are able to maintain their rhythms by mutual synchronisation, although such population effects have been demonstrated in some other organisms (Broda et al. 1985; Roenneberg and Mittag 1996). The occurrence of the eclosion rhythm in DD is apparently caused due to the resetting effect of the LL/DD transfer, as in *D. pseudoobscura* described earlier in this chapter. Such resetting becomes plausible because the populations do entrain to LD cycles. The underlying assumption here was that during the 600 generations of LL the individuals continued to possess circadian rhythmicity. Further studies confirmed that the locomotor activity in individual flies continued to express a circadian rhythmicity in both LL and DD after being raised for 600 generations in an aperiodic environment. The populational rhythm of eclosion damps out in LL, whereas the locomotor activity of individual flies continued to possess a circadian rhythmicity suggesting that maintenance of such rhythmicity is important in regulating vital biochemical processes within the individuals. The results of these experiments strongly indicate that there is an intrinsic adaptive significance to possessing a circadian clock and that this adaptive value derives

primarily from the periodicity *per se* and not its phasing properties with reference to external cycles in the environment (Sheeba et al., 2000).

3.9 A case for multiple oscillators

Evidence has accumulated in several organisms which indicate that multiple oscillators might underlie the different circadian rhythms operating in the same organism. In the marine dinoflagellate *Gonyaulax polyedra*, which exhibits circadian rhythms in photosynthesis, cell aggregation, superoxide dismutase production, phototaxis, bioluminescence and cell division (Hastings and Sweeney, 1959; Roenneberg, 1996), two oscillatory subsystems sensitive to different wavelengths of light – one controlling aggregation and the other controlling bioluminescence – have been demonstrated (Roennerberg, 1994). Not only may different physiological functions be separated temporally, as in *Gonyaulax,* but also the rhythm in the same function may split into two rhythms with *different* frequencies. Hoffmann (1969; 1970; 1971) observed such splitting when recording the locomotor activity of *Tupaia glis* after he lowered the LL from 10 to 1 lux - the two components free-ran with different frequencies but fused again when he raised and restored the light intensity. The locomotor activity of the Arctic ground squirrel showed a break-up of the activity band into two components, each of which free-ran with different periods when kept in LL for several cycles (Pittendrigh, 1960). A similar phenomenon was observed in hamsters kept in LL for several days (Pittendrigh, 1974). In pinealectomised birds (Takahashi and Menaker, 1982), several separate oscillators in an individual could be independently entrained to LD cycles. Similarly, Ishizaki and colleagues showed that there are two independently light entrainable pacemakers in the saturnid moths – one in the forebrain and the other in the prothoracic gland (Pittendrigh, 1993). In the fleshfly *Sarcophaga argyrostoma,* at least three distinct oscillators are believed to regulate initiation and duration of larval wandering, diapause induction, pupal eclosion rhythms, adult locomotor activity, and the deposition of cuticular growth layers on thoracic apodemes (Saunders, 1986). The view emerging from these studies is that different cellular functions or groups of functions are likely to be controlled by separate circadian oscillators, and that these oscillators are internally coupled such that they influence each other, making up a multioscillator system (Scully and Kay, 2000).

In the fruitfly *Drosophila melanogaster,* circadian rhythmicity has been reported at various levels of organisation. A number of genes like *per, tim, clock* and *cry,* exhibit oscillations in the concentration of mRNA and/or their protein products (Zordan et al., 2000). In individual flies locomotor activity and oviposition have been observed to be rhythmic (Helfrich, 1985) and pupal eclosion also follows a circadian pattern so that the population can act as an orchestrated chronometer (Sheeba et al., 1999). The best-studied circadian

rhythms in *Drosophila melanogaster* are those of eclosion and locomotor activity (Helfrich-Förster, 1996). Until very recently (McCabe and Birley 1998; Sheeba et al., 2001) there was no clear evidence of the endogenous circadian origin of the oviposition rhythm in *Drosophila melanogaster*. In this species oviposition is supposed to be the outcome of two physiological processes – vitellogenesis and egg retention. It has been assumed that the timing of vitellogenesis, controlled by the circadian pacemaker, could play a major role in the expression of free-running oviposition rhythms.

In a detailed study using *Drosophila pseudoobscura*, the free-running period, and the PRCs of the eclosion and locomotor activity rhythms were found to be different, suggesting that separate circadian pacemakers control these two rhythms (Engelmann and Mack, 1978). To the best of our knowledge such detailed studies have not been carried out in *D. melanogaster*, although a difference in the value of the period length for eclosion and locomotor activity rhythms has been reported from the same laboratory (Helfrich, 1985). Interestingly, the oviposition rhythm in *Drosophila* has not been experimentally observed to investigate the possible role of multiple oscillators in controlling circadian rhythms, although the lack of cycling of *per* mRNA level in the ovary of *D. melanogaster* (Plautz et al., 1997) suggests that oviposition may be controlled by a separate circadian oscillator. We therefore investigated if multiple oscillators control various circadian rhythms in *D. melanogaster*, taking for this purpose (a) population level rhythm (eclosion) and (b) individual level rhythms (locomotor activity and oviposition), the underlying molecular mechanisms of which have been fairly well studied. The laboratory populations had been reared in LD 12:12

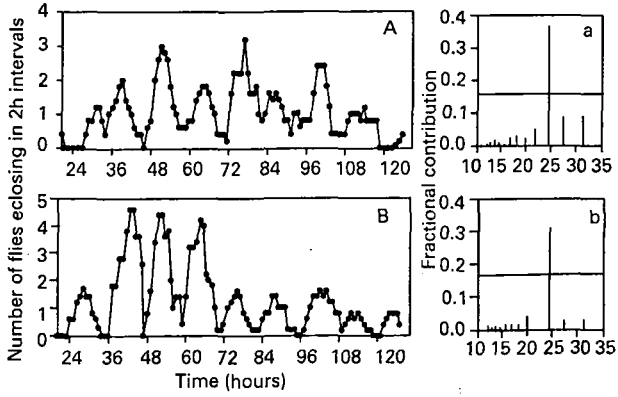

Fig. 3.17 Time series data of number of eclosing flies in two representative vials in DD (A, B). The corresponding periodograms show a significant contribution of 23.6 h periodicity (a, b). (After Sheeba et al., 2001).

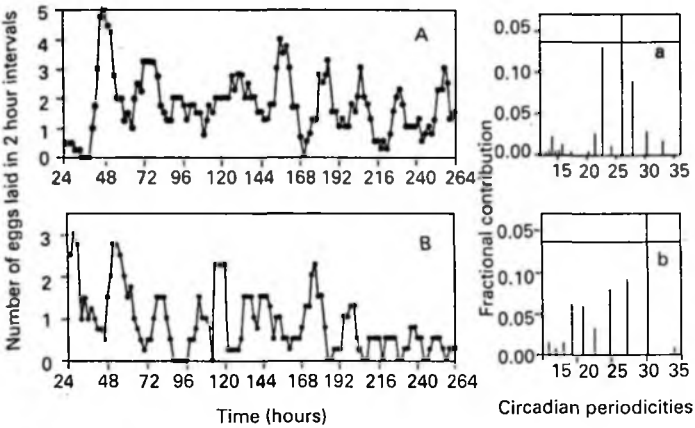

Fig. 3.18 Time series data of number of eggs laid by two representative females in DD (A, B). The corresponding periodograms show a significant contribution of 26 h and 30.22 h periodicity (a, b). (After Sheeba et al., 2001).

h cycles for ca. 35 generations. The eclosion, oviposition and locomotor activity rhythms in these flies were assayed in DD and LD 12:12 h and the *period* and *phase angle values* of these rhythms were recorded and compared with one another.

The value of the period length of the eclosion rhythm in DD was observed to be 23.64 h (Figs. 3.17 and 3.18) and was significantly shorter than the period length of the locomotor activity rhythm which i n DD was 24.73+0.29 h.

The period length of the eclosion rhythm in DD was also significantly shorter than the period length for the oviposition rhythm, which in DD was 27.66+2.16 h. The period length of the oviposition rhythm was significantly greater than the period of the locomotor activity rhythm in DD (Fig. 3.19). All three rhythms (eclosion, locomotor activity and oviposition) entrained to LD 12:12 h cycles (Figs. 3.20 and 3.21).

The periods of the eclosion, locomotor activity and oviposition rhythms in LD 12:12 h were not significantly different from 24 h. Multiple comparisons showed that "phase angle values" for all three rhythms were significantly different from each other ($p<0.001$, for all three comparisons). While the locomotor activity rhythm phase leads "lights on" of the LD 12:12 h cycle, the eclosion rhythm phase lags it and the oviposition rhythm is almost 180° out of phase compared to eclosion and oviposition rhythms. Based on these results, we conclude that different circadian oscillators control the eclosion, locomotor activity and oviposition rhythms in *D. melanogaster* (Sheeba et al., 2001).

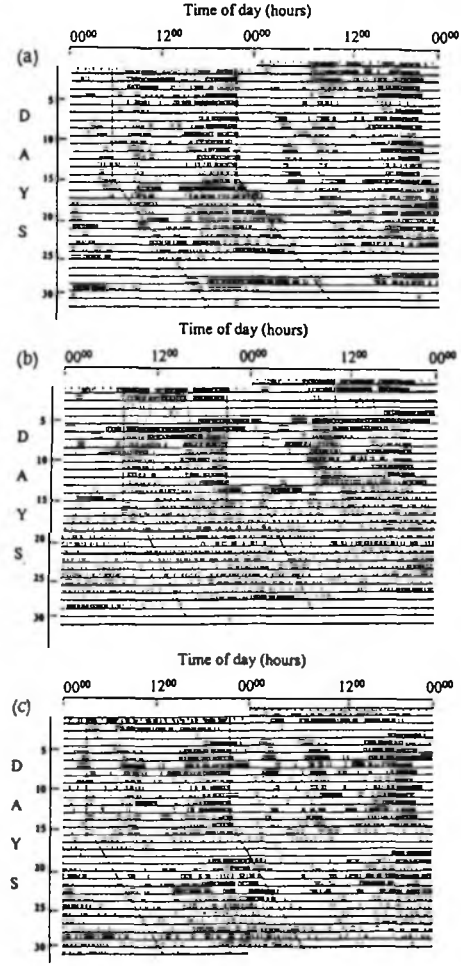

Fig. 3.19(a)–(c) The locomotor activity records of flies for the first 15 days in LD 12:12 h cycles, followed by the next 15 days in DD. The phase angle differences expressed as the average time difference between the onset of locomotor activity and 'lights on' were 4.77 h, 0.077 h and 5.88 h, respectively, while free-running periods are 24.59 h, 24.66 h and 24.25 h, respectively. The lights in the LD cycle were switched on at 08.00 h and switched off at 20.00 h. (After Sheeba et al., 2001).

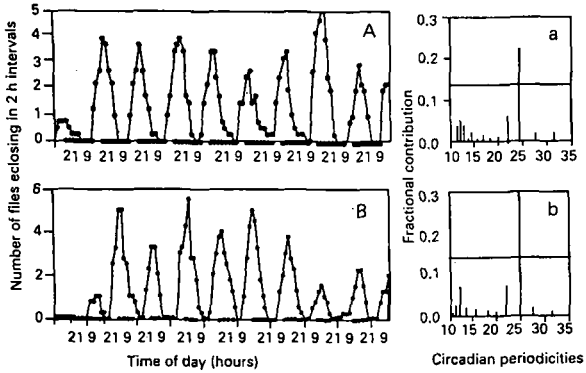

Fig. 3.20 Time series data of eclosion rhythm of two representative vials in LD 12:12 h cycles (A, B). The corresponding periodograms show that eclosion in the two vials occurred with 24 h periodicity (a, b). The eclsion rhythm was found to entrain to the LD 12:12 h with a cycle-to-cycle period not significantly different from 24 h. The 'phase angle' value of the eclosion rhythm in LD 12:12 h, was estimated as the average time interval between peak eclosion and 'lights on' over 10 consecutive days. The lights in the LD cycle were switched on at 08.00 h and switched off at 20.00 h. (After Sheeba et al., 2001).

In several rhythm mutants of D. melanogaster such as per, tim and disco (Konopka and Benzer, 1971; Dushay et al., 1989) the locomotor activity and eclosion rhythms have been found to be affected in a similar manner. In another study, the period lengths of the oviposition and activity rhythms of per mutants and wild type D. melanogaster were found to be significantly correlated and it was concluded that the per gene influences the circadian periodicity of both the locomotor activity and oviposition in a similar manner (McCabe and Birley, 1998). Several other mutations, however, such as ebony and lark affect only one of the two rhythms: eclosion and locomotor activity. While ebony mutants show arrhythmic adult locomotor activity and a normal eclosion rhythm, lark has an arrhythmic eclosion rhythm and periodic locomotor activity (Newby and Jackson, 1991; 1993), suggesting that these mutations are perhaps affecting clock-controlled-genes (CCGs) and not the central pacemaker. The results of these studies suggest that eclosion, locomotor activity and oviposition rhythms in D. melanogaster share common pathways in pacemaker regulatory mechanisms which when modified by mutation proportionally affect the rhythms. In our own studies, summarised here and still under progress, we found that period length and phase angle property values for the eclosion, locomotor activity and oviposition rhythms of D. melanogaster are significantly different from one another (Fig. 3.16).

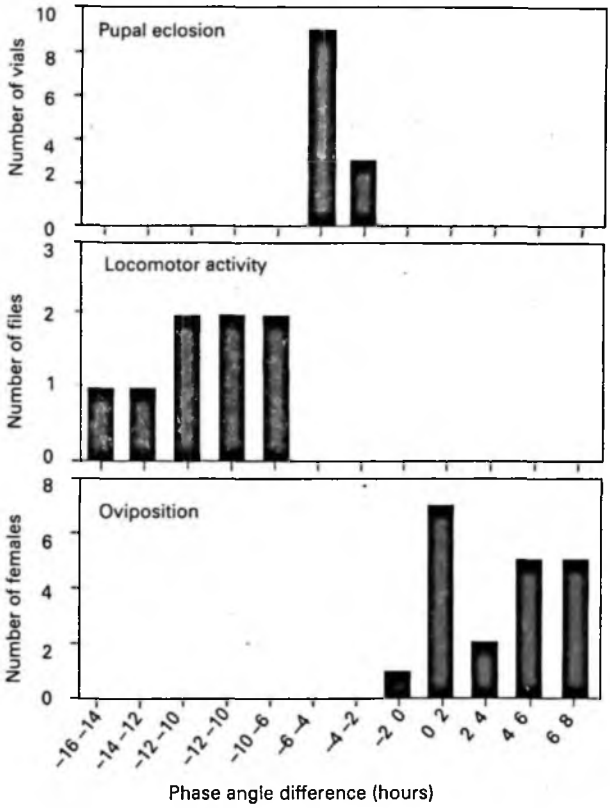

Fig. 3.21 Frequency distribution between the phase angle differences in LD 12:12 h cycles for eclosion, oviposition and locomotor activity rhythms. The *x*-axis represents class intervals with a range of 2 h starting from – 16 to +8 h. (After Sheeba et al., 2001).

The fact that these rhythms can be modified proportionally in some mutants rules out the involvement of independent circadian pacemakers for these three rhythms. However, it may not be unreasonable to assume that separate circadian oscillators, which are not part of the common pacemaker mechanism, control the rhythms in eclosion, locomotor activity and oviposition. Careful experiments on long and short period *D. melanogaster* mutant strains may throw more light on the role and possible involvement of multiple circadian oscillators in modulating the several rhythms in eclosion, locomotion and oviposition processes.

3.10 Developmental plasticity

In a recent series of experiments on the developmental plasticity of the locomotor activity of D. melanogaster we (Sheeba et al., 2002) used four replicate outbred populations of D. melanogaster to study if light regimes experienced during pre-adult (larval and pupal) and early adult stages influence the free-running period of the circadian locomotor activity rhythm of adult flies. In a series of two experiments, four different populations of flies were raised from egg to eclosion in LL, in LD 12:12 h cycles, and in DD. In the first experiment, the adult male and female flies were directly transferred into DD and their locomotor activity was monitored, while in the second experiment the locomotor activity of the emerging adult flies was first assayed in LD cycles of 12:12 h for 15 days and than in DD for another 15 days. The τ_{DD} of the locomotor activity rhythms of flies that were raised in all the three light regimes, LL, LD 12:12 h and in DD were significantly different from one another. The τ_{DD} of the locomotor activity rhythm of the flies, which were raised in DD during their pre-adult stages, was significantly shorter than that of flies that were raised as pre-adults in the LL regime, which in turn was significantly shorter than that of flies raised in the LD 12:12 h regime. The pattern was consistent across both the experiments. The results of these experiments serve to emphasise the fact that in order to draw meaningful inferences about circadian rhythm parameters in insects, adequate attention should be paid to the control environment and specifications have to be given about the environment in which pre-adult rearing takes place. Other authors have also reported findings that the period of the circadian pacemaker varies in response to different environmental conditions experienced, typically referred to as "after effects" (Pittendrigh, 1960; Sokolove, 1975; Christensen, 1978; Page and Block, 1980). The after-effects of LD cycles have been speculated to be of functional significance in helping organisms to perform various physiological and behavioural functions at appropriate times even when the environmental LD cycles are transitorily masked, for example, due to cloud cover.

In conclusion, it is striking that all the earlier body of work on circadian rhythms, including the elegant experiments of Beling (1929), Kleinhoonte (1929), Bünning (1932), Kalmus (1934, 1935) and the subsequent work of Aschoff, Pittendrigh, Menaker, DeCoursey, Hastings, Sweeney, Winfree, and my own work described here and those of my contempoaries cited, have continued to remain relevant even in the era of molecular biology.

4. Biological Clocks and Behaviour of Insect Bats

❄

"The sun has set when they began their flight
within a darkened cave they'd grown more bold.
A cloud of bats erupts into the night
their leath'ry wings now lick the air so cold."

Nicole Pawlucki

❄

4.1 Working with bats

Bats are fascinating animals to work with. Very little is known about their biology and behaviour because of their nocturnal habits and the largely inaccessible places they live in. For the same reasons one needs special devices to study bats and these devices are not readily available in the market. It is also fairly difficult to have insect bats in captivity for they have to be hand-fed; preferably during the darkness fraction of a 12–12 h LD cycle, for it is only then that they are awake, active and feed readily. In European laboratories, bats are fed with mealworms but in India, they are fed with de-gutted cockroaches with the cuticle and wings removed. This is not their staple or natural diet but they learn to accept this after a few days of force-feeding on the part of the researcher. For these reasons they are *not ideal* animals for studies on circadian rhythms. Field mice and squirrels are sturdier to maintain in the laboratory and better suited for work on biological rhythms.

The world is a more fascinating place after sunset – swarming with myriads of insects, and fragrant with flowers which open only at night, advertising themselves to pollinating agents and the alluring smell of ripening fruits. This nightly world is also the arena of the bats called metaphorically the *birds of the night.* Insect bats, without competition from birds who are generally active at daytime, harvest the rich fare of a sea of insects swarming the skies. Since bats roost away from the prying eyes of humans – in caves, crevices of boulders, holes in trees, abandoned human

habitations, churches, temples and such structures – and fly only during night, they are viewed as mysterious creatures. They are easily the most misunderstood mammals.

Bats, like rodents, are the largest group of mammals in the world with over 950 species and wide zoogeographical distribution. The Latin name for bats is *Chiroptera* which means "hand-winged", as the forelimbs of bats are modified into simple wings. There are two sub-orders: (1) *Microchiroptera* (the insect bats) and (2) *Megachiroptera* (the fruit bats). In India there are some 35 species of *Microchiroptera* and 5 species of *Megachiroptera*. In the Madurai region (9°58'N, 78°10'E) we have investigated 9 species of insectivorous bats which *echolocate* prey by means of ultrasonics, and 3 species of fruit bats, which rely on eye sight and a sense of smell to locate ripening fruits.

Fruit bats

The most prominent fruit bat seen practically all over India (except in the Himalayas) is the flying fox, *Pteropus giganteus*, which is restricted to India, Africa and Australia. In spite of their roosting on trees in huge colonies and being very visible, the biology and behaviour of these bats have not been sufficiently well investigated (see however Neuweiler, 1969). Flying foxes roost in there hundreds in bamboo clumps and trees such as mango, jackfruit, tamarind and banyan. They seem to prefer to roost close to human habitations unmindful of the noise but are interestingly sensitive to deliberate disturbances. Even after their nightly foraging and toil they do not seem to enter deep sleep during the daytime; screeching at one another all through the day, with individual bats fluttering out from their perch for brief reconnaissance flights and returning to their inverted comfort. It is difficult to count these huge creatures as they fly in groups of 20–30 animals in unending succession. Darkness invariably sets in before the last of them leaves the roost. This is an interesting feature. There are no known predators of flying foxes even though we had on one occasion witnessed the ingestion of a fruit bat by a python – it made a meal of the whole flying fox save the diaphanous wings! (Marimuthu and Chandrashekaran, 1991). Yet the powerfully built flying foxes wait for sunset and dusk, like all other less well-endowed and smaller insect bats, to begin their foraging in far-flung fruit orchards.

4.2 Echolocation and hearing in bats

The Italian anatomist Lazzaro Spallanzani in 1793 found that insectivorous bats can fly blindfolded. It is interesting to note how this discovery was made. Spallanzani was sitting in his study late one evening with a pet owl

and a pet bat when a sudden gust of wind snuffed his candles and enveloped the room in darkness. The owl, which must rely on eyesight, sat still but the bat flew around without bumping into objects. Spallanzani then blindfolded the bat and noted that it could still fly around with great skill. He plugged the ears of his bat and discovered that now it became helpless and clumsy in its flight. Spallanzani speculated that bats saw by some means other than their eyes. To discover what it was Spallanzani blinded the bats. He reported in a letter to a colleague, "(The blinded bat) can be made to fly freely in a closed room either during the day or at night. During such flight, we observe furthermore that before arriving at the opposite wall, the bat turns and flies back dexterously avoiding obstacles such as walls, a pole set across his path, the ceiling, the people in the room, and whatever other bodies may have been placed about in an effort to embarrass him. In short he shows himself just as clever and expert in his movements in the air as a bat possessing its eyes."

One of Spallanzani's letters was read a few months later at a meeting of the Geneva Natural History Society, and it intrigued a surgeon entomologist called Louis Jurine. Jurine repeated the Spallanzani experiments and added some of his own. In 1794 he reported to the society that if the ears of a bat were tightly stopped with wax or other substances, the creature blundered into obstacles as if drunk. "The organ of hearing," Jurine concluded, "appears to supply that of sight in the discovery of bodies... and enable them to avoid those obstacles which may present themselves." In the 1790s the idea that bats "see" by hearing was scientific heresy. A zoologist wrote in 1809 that it "requires more faith and less philosophic reasoning, than can be expected of the zootomical philosopher, by whom it might be fairly asked "Since bats see with their ears, do they hear with their eyes?" For more than a century that scornful question seemed to have been the final verdict on the subject. So thoroughly were the Spallanzani and Jurine experiments discredited that few investigators of later years were even aware of their work (Stevens and Warshofsky, 1965; Chandrashekaran, 1980).

In the 1920s, following the invention of echoranging by the military, the English physiologist Hamilton Hartridge suggested the possibility that bats use a similar orientation system. In 1938 Donald Griffin, then a student at Harvard, reopened the subject. He theorised that bats emit very high frequency ultrasonics, which bounce off objects and return as echoes, which are heard by the bats. Donald Griffin (1958) called such object location "echolocation" .

Echolocation can be fomally defined as a method of self-information in which one organ is used to emit the sound signal (the *sònar* apparatus) and another organ is employed for receiving that sound signal (the *radar* mechanism) by the same animal. Spallanzani was not entirely wrong. Bats made their way in the darkness by literally "listening in the dark" (Griffin, 1958). The power of flight and the ability to echolocate prey are behind the tremendous evolutionary success of insect bats. Bats skillfully navigate in

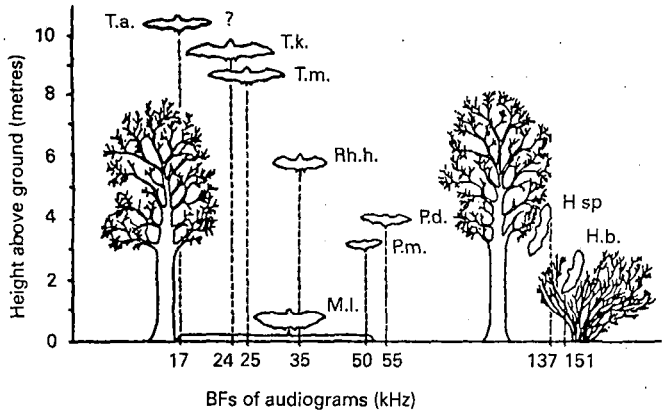

Fig. 4.1 Relationship between the 'best hearing frequency' (BF) of species-specific audiograms (abscissa) and preferred foraging areas (ordinate) for echolocating bats of Madurai. H. sp. : *Hipposideros speoris;* H.b. : *Hipposideros bicolor;* M.l.: *Megaderma lyra*; P.d.: *Pipistrellus dormeri*; P.m.: *Pipistrellus mimus*; Rh.h.: *Rhinopoma hardwickei*; T.a. : *Tadarida aegyptiaca*; T.k.: *Taphozous kachhensis*; T.m.: *Taphozous melanopogon.* The question mark indicates that the preferred foraging area of *Tadarida aegyptiaca* is not precisely known. (After Neuweiler et al., 1984).

pitch darkness over hill and dale and elegantly hunt for insects within brambles, thicket and bushes.

Neuweiler (1984) has summarised some of the findings, which emerged out of our collaboration in Madurai. The bats foraged in three modes: (1) surface gleaning, (2) foraging within foliage and (3) open air foraging. It was established by Neuweiler, through a combination of sophisticated neurophysiology experiments and field ethology studies that the pattern of the burst or *train* of ultrasonic pulses emitted by the Madurai bat species varies according to the species and is *adapted* to its feeding habitat. Figure 4.1 illustrates data obtained on echolocation experiments performed on the *eight* species of insectivorous bats that forage in the Madurai Kamaraj University campus.

What differentiates the auditory system of an echolocating bat from that of a non-echolocating mammal? Neuweiler (1990) writes: "This basic question has still no unequivocal answer. Sensitivity to ultrasonic frequencies (frequencies >20 kHz) would be the wrong answer. Audiograms obtained by neurophysiological or behavioural methods disclose that all small mammals, including non-echolocating fruit-eating bats (megachiropterans), hear well above 20 kHz. Figure 4.2 demonstrates that the range of frequencies heard by mice, cotton rats, non-echolocating fruit-eating bats, and bat

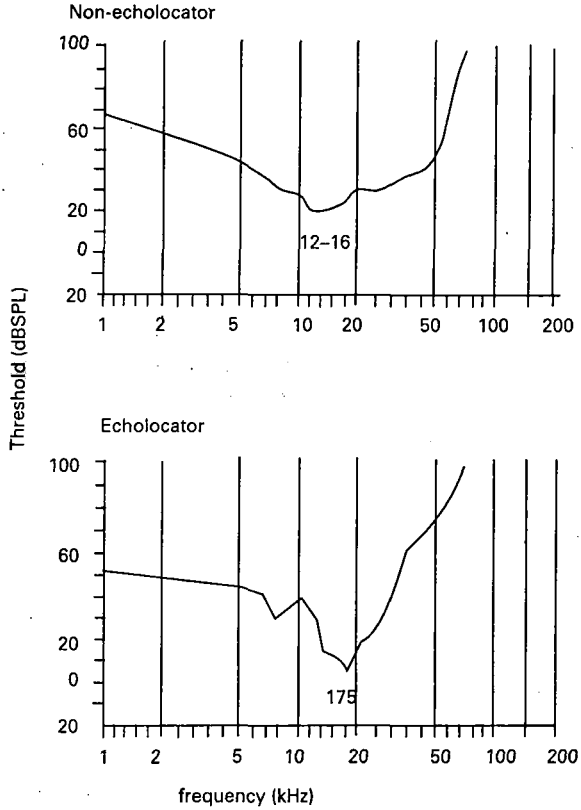

Fig. 4.2 Audiograms of the non-echolocating, fruit-eating bat *Cynopterus sphinx*, and the echolocating, insectivorous bat *Tadarida aegyptiaca*; as derived from threshold measurements of evoked potentials in the inferior colliculus (After Neuweiler et al., 1984).

species that exclusively detect and catch flying insects by echolocation are indistinguishable.

In each species the audiograms extend far into the ultrasonic spectrum." There is no echolocation without a preceding vocalisation. This fact uniquely distinguishes audition in echolocation from that in communication or from listening to noises generated by alien sources. Therefore, in auditory imaging, information on the nature of the animal's surroundings may be gained only by comparing the emitted signal with the returning echoes. In recent years, the general hypothesis that the emission of echolocation sounds

triggers specific auditory mechanisms that facilitate echo detection and analysis has been strongly backed by neurophysiological and behavioural experiments (Marimuthu and Neuweiler, 1987).

The next question posed would be: Why are high frequency sounds used? The fact is that in this range, interference from other sources of sound would be minimal, although high frequency sounds do not travel very far in air. Energy absorption increases exponentially with frequency, and a bat echolocating at 30 kHz may have a range of *ca.* 30 m, decreasing to 10 m at 100 kHz and 4 m at 200 kHz. The Madurai bat *Tadarida aegyptiaca* emits search signals of 18 kHz and may therefore effectively scan air spaces as deep as 50 m, whereas in *Hipposideros bicolor,* which emits signals at 150 kHz, "the auditory world will be "pitch dark" at distances >5–6 m" (Neuweiler, 1990)

As a rule fruit bats do not need echolocation to detect objects or find food. Megabats of the genus *Rousettus* echolocate within their cave producing shrill sounds by clicking their tongue. The pulses are short (1–2 ms), and of high frequency 10–60 kHz. They are the deftest navigators among fruit bats. The biggest colony of *Rousettus lechenaulti* I have seen contained over 20,000 individuals, packed into the corners and ceilings of a temple in ruin near Palayamkottai (8°44' N; 77°42' E), in Tamil Nadu. Unlike the other two fruit bats, *Pteropus giganteus* and *Cynopterus sphinx, Rousettus* (also called dog bats) never roosts on trees.

4.3 Are bats blind?

A question frequently posed is if bats were blind. The answer is decidedly "no". As blind as a bat may be a good alliteration but is bad biology. Some microchiropteran bats have very small eyes and acuity of vision may be poor compared to fruit bats, which have good eyesight. However, they do see big objects and landmarks. An important function their eyes most certainly perform is the entrainment of the circadian rhythm to their flight/rest activity. The dim light of dawn and dusk achieves this daily setting of the clock. In fact the dimmest intensities of light that can entrain (0.0001–0.0006 lux for *ca.* 90 min per cycle) (Fig. 4.3) (Joshi and Chandrashekaran, 1982) and shortest flashes of light (Fig. 4.4) (Joshi and Chandrashekaran, 1984) that can phase shift a circadian rhythm (1/16,000 s or 0.0625 ms) were reported from our laboratory for the cave bat *Hipposideros speoris*.

Hearing of course in microchiropteran bats is much more efficient than seeing. Morphologically the auditory regions of the brain of insect bats are disproportionately large compared to optic regions. The enlarged auditory region enables insectivorous bats to receive, process, store and retrieve information about the environment on the basis of soft echoes.

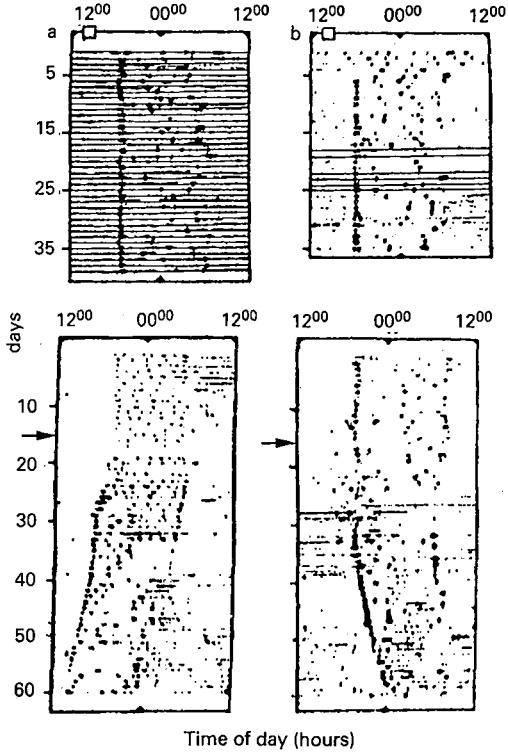

Fig. 4.3 Upper panel: Entrainment of the flight activity rhythm in two *Hipposideros speoris* male bats recorded inside a natural cave for (a) 39 days and (b) 35 days in response to *ca.* 1.5 h of light of 0.0001 to 0.0006 lux intensity streaming in at midday hours indicated by the small box at top. (Actogram raw data;. blotches: activity; horizontal traces: rest.) Onset of activity every day coincided with local sunset time.
Lower panel: Pattern of activity of two male *Hipposideros speoris* bats for a period of 60 days inside the natural cave. Entrainment ensues in response to the very dim light for (a) 18 days and (b) 33 days. The light leak was fixed on day 18 (arrow). In (a) one bat the rhythm began to free-run immediately, in (b) the other the rhythm began its free-run after day 33. (After Joshi and Chandrashekaran, 1982).

4.4 Social synchronisation of the circadian rhythm in *Hipposideros speoris* bats

One of the most spectacular sights to coincide with the setting sun in many

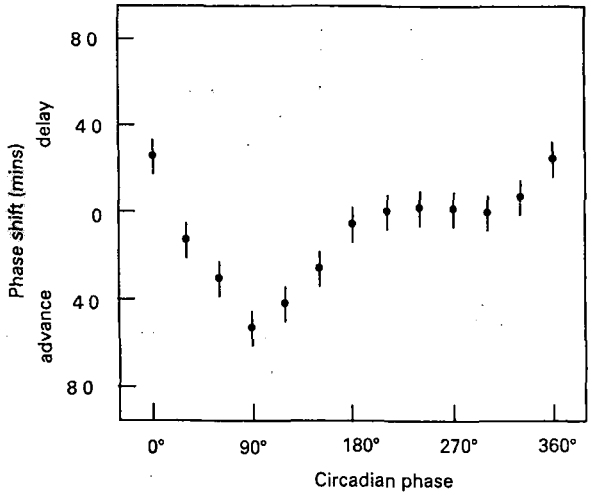

Fig. 4.4 Phase response curve for the circadian flight activity rhythm of the bat *Hipposideros speoris* exposed to brief light flashes of 0.0625 ms at various circadian phases. The phases are expressed in degrees between 0° and 360°. 0° denotes onset of activity, and therefore, onset of subjective night. The light flashes were administered at 30° intervals. The advances were observed in the course of subjective night, and delays around onset of activity, and little or no phase shifts during subjective day phases. Solid circles represent the means, and vertical bars SD. N= 4 or 5 bats. (After Joshi and Chandrashekaran, 1985).

parts of the world is the exodus or out-flights of huge colonies of bats from their caves. In fact, in the Bracken caves in Central Texas a lot of people assemble just to see millions of free-tailed *Tadarida brasiliensis* bats pour upwards out of the cave for minutes on end. Clouds of bats emerge and sway away and disperse to distant foraging sites. This is the largest known bat colony in the world and it is estimated that some 20 million Mexican free-tailed bats live in the caves. The combined body mass of the bats living in the Bracken caves amounts to a stupendous 270 tons. By sharing their body heat, the bats are able to raise the cave's temperature by over 20°C. In any nursery roost, warmth is necessary for the survival and growth of the young, but only species that congregate in very large numbers can so dramatically heat an entire cave. Nevertheless even these bats must migrate up to 1400 km or more south into Mexico to find warm caves and food in winter.

In Phu Phaman in Khon Kean province, Thailand, some 450 km north-east of Bangkok, tourists flock to watch a colony of bats emerge from a gigantic cave whose mouth is roughly 150 ft. × 100 ft. in dimension. It is a spectacular sight, with the flow of thousands upon thousands of bats out of the cave continuing steadily for atleast *seventeen* minutes.

In tropical and sub-tropical areas, where caves are relatively warm and food and water often nearby, bats can use nearly any cave that is safe from predators. There is a huge hill rock complex in Madurai (9°58'N lat; 78°10'E long) called Samanar hills. Samnar means "Jain" and it is so called since on the northern rock-face there are beautiful stone sculptures made by Jain monks dating back to the sixth century AD. The hill rock campus is about 70 m at its highest and mostly granite in composition and is situated 8 km east of the University campus. There are over seven caves in this hill, one of them 40 m deep being a true cave with just one opening. A colony of *Hipposideros* speoris, numbering ca. 600 bats, lives inside the cave evenly distributed in its many labyrinths and pockets.

These bats show great space memory and have a fixed *personal* space in which they roost. Unlike the Mexican free-tailed bat these hipposiderid bats do not huddle. The males urine-mark the spot in the ceiling or the sides of the cave where they roost and relocate this spot morning after morning with amazing accuracy when they return after the nightly foraging. There is hierarchy among males in roosting, which is very clearly seen in laboratory experiments. Females do not urine-mark and it is still not clear how they select their roosting spot. Most probably the females take their position relative to neighbouring males (Selvanayagam and Marimuthu, 1984). The bats are so spaced out that while hanging from the ceiling they

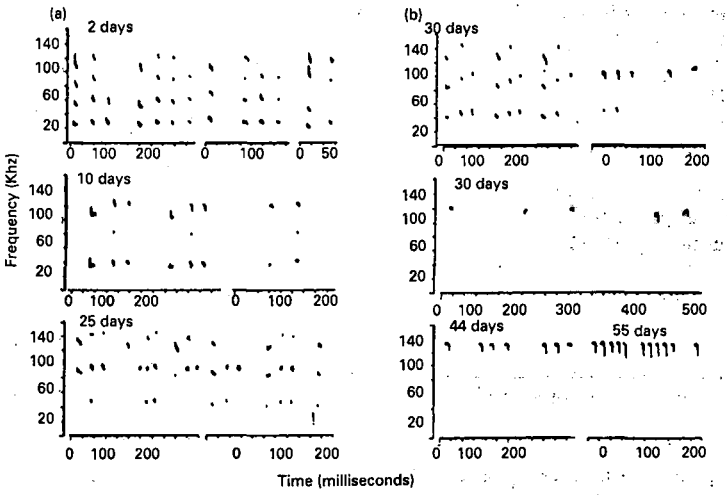

Fig. 4.5 (a) Sonagrams of sounds of the bat *Hipposideros speoris* emitted two, ten and twenty five days after birth when the bat was not yet able to fly. (b) Sonagram of sounds of volant *H. speoris* thirty, forty four and fifty five days after birth.

can stretch their wings and pirouette and still not touch the wings of a neighbour behaving in a similar fashion.

Most bats, even echolocating insect bats, are capable of producing audible vocalisations in addition to the trains of ultrasonic pulses each lasting about a few ms. Such audible vocalisations are mostly produced when they socially interact with members of their own colony. *H. speoris* adult bats are, however, "silent" to humans, who cannot hear frequencies beyond 10 to 11 kHz, depending on the age. *H. speoris* produce ultrasonics in the range of 134 to 137 kHz. As pups until the age of 48–50 days (when they become sub-adults) they produce squeaks, which are audible to human ears owing to the emitted harmonics (Habersetzer and Marimuthu, 1986). Figure 4.5 illustrates the kind of vocalisations these bats emit in the course of their ontogeny.

The sound emission of juveniles aids mothers in finding their young ones. Mothers located their infants even when the juveniles were displaced from where they had left them behind. By the time they are 50 days old they can fly as well as emit ultrasonics characteristic of adults with the constant frequency (CF) and frequency modulated (FM) components in place (Habersetzer and Marimuthu, 1986).

Like most other species of bats all the members of the *Hipposideros speoris* colony flew out in great synchrony much like a cloud burst and scattered after flying in formation over a distance of ca. 500–600 m. Such synchronous exiting has been called emergence by *coup* and may be a strategy bats adopt to put off predators. They then dispersed, mostly in pairs and forage for insects under streetlights and foliage. There is pair bonding for much of the year but it is not known if such pair bonding is for life. Having left the cave soon after sunset when light intensities are typically below 0.5 lux the bats return individually at varying hours any time before sunrise. The synchrony of the colony outflight in two species of bats, *Rhinopoma hardwickei* and *Taphozous melanopogon,* is shown in Fig. 4.6.

It can be seen that the cave empties of all adult bats in a narrow window in time of 20–30 min. We have suggested that the onset of activity of these bats may be under the control of the circadian clock but the termination of foraging might be regulated by ecological and environmental factors such as wind velocity, precipitation, foraging success and the like (Chandrashekaran et al., 1983). Mother bats that leave their young in a creche are known to return to the cave intermittently to check on the well-being of their young.

The sequence of events culminating in such regular out-flights soon after sunset is as follows. The sunset in Madurai was around 1900 h – the longest day being 13.5 h and the shortest day 11.5 h. The out-flight of the various species of bats in the region did mirror the small changes in time of sunset over the seasons. Watching through an infrared nightscope inside the cave 40 m deep we noticed that the bats wake up well before sunset – around 17.30–18.00 h. They then yawn, stretch, preen and start flying about. Prior to out-flight the bats fly through the labyrinthine cave, right up to the cave mouth

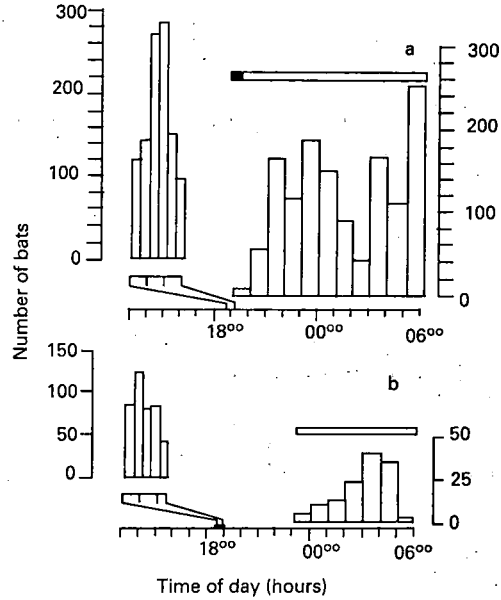

Fig. 4.6 Pattern of the "onset" and "end" of the nightly foraging activity of two colonies of bats belonging to the species (a) *Rhinopoma hardwickei* and (b) *Taphozous melanopogon*. In *Rhinopoma* the out-flight of the members of the colony lasted only 26 min (18.42–19.08 h) but the return flight was spread over 10 h 18 min (618 min). In *Taphozous melanopogon* the outward flight lasted 22 min (18.34–18.56 h) and the return flight lasted 6 h 14 min (374 min). The numbers of bats flying out and returning do not tally because bats are known to take different routes, especially during out-flight.
(After Chandrashekaran et al., 1983).

(the light sampling chamber) and fly about waiting for the outside world to be sufficiently dark (ca. 0.3 lux). Generally, a solitary bat or two darts outside the cave but dart immediately back into the cave. Most probably they then alert all the other bats of the state of darkness outside and then begins the spectacular exodus. Such 'light sampling' behaviour prior to onset of foraging flight has been reported in the European cave-dwelling bat *Rhinolophus ferrum equinum* (DeCoursey and DeCoursey, 1964). We have noticed, as indeed had another group of Dutch scientists for their bat *Myotis* sp. (Voute et al., 1974), that *Hipposideros speoris* bats inhabiting the innermost recesses of the cave, which are mostly weaning females and sub-adult males and females, are the earliest to fly out.

The interesting feature of much of the Samanar Hill cave was that the temperature within was a constant 27° ± 0.1°C, the relative humidity a

constant 98% and the darkness absolute. An optometer, on the energy mode of time × intensity, showed a "0" reading after 1000 s exposure of the photo-element. In other words, the cave environment was entirely lacking in time cues.

The first question we asked ourselves was whether each bat had to sample light individually for itself? If not how would those bats living deep in the cave, where the darkness was absolute with no time cues, know that the sun has set? Do bats volunteer the information of sunset to those living in the interior?

We performed the first experiment in which we kept *three* male bats in three flight activity cages for an extended period of 50 days. The cages were made of light aluminium frame of 50 × 50 × 50 cm dimensions with mosquito net covering and a sleeve. The cages with writing ink stylets were suspended from a beam by means of a spring. Every time a bat flew, the cage jiggled and traced its excursions on a hand wound thermo-hygrograph drum. The bats were hand fed with degutted soft parts of minced cockroaches. For our operations, we used a battery torchlight fitted with monochromatic filters that permitted only red light >610 nm, which was "safe light." Otherwise the bats were bathed by the perpetual darkness of the cave 40 m away from its opening.

Evening after evening all three captive bats of our first experiment inside this natural cave, started their nightly "foraging" activity around 1900 h to perfectly coincide with the out-flight of the conspecifics of the colony. The actogram is presented in Fig.4.7.

The three bats were less active during the hours when the cave had emptied of bats and even responded to stray returning bats. Activity picked up when the bats were returning in larger numbers in the wee hours of the morning after foraging (Marimuthu et al., 1978; 1981). In our first scientific communication on social synchronisation of circadian rhythms, we stated that it was not clear how this synchronisation was achieved, but suggested the involvement of (1) pheromones, (2) wing-beat of conspecifics flying out and (3) acoustic transmission, or a combination of these. Ultrasonics have been implicated in the context of social behaviour in bats only in one other instance, i.e., in the case of the males of the Indian false vampire *Megaderma lyra* "serenading" the female during courtship.

We were reasonably sure that free-flying conspecific bats were telling the three captive bats the time. In other words there were social informers of time. In which case, what would happen if a solitary bat were held in a solitary cave *without* social informer conspecifics? Our biggest problem was to find a solitary cave, which was habitable for bats and suitable for our experiment, which has not been colonised by bats already. When finally we did find a cave without bats, we performed the experiment placing a solitary male *Hipposideros speoric* bat in a flight activity cage inside this cave and recorded its flight/rest activity. The solitary bat was indeed

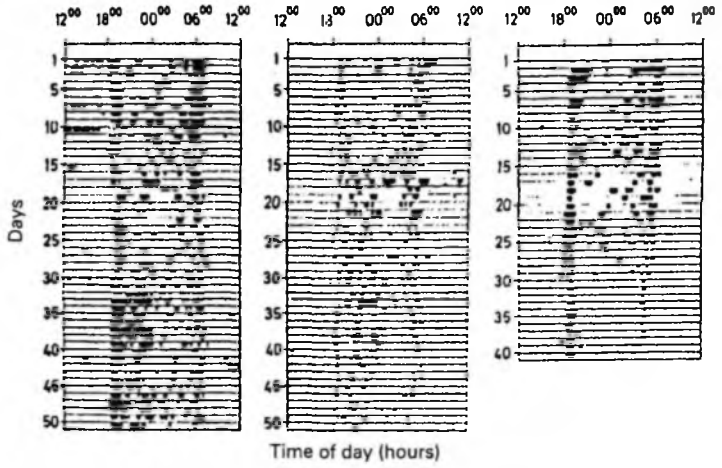

Fig. 4.7 The flight activity patterns of *three* captive *Hipposideros speoris* male bats recorded for 40 days in one case and 50 days in the other two cases. The recordings were performed with aluminium framed flight activity cages containing the bats, suspended from a rigid frame by springs. The bats were hand-fed through a sleeve. The site of the experiment was 40 m from the cave mouth, bathed in absolute darkness and a constant temperature of 27° C and invariant humidity of 95–98 %. The onset of activity in all three bats coincided with the out-flight of the free flying conspecifics. The free flying bats apparently 'tell' the captive bats the time of sunset (after Marimuthu et al., 1981).

helpless (Fig. 4.8) and its circadian activity rhythm free-ran (Marimuthu et al., 1981).

The bat began its nightly flight ca. 20 min earlier in each cycle. After 50 days in captivity and free-run, the experimental bat began its subjective *nightly* activity just when the free-flying conspecifics were returning to their daytime roost in the early hours of the civil morning!

It may be seen in Fig. 4.8 that the activity onset was very close to 1900 h for the first 10 days of the experiment, which is very close to sunset. We started suspecting that the solitary bat was still aware of the time of sunset by other means. On daytime inspection for any light leak, it turned out that during the day, crows and myna birds could be heard from a nearby watering hole. During the night, the crickets residing in the cave stridulated. Maybe the bird-songs and the stridulation of crickets were providing the day/night information and thus entraining the flight activity rhythm of the solitary male bat. Since we were not very sure of such interspecific acoustic entrainment in this case, the experiment was continued, and to our surprise, the bat launched into a free-run of its rhythm from the eleventh day onwards. Clearly, the birds and the crickets were of no direct use in the temporal orientation of the captive bat.

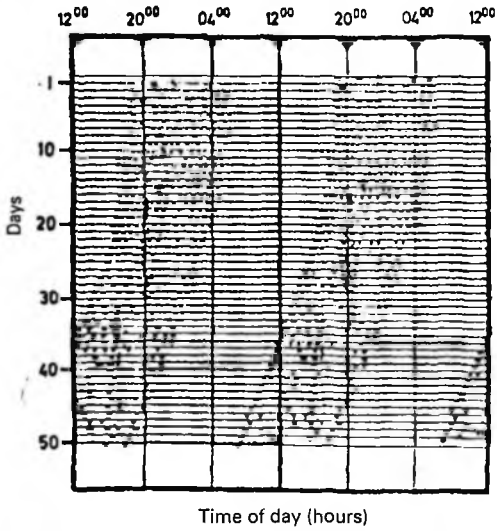

Fig. 4.8 . The flight/rest activity pattern of a solitary male *Hipposideros speoris* bat in cave without conspecifics of its colony, recorded over a period of 50 days. There are no social 'informers' and therefore no social entraiment. In the darkness of the cave the rhythm of the bat free-runs with a period shorter than 24 h. Other details as in Fig. 4.7. (After Marimuthu et al., 1981).

These observations led us to pose the next major question. If in the experiment, the bat ignored the day/night acoustic inputs of birds and crickets, could it be that *social synchronisation of circadian rhythms* was possible only by the presence and stimuli given away by conspecifics? In other words, is this phenomenon species specific? It must be pointed out that non-species specific entrainment of the circadian rhythmicity has been reported for birds and is suspected to be effective even in entrainment of human circadian rhythms. In one of the early experiments attempting to socially entrain the biological clock in the perch-hopping activity of the common sparrow *Passer domesticus,* Gwinner (1966) made use of a conspecific song. The circadian rhythm was successfully entrained by playing back the bird-song for four and half-hours each day. Similar results were got for two other species of birds the same year and the results generated much scientific enthusiasm since it all appeared to make much ecological and behavioral sense. However, a year later it was found (Lohmann and Enright, 1967), rather unromantically, that cycles of mechanical noise administered by a loud buzzer for a few hours in a 24 h cycle, entrained activity rhythms of three species of passerine birds, demonstrating the phenomenon of non-species specific entrainment at its best.

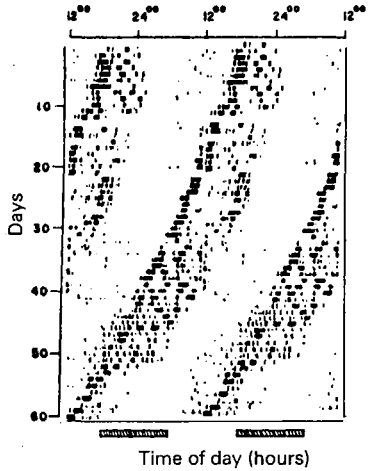

Fig. 4.9 The free-running rhythm in flight activity/rest pattern of a captive alien male *Taphozous kachhensis* (an emballonurid bat of ultrasonic emission frequency of 83.5 kHz) recorded in the *Hipposideros speoris* cave with ca 600 free living bats (the ultrasonic emission frequency of this hipposiderid bat is 134.5 kHz). The rhythm free-ran with period < 24 h in the darkness of the cave. Blotches represent the activity bouts of the bat. Original felt tip tracings reproduced. (After Marimuthu and Chandrashekaran, 1983).

In order to further probe the question of species-specificity, we performed an experiment in which we introduced a rank *alien* species of bats, a male emballonurid *Taphozous nudiventris kachhensis* into the *Hipposideros speoris* cave and monitored its daily flight/rest activity. The circadian rhythm of the *alien* bat could *not* be entrained by the combined efforts of some 600-hipposiderid resident bats. The circadian rhythm of the introduced alien bat impressively free-ran. The results are presented in Fig. 4.9 (Marimuthu and Chandrashekaran, 1983).

We called this kind of non-entrainment as resulting from "a communication gap" and can back this up with results obtained from neurophysiological experiments. *Taphozous nudiventris kachhensis* emits ultrasonics in the region of 80 kHz whereas, as pointed out earlier, *Hipposideros speoris* emits in the region of about 134 kHz. When electrodes were implanted in the lower colliculus region of the brain of *Taphozous nudiventris kachhensis* and the recorded ultrasonics of *Hipposideros speoris* was beamed into the ear of the former, the absence of "action potentials" indicated that the message was not being *heard*. These results appear to favour some manner of species-specificity in social synchronisation of circadian rhythms in bats. They apparently get the message of sunset only when told in their ultrasonic *language* by free-flying conspecifics.

We have seen that all circadian rhythms free-run in LL and/or DD. From the foregoing results it is clear that in the natural DD environment of the cave, bats seem to tell one another time, at least at the time of synchronous out-flight. Therefore, there are no free-running circadian rhythms just as there are no free-running circadian rhythms in nature under normal conditions. But having established that a captive bat maintained in isolation in the darkness of a cave (unnatural environment) free-runs, we wanted to test a situation where there were conflicting zeitgebers. We wanted to know how bats dwelling in darkness would react to imposed LL inside their cave. This was a very difficult experiment (as indeed most nature/cave experiments are) to perform. Because for one, such a situation never takes place in nature. We tried it in the laboratory and found that both DD and (permissive intensities of) LL induce free-running in circadian rhythms.

Producing the same conditions in the cave, required some ingenuity. We created artificial LL with car batteries and an incandescent bulb inside the *Hipposideros speoris* cave and measured the flight/rest activity of three male captive bats. The light bulb was so positioned that the ambient intensities of light at the activity cages level were between 10 and 15 lux. Figure 4.10 shows that the circadian rhythm in the flight activity of all three bats free-ran (Marimuthu and Chandrashekaran, 1983) with a period (this time) >24 h, an effect LL often imposes on night-active animals.

In day-active animals LL would on the contrary decrease the period. These effects were codified by Aschoff (1960; as described in Chapter 1) and are referred to as *Aschoff's rule.* The LL inside the cave could have driven away

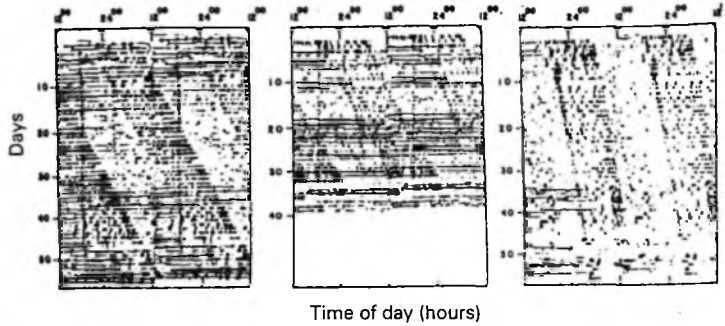

Fig. 4.10 The flight activity pattern of three captive male *Hipposideros speoris* bats recorded for (a) and (b) 55 days and (c) 55 days inside a cave in LL created with incandescent bulbs and automobile lead acid batteries Bats (a) and (c) did seem to wake up and move about for a short while when the colony out-flight was in full swing but later the activity subsided. LL clearly abolished the social synchronization of the circadian rhythms in all three bats, whose rhythms then free-ran with a period > 24 h. (After Marimuthu and Chandrashekaran, 1983).

all bats from the site of the experiment, in which case an effective social communication and consequent entrainment may *not* be expected. But careful examination of Fig. 4.10 reveals that all three bats did wake up around 1900 h and fly around for a while as the colony was leaving the cave but then relapsed into sleep and were aroused on their own subjective free-running circadian schedule (Marimuthu and Chandrashekaran, 1983). We conclude that the circadian clocks are somehow uncoupled by LL from the 24 h social inputs of the colony and that LL abolishes entrainment. These results, though not anticipated or known for the circadian rhythm of any other animal, are of extreme physiological importance and interest. Most probably the LL interferes with the nightly secretion of melatonin and by means of this causes a lack of entrainment by social information. It must be pointed out that in spite of years of investigation we still are not clear about the exact nature of the social cues (acoustics?, pheromones(s)?, wing-flapping noise during flight exodus?) that entrain the circadian rhythms of flight/rest activity in *Hipposideros speoris*.

One of the earliest reports to impute social synchronisation among conspecifics was for the mice of the genus *Peromyscus* (Halberg et al. 1954). Subsequently, similar effects have been claimed for blinded mice, male chevrotan antelopes, wolf-coyote hybrids, colonies of the the beaver *Castor canadensis*, macaque monkeys and sexual cyclicity of female mammals (Chandrashekaran, 1982). The phenomenon of *social synchronisation of circadian rhythms* deserves to be better studied – preferably employing social insects such as honeybees and ants. It has been shown in honeybees (Frisch and Aschoff, 1987) that all members of a beehive free-run with the same period in LL conditions. Social insects also promise to be ideal material for investigations of circadian consequences arising as a result of the evolution of a social structure, in their physiology and behaviour.

5. The Biological Clock of the Field Mouse *Mus booduga*

❄

Where has he gone, my meadow mouse,
My thumb of a child that nuzzled in my palm ? —
To run under the hawk's wing,
Under the eye of the great owl watching from the elm-tree,
To live by courtesy of the shrike, the snake, the tom-cat.

Theodore Roethke

❄

5.1 Role of LD cycles

By 1984, we at MKU started working on the field mouse *Mus booduga*. This sturdy little creature was found in great numbers in burrows of paddy fields around the university campus. In chronobiology, working with rats and mice, whether captured from the wild or laboratory bred Wistar strains, has many advantages over working with insectivorous bats,. Our captive bats used in the experiments had to be carefully hand-fed with soft insect parts every day. On the contrary, the field mouse *Mus booduga* was robust and even in captivity seemed to live well on millets and grains and clean drinking water, fortified with a few drops of multi-vitamins. They were placed in cages attached to activity running wheels in which the animals exercised. The cages were irregularly cleaned at staggered hours once every 3–4 days with minimum disturbance to the animals.

Notwithstanding the importance of light in circadian entrainment, reports on the effect of the spectral composition of light on circadian processes remains scanty. The limited data available suggest the existence of important inter-species differences in pigments, receptors and pathways (Gordon and Brown, 1971; McGuire et al., 1973). However, in mammals, the action spectra for entrainment and phase shifting were found to resemble the spectral sensitivity curve for rhodopsin with a single peak in the blue-green region. The existence of two different classes of photoreceptors, sensitive to either short (430 nm) or medium (520 nm) wavelengths, in the nocturnal cave-dwelling bat,

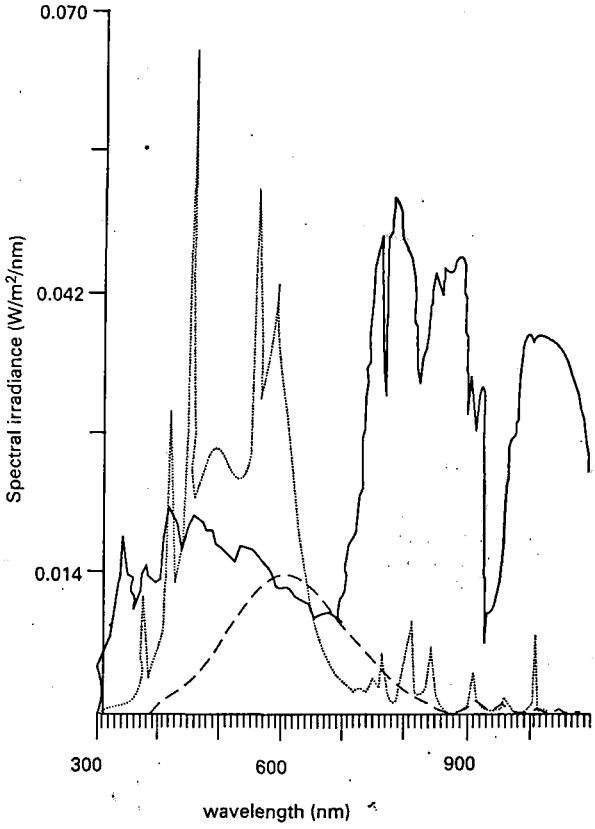

Fig. 5.1 Spectral irradiance curves constructed for diffuse daylight (uninterrupted line), incandescent light (broken line) and fluorescent light (dotted line). (After Sharma et al., 1997).

Hipposideros speoris, has been postulated on the basis of data obtained in our laboratory (Joshi and Chandrashekaran, 1985). Most PRCs are, however, constructed under laboratory conditions with incandescent or fluorescent light pulses. "Natural daylight" is much richer in its spectral composition (Fig. 5.1) compared to artificial sources of light.

We had in fact constructed the first "daylight PRC" for a mammalian system (Joshi and Chandrashekaran, 1983), for the circadian rhythmicity in the flight/rest activity of the insect bat *Hipposideros speoris* working the

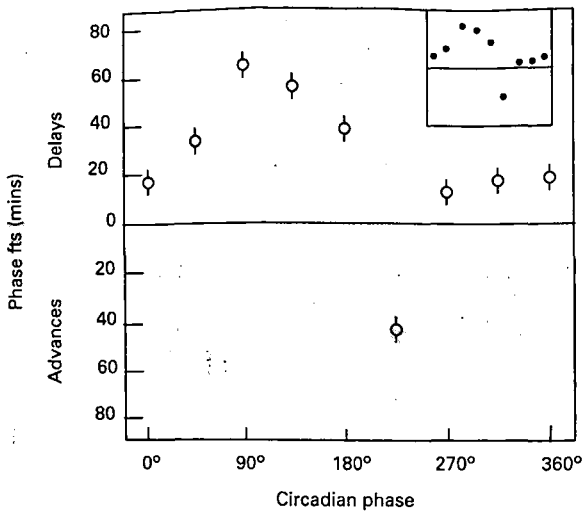

Fig. 5.2 PRC obtained on several bats (main PRC) and for a solitary male bat over a protracted period of 156 days (PRC in the inset). Open circles represent the mean and vertical lines represent the S.D., $n = 4$ or 5 bats.

whole time in a natural cave. We used diffuse daylight of ca. 1000 lux, naturally occurring close to the cave mouth for 15-min pulses, which we administered to the free-running circadian rhythms of bats maintained in flight activity cages in the natural DD of a cave. The actual spot in which the diffuse daylight of ca 1000 lux occurred, changed in the course of the day depending on the inclination of the sun. The time course and waveform of the daylight PRC resemble that of a PRC constructed for other nocturnal mammals in the laboratory as may be seen from Fig. 5.2.

Both delay and advance phase shifts were discrete and direct without any transients but the phase shifts did effect changes in the period. In general, delays lengthened the period and advances shortened it. In Fig. 5.2 are illustrated the PRC data obtained on several bats over 870 bat-days in the laboratory. The "inset" in the figure sets forth the PRC data obtained on the flight activity rhythm for a single sturdy male *Hipposideros speoris* bat in an extended experiment which lasted 156 days. It may be seen that the single bat PRC and the PRC constructed from the data obtained on several bats possess the same time course and waveform.

We also constructed the first entirely "daylight PRC" in the laboratory for a nocturnal mammalian system employing the locomotor activity rhythm of *Mus booduga* and compared its waveform and time course with the PRC constructed for this and other organisms using incandescent and fluorescent

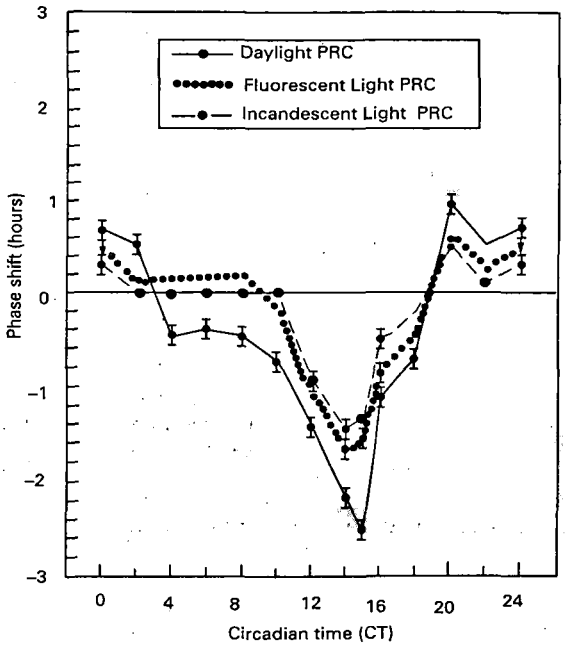

Fig. 5.3 PRC constructed for the circadian rhythm in the locomotor activity of *Mus booduga* with pulses of 15 min duration and 1000 lux intensity, with phase shifts evoked by three different kinds of light stimuli. (After Sharma et al., 1997).

light (Sharma et al., 1999). The diffuse daylight of ca. 1000 lux for the 15-min pulses was obtained by appropriately moving the plexiglass cage with the animal within, inside a shed. With the mouse circadian system, we observed significant quantitative differences between the 15-min 1000 lux daylight PRC and the PRC for incandescent and fluorescent light pulses of the same duration and intensity as shown in Fig. 5.3.

Among other subtler differences, the magnitude of advance as well as delay phase shifts evoked by daylight pulses were more pronounced at most phases. We attribute these differences to the variations in the spectral intensity distribution of the different components of the light stimuli (illustrated in Fig. 5.1), since all other factors in these three sets of experiments such as sample size, free-running period of the locomotor activity rhythm, duration and intensity of light pulses, etc., were comparable. Also, the influence of low doses of UV-radiation or far-red components or of both cannot be ruled out. The phase response characteristics of circadian

rhythms contained in daylight PRCs may mirror more faithfully events involved in entrainment by natural LD cycles.

Light of wavelength beyond 650 nm was found to be safe when used for brief durations. So much so that we used red light >650 nm as "safe light" in DD experiments to change food, water, clean the cages and other minor operations. Red light of wavelength >610 nm and of intensity <150mW/cm^2 given in the form of red light/darkness cycles of 12:12 h caused negative masking and the same wavelength of red light of intensity >150 mW/cm^2 caused entrainment of the locomotor activity rhythm of *Mus booduga*. Such intensity-dependent masking and entrainment were also obtained with white incandescent light (Viswanathan and Chandrashekaran, 1985a). Shorter flashes of red light of wavelength >610 nm, which we routinely use for cleaning, and care of the animals do not appear to affect the period of the rhythm. And the same may be said of the continuous red light of sufficiently low intensities used in our so-called DD experiments.

We also know that the circadian systems of mice and the golden hamster are sensitive to both green light (ca. 500 nm) and near-UV radiation. The effect of 15 min pulses of different wavelengths of monochromatic light (blue: 480 ± 11 nm; green: 549 ±11 nm; and red: 649 ± 11 nm) administered to the circadian rhythm of *Mus booduga* at CT 14 and CT 20 h were investigated. CT 14 and CT 20 h are the two phases at which maximal delay and advance phase shifts are evoked by white light pulses. Pulses of all three wavelengths evoked qualitatively similar responses in terms of signs of the phase shifts (Geetha et al., 1995; Geetha and Subbaraj, 1996). DeCoursey (1986) reported that for the flying squirrel *Glaucomys volans* 15-min light pulses of monochromatic light of 500 and 620 nm evoked similar responses in terms of the phase shifts of the locomotor rhythms, and that the amplitude of the green light PRC exceeded that of the red light PRC. In our laboratory also we found (Geetha and Subbaraj, 1996) that green light pulses evoked larger phase shifts – both delay and advance – than red light pulses; we also observed that the threshold of response to green light was much lower than that for red light.

Since it was not known for certain if the circadian clocks of mammals were sensitive to near UV irradiation at all phases, we decided to investigate the phase-resetting effect of low doses of UV-A (wavelength between 25 and 400 nm) on the locomotor activity rhythm of *Mus booduga* at all circadian phases of the cycle. Figure 5.4 sets forth PRCs constructed for diffuse daylight stimuli of 15-min duration and for 5.06 W/m^2 intensity UV-A stimuli of 30-min duration (Sharma et al., 1999).

The UV-A PRC qualitatively resembles the diffuse daylight PRC although the small differences in amplitude may be of ecological significance in the entrainment of circadian rhythms in natural LD cycles. It would appear proper to conclude that the photoreceptors in *Mus booduga* involved in phase shifting and entraining the circadian rhythm are sensitive to variations in the spectral composition of light stimuli. In addition to natural daylight

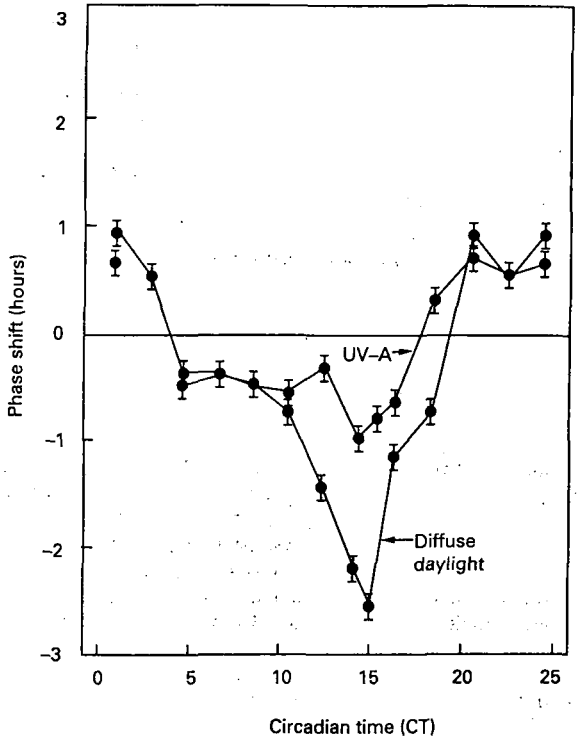

Fig. 5.4 PRC constructed for the circadian rhythm in the locomotor activity of the field mouse *Mus booduga* evoked by diffuse daylight stimuli of 15-min duration and 5.6 W/m² intensity (●) and UV-A stimuli of 30-min duration and 2.5 W/m² intensity (▲). Error bars represent 95% confidence interval about the mean phase shift for 6 to 7 animals. (After Sharma et al., 1999).

and the incandescent and fluorescent white light employed in most laboratory experiments, animal circadian rhythms also respond most effectively to blue and green light with the responses, in terms of phase shifts and entrainment, declining sharply beyond 490 nm.

Krüll (1976; 1985) reported that in the Arctic snow buntings *Plectophenex nivalis,* during the continuous daylight of the long summers, the colour temperature, which changes with differences in the spectral quality of daylight, is the only zeitgeber entraining the circadian rhythms. Krüll (1976) also speculated that colour temperature may play a role even in non-arctic latitudes and emphasised the importance of colour temperature in

investigations involving artificial illumination for entraining circadian rhythms. We performed experiments to investigate if 12:12 h variations in the colour temperature of 4000° K and 2700° K would entrain the circadian rhythm in the locomotor activity of *Mus booduga* and found that the variations used in the colour temperature in this experiment were inadequate. We concluded that the intensity of light but not the colour temperature was the principal entraining component for the circadian rhythm of this tropical mouse (Geetha et al., 1995).

5.2 Maternal entrainment of pups' rhythms

Earlier work in Japan and USA showed that neural and endocrine rhythms in infant mice and rats are maternally coordinated even when they were in the fetus. Interesting as these findings were, they do not come as a surprise for the fetus is very much a part of the mother. Most mammals are said to be *altricial* at birth, which means that for at least the first week or two, they are helpless and totally dependent on the mother for survival. The most altricial mammals are humans – needing parental care for months after birth. The opposite state, of being ready to fend for oneself very early after birth, is called the *precocial* state as in chicken and some other birds.

The pups of the field mouse *Mus booduga* found in our paddy fields are altricial at birth with eyes closed for 12–14 days after parturition. They however, grow fast and are already sub-adults at the age of 33–35 days, venturing out of their burrows to forage. We were interested in finding out how these helpless pups knew the time of day and night. In other words did these pups have functional circadian clocks? If they did, were they in a state of entrainment or were they free-running?

It should be remembered that the burrows in which these mice are born are 1–2 m deep and are very narrow thus providing a perpetual DD much as the bats had in their caves. It is important that they have functional clocks for when they venture out of their burrows as sub-adults (day 33–35 after parturition) they must time it to coincide with the onset of darkness outside, i.e., soon after sunset. If they come out earlier they will be eaten up by birds of prey. It is literally a matter of life and death to know time accurately. In a series of experiments, we set out to find out how the altricial pups inside the burrows obtained their information about the time of day and night.

In a trial experiment pregnant *Mus booduga* were brought to the laboratory and allowed to litter in DD in a well-ventilated chronocubicle 8' × 8' without windows. Two pups were then isolated and placed separately in two cardboard containers with sterile nesting material. The pups were arbitrarily designated pup B and C. The mother was physically picked up and offered to pup B from 06.00 to 18.00 h and to C from 18.00 to 06.00 h. The two pups thus experienced presence–absence (PA) cycles of the mother mouse for

12:12 h, suckling of course when they had the mother presented to them. These PA cycles of 12:12 h were continued for 21 days, at the end of which they were sturdy enough to run delicately mounted activity wheels. The pups were introduced into the activity wheels on day 21 of their life.

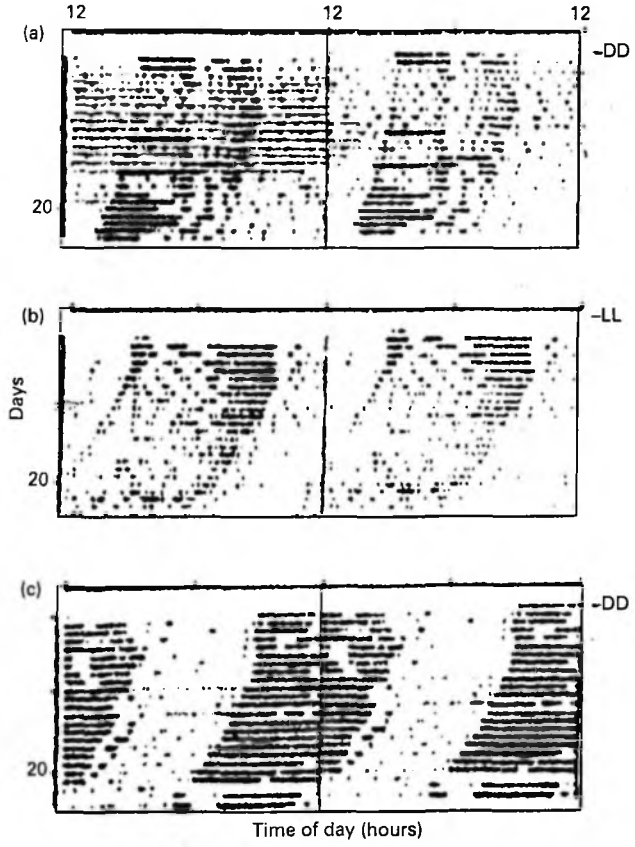

Fig. 5.5 Double-plotted wheel running activity of a field mouse *Mus booduga* mother in (a) DD and two of her pups, (b) one in LL and (c) the other in DD. Activity bouts were obtained as bands on an Esterline Angus 20-channel event recorder and periods of rest traced mere lines. Day 1 of the recordings is day 16 of the animals after parturition. Note that free-runs of the activity rhythms of the LL pup B and the DD pup C start ca 12 h (180°) apart (After Viswanathan and Chandrashekaran, 1985b).

The results we obtained on the rest and running activity of the pups were fascinating (Viswanathan and Chandrashekaran, 1985b) and are set out in Fig. 5.5. The presence-absence cycles of the mother resulted in (maternal) entrainment of the circadian rhythms of the pups. With the result that from day 21 onwards, the pups ran the wheel (wakefulness) when the mother was absent and were inactive (sleep) when the mother was present. Since the PA cycles were discontinued after day 21 the locomotor activity rhythms, as seen in Fig. 5.5, free-ran as also the rhythm of the mother.

There are many stressful factors that come into play in these experiments. Most important of them is that the mother is subjected to non-stop suckling by the pups, which obviously never happens under natural conditions. Another artificial component is the activity-rest rhythms of the mother itself under the DD conditions of the experiments. Her locomotor activity would have free-run and thus one or the other of the pups would have experienced a restless mother. These important factors need to be investigated in greater detail. These nocturnal mice interpreted presence of mother as representing subjective day and absence as representing subjective night. Thus PA cycles replaced LD cycles. The upper panel in Fig. 5.5 (a) illustrates the activity–rest rhythm of the mother; the middle panel, the activity-rest rhythm of pup B and the lower panel, the activity–rest rhythm of pup C, all three free running in DD. Please note that when pup B ran, pup C slept and *vice versa* since the PA cycles of the mother were experienced by the two pups 180° displaced relative to each other. We had succeeded in replacing the LD cycles of nature with the PA cycles of the mother, which was perhaps what was happening in nature.

5.3 Entrainment or masking?

In the next series of experiments, we wanted to confirm that the entrainment of the clocks of pups by the cyclic presence-absence of their mother arises through a genuine circadian mechanism, and not as a result of "masking". Masking is a phenomenon where an external factor directly influences the expression of the clock, as for instance if the external agent directly suppressed activity by its presence or released activity in its absence. An example will make the situation clear. Separation of the mother from infants in guinea pigs, monkeys and rats results in an increase in sleep disturbance and enhanced activity and rest is restored when the mother is returned (Viswanathan, 1987). This is a typical masking effect, in which the circadian clock does not appear to be involved. One way to test if masking is involved is to perform experiments in which the zeitgeber (in this case, the PA cycles of the mother) period (T) is varied widely from $T = 16$ to 32 h. If masking is involved, the pups would be active in the absence of the mother and cease activity altogether (rest) when the mother is presented, regardless of the value of T. Circadian entrainment, on

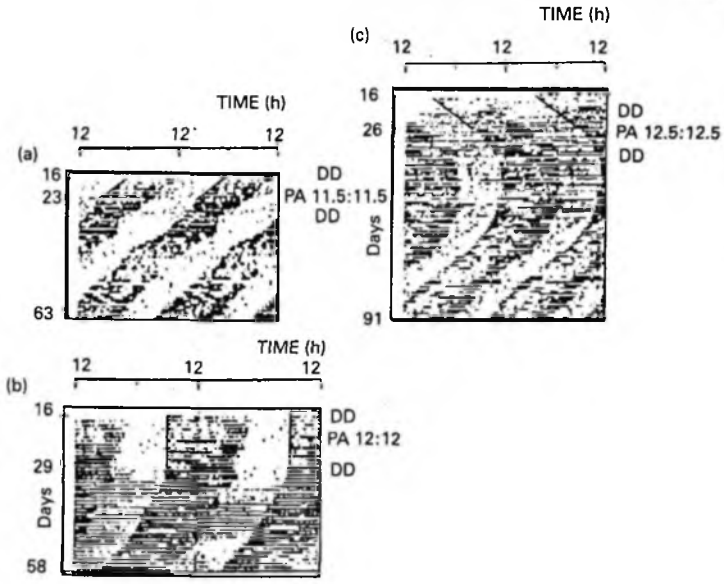

Fig. 5.6 Double-plotted wheel running activity records of *Mus booduga* pups exposed to various presence/absence (PA) cycles of mother. (a). 11.5 : 11.5 h (T = 23 h), (b). 12 : 12 h (T= 24 h) and (c). 12.5 : 12.5 h (T= 25 h). All three PA cycles successfully entrained the circadian rhythms of pups. (After Viswanathan and Chandrashekaran, 1988).

the other hand and by definition, will be effective only within limits, called *limits of entrainment*. For example, in plants, leaf movement rhythms entrain to LD cycles as short as T= 19 h up to cycles as long as T = 29 h. In such a case, the limits of entrainment are 19 h and 29 h. One more defining feature of "masking" is that the masking factor would temporarily impose its own period on a free-running rhythm. The resulting situation would much resemble genuine circadian entrainment. But when the periodically masking factor is removed, the free-running rhythm would resume, as though the basic oscillator was indeed free running all along, at the precise phase of the rhythm that existed prior to exposure to the factor. In other words, masking factors are much like *forces that act on the overt rhythm, which is likened to "the hands of the* clock," without affecting the clockwork (Aschoff et al., 1982).

We performed experiments in which we imposed PA cycles of the mother with T = 22 h (11:11 h) and were surprised to find that the circadian rhythms in the locomotor activity of the pups did *not* entrain. Figure 5.6 shows that PA cycles of 11.5:11.5 h (T = 23 h), 12:12 h (T = 24 h) and 12.5:12.5 h (T = 25 h) perfectly entrain the locomotor activity of the pup.

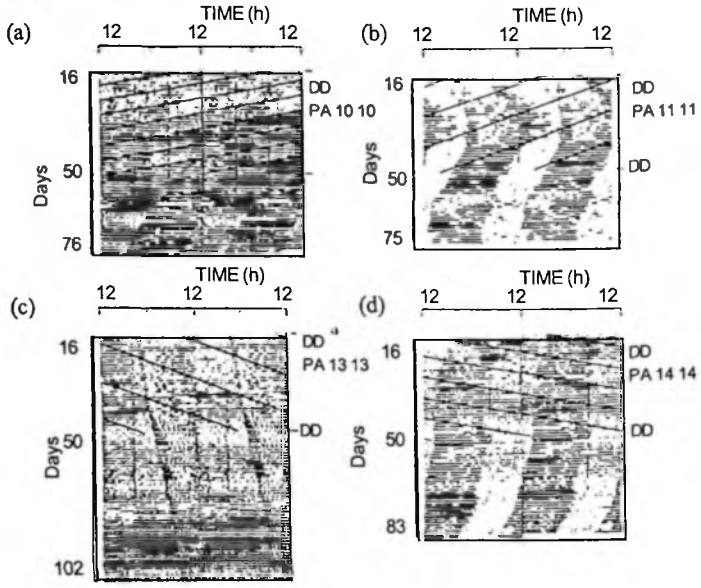

Fig. 5.7 Double-plotted wheel running records of *Mus booduga* pups exposed to various presence/absence (PA) cycles of mother. (a) 10 : 10 h (T = 20 h), (b). 11 : 11 h (T = 22 h), (c). 13 : 13 h (T = 26 h) and (d). 14 : 14 h (T = 28 h). Guidelines indicate when the mother was presented in each cycle and such cyclic presentation of mother was discontinued on day 50. None of the PA cycles entrained the circadian rhythms of pups. (After Viswanathan and Chandrashekaran, 1988).

These durations of PA were close enough to what happens in natural situations. Figure 5.7, on the other hand, illustrates that PA cycles of 10:10, 11:11, 13:13 and 14:14 h, being too far removed from the natural day length, *do not entrain* the circadian rhythms of pups.

Therefore in terms of maternal entrainment by T, cycles of PA of 20 h and 22 h do not entrain; PA cycles of 23 h, 24 h and 25 h entrain but PA cycles of T = 26 and 28 h *do not* entrain. We conclude that *the limits of maternal entrainment* lie within a narrow range in time of T = 23 h to 25 h (Viswanathan and Chandrashekaran, 1988). T values below 23 h and beyond 25 h do not entrain. When we reported this phenomenon in 1985, it was the first report of post-parturition maternal entrainment of any circadian clock and demonstration of *limits of entrainment* for a non-photic, social (behavioural) zeitgeber.

5.4 How much mother for entrainment?

In a further series of experiments we investigated the duration over which the mother must be present/absent for successful entrainment of the circadian clocks of her pups. In LD experiments it is known that LD of 1:23 h would be effective (in fact just a few minutes of bright light in a 24 h DD is sufficient to entrain circadian rhythms in some animals), but its reciprocal of LD of 23: 1h cannot entrain. At least 6 h of darkness is a pre-requisite for stable LD entrainment. In imitation of such LD entrainment, we performed the following experiments with PA cycles of 6:18 h; 8:16 h; 10:14 h; 14:10 h; 16:8h and 18:6 h. The extreme PA cycles of 6:18 and 18:6 h did *not* entrain (Fig. 5.8).

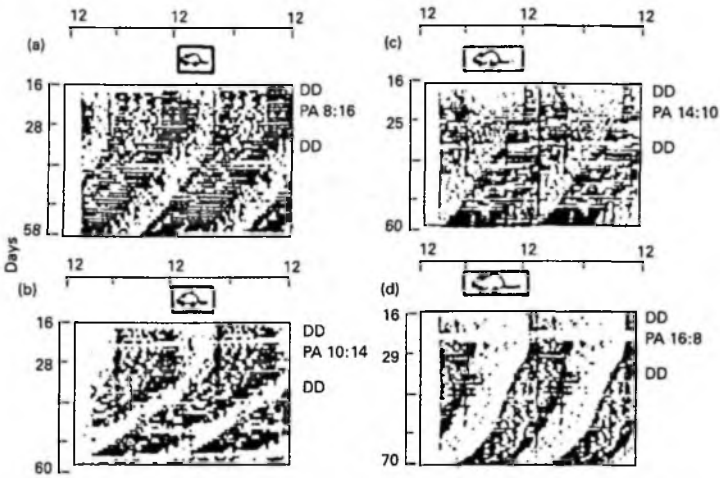

Fig. 5.8 Double-plotted wheel running activity records of *Mus booduga* pups to the presence/absence cycles of mother with systematically varied PA ratios. (a). PA cycles of 8 : 16 h (T= 24 h), (b). 10 : 14 h (T = 24 h), (c). 14 : 10 h (T = 24 h), (d). 16 : 8 h (T = 24 h). PA cycles in which the mother was presented for at least 8 h and up to 16 h entrain (After Viswanathan, 1990).

The results were again novel and exciting (Viswanathan and Chandrashekaran, 1988). PA cycles of 8:16–16:8 h and other intermediate combinations of PA cycle successfully entrain

In other words the mother mouse must be present for at least 8 h for stable entrainment. She may stay on for 16 h in a cycle and entrainment still occurs. Just a 6 h presence is "too little" mother and an 18 h presence is "too much" mother. In other words, the mother must be present for *one-third*

and up to *two-thirds* the cycle for successful entrainment. This discovery was also a first report of its kind. The adaptive or ecological significance of these ratios of PA of the mother mouse is not immediately clear. But such situations do not arise in our latitudes and, it must be remembered that in Madurai, 6 and 18 h nights or days do not occur at any time of the year. In most animal houses and laboratories, the need for the kind of "personal space and time" we are reporting here is not sufficiently appreciated. It is interesting that too little or too much of the presence of the mother induces free runs in the circadian rhythm much as LL and DD do, and does not suppress or abolish the rhythm. Precisely how this continuous presence of the mother beyond 16 h and below 8 h induces free-runs in her pups, and abolishes maternal entrainment is not known in the physiological and endocrinological contexts.

5.5 Conflicting zeitgeber experiments

Experiments were performed to investigate the role of LD cycles in the entrainment of the circadian rhythms, which would anyhow have to take over at later stages of development. These experiments may be called "conflicting zeitgeber experiments." Owing to ontogenic and methodological expediency, the locomotor (wheel running) activity of the pups could be studied only after day 16 following parturition. A total number of 8 mothers and 16 pups were used in these experiments (Viswanathan, 1989). The day of birth was designated as day 0. Starting on day 5, two pups of either sex were selected from each litter, named A and B, and placed in separate plastic boxes 21 × 15 × 13 cm. The mothers were presented for 12 h periods alternately to the pups thus creating PA cycles of 12:12 h, as in the first series of experiments. The A pups were berthed with their mothers 12.00–24.00 h and B pups 24.00–12.00 h. This time around there were additionally LD cycles of 12:12 h which were displaced by 6 h *vis a vis* the PA cycles. In other words, the pups experienced the *behavioural* and the *physical* zeitgebers out-of-phase relative to each other by 90° or 6 h. When the light came on at 06.00 h, the mother was presented only at 12.00 h for A. On day 16 the pups were introduced into the activity running wheels and the locomotor activity was monitored. The PA cycles were continued until day 36 and the recording of the locomotor activity of pups was continued for a total period of two months. The mother had to be tethered, as in earlier experiments, by a 10 cm aluminum chain to prevent her from entering into the activity wheel. The mother/infant interactions, including suckling, thus took place in the nesting cage attached to the wheels. Food and water for the mother were available at all times.

Results of these experiments reveal that the onset of locomotor activity of A and B pups on day 16 (first day of activity recording and onset of LD) was 180° off course relative to each other, as was to be expected. The results clearly indicate that until day 16, the rhythm solely entrained to the PA

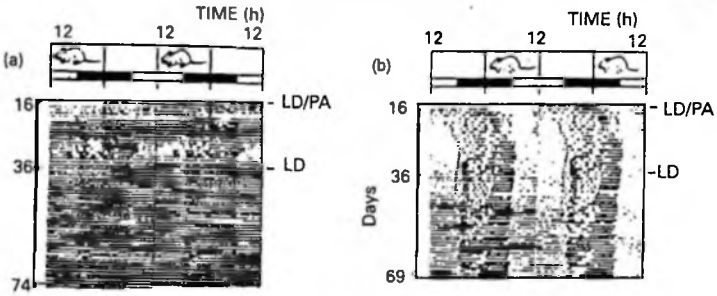

Fig. 5.9 Double-plotted wheel running activity of two pups (A) and (B) exposed to conflicting zeitgebers (PA cycles versus LD cycles) (After Viswanathan, 1989).

cycles of the mother. From day 17 onwards the influence of LD cycles on the activity rhythm becomes manifest, i.e., the *physical* zeitgeber starts to override the influence of the *behavioural* zeitgeber (Fig.5.9). By day 20–22 complete classical entrainment to LD cycles ensues (Viswanathan, 1989). To the best of our knowledge these experiments are the first experimental demonstration of a circadian rhythm switching over from a state of entrainment by a behavioural zeitgeber to a state of entrainment by the ubiquitous physical i.e., LD zeitgeber. The results clearly show that the developing animals during the early stages of their ontogeny rely solely on the PA cycles of the mother for zeitgeber function even though the LD cycles are generally available. The PA cycles of the mother probably provide the developing pups with a state of internal circadian temporal order. Such early behavioural maternal entrainment may have considerable physiological significance. Without the PA cycles of the mother, the pups would develop circadian rhythms uncoordinated to the environmental LD cycles and render the animals vulnerable to environmental hazards such as predation. The mouse requires functional eyes and a properly developed retina to be able to entrain its activity-rest cycles to LD cycles. The absence of entrainment to LD cycles in the first two weeks after parturition could be due to the non-functioning of eyes in these altricial pups whose eyes do not open until 12–14 days. The very low intensities of light that might have penetrated into the darkness of the burrows of the mice during daytime is unlikely to have' reached the retina, and therefore, could not have entrained the circadian rhythms of pups. Entrainment of mammalian circadian rhythms can occur only when their retina perceives light.

In a further series of experiment the PA cycles experiments were performed in LL of 10–20 lux. Figure 5.10 illustrates the results of an experiment that clearly demonstrates that the spectacular maternal entrainment of the circadian clocks of PA of 12:12 h breaks down in LL of just 20 lux (Viswanathan and Chandrashekaran, 1987).

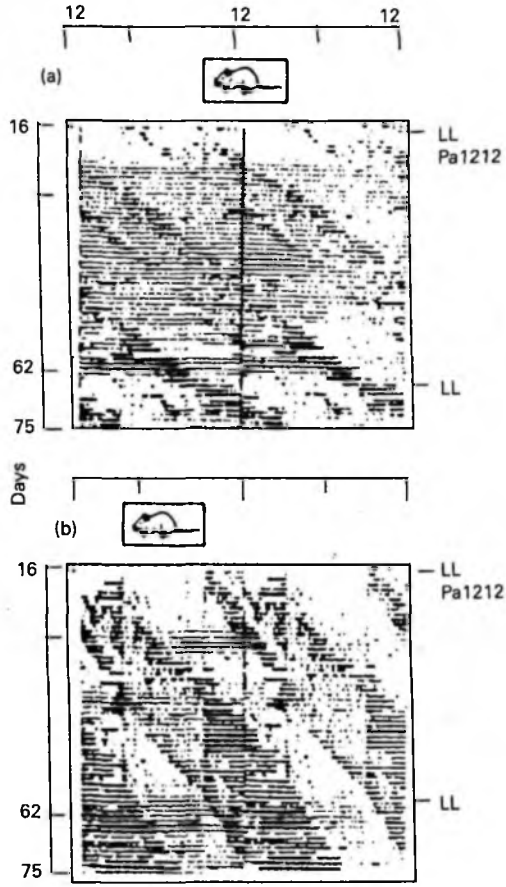

Fig. 5.10 Double-plotted wheel running activity record of *Mus booduga* pups A and B exposed to PA cycles of mother in LL of 15–25 lux. The impressive maternal entrainment is abolished in LL. The activity rhythm free-runs in LL with periods > 24 h (Period of pup A = 24.61 h, B = 24.49 h). (After Viswanathan and Chandrashekaran, 1987).

This is reminiscent of a similar break down of the social synchronisation of the circadian rhythms in bats in LL. One possibility is that the PA cycles do indeed entrain the underlying circadian clock both in the case of bats as well as in the case of *Mus booduga*, but the overt activity rhythms ("hands

of the clock") are uncoupled from the clock-works. If what is happening to the circadian rhythms in bats and mice (not a far-fetched analogy, given the great *clock gene* homology between mice and men) in LL should also hold good for humans, then our findings are of paramount importance to lighting conditions in hospitals and maternity wards. The obvious conclusion is that continuous lighting must at all costs be avoided in maternity wards lest the mother's soothing influence on her (altricial) child is lost.

6. Human Circadian Rhythms

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Our thoughts and sentiments answer to the revolution of the seasons, as two cog-wheels fit into each other. We are conversant with only one point of contact at a time, from which we receive a prompting and impulse and instantly pass to a new season or point of contact.

Henry David Thoreau

❄

6.1 · Sleep and wakefulness

Even though it is a daily recurring phenomenon, and therefore a routine of life, sleep has always aroused curiosity and speculation about its nature, cause and necessity. Even today a satisfactory definition of sleep is lacking. The notion that sleep is the antithesis of wakefulness has prevailed ever since Aristotle. But it would be proper to say that more information on sleep has been gathered in the last 60 years than in the preceding 6000 years. The recent discoveries about sleep have been made possible by developments in the fields of neurophysiology and brain science. Sleep research, in turn has enabled neuroscientists, psychiatrists and sleep researchers to explore the physical basis of human consciousness.

Roughly, animals spend 1/3 of the 24 h geophysical day in rest (sleep). This is also true of humans. The duration of human sleep is known to vary with age. Infants need and do spend more time in sleep. There is vast literature on sleep most of which is discursive and descriptive. Nathaniel Kleitman (1923; 1949), in his classic *Sleep and Wakefulness* (1963) which was written in 1939 and revised in 1963 and 1987, gives 4337 references! Sleep is one topic on which many people consider themselves to be an authority because of personal interest and first-hand experience. Next only to the common cold, difficulty in sleeping is perhaps the most widespread health complaint, which can range from transient insomnia to intrusive nacrolepsy which causes sudden and uncontrollable slumber. There are well over 50 sleep or sleep-related disorders, among them, head banging, sleepwalking, nightmares, teeth grinding, kicking legs, sleep apnea and

other breathing disorders. There are hundreds of sleep clinics the world over. Sleep science is a relatively new area but burgeoning and gaining in importance rapidly. There are national societies for sleep research in Austria, Japan, China, India, Israel, Italy and many more countries and a European Sleep Research Society.

In sleep as defined by sleep researchers, the brain remains active even if it does not process information received from the receptors. Since non-mammalian vertebrates never really stop reacting to stimuli from the external world, these animals are said to *rest* and not sleep. Sleep and *electroencephalographic* (EEG) studies have been carried out for birds and many mammals. In spite of important differences in their sleep pattern, humans, rats and hamsters show similar trends in their EEG spectra (Tobler and Borbely, 1988). Behavioural sleep has been reported for Asian elephants in captivity. A Swiss scientist (Tobler, 1992) found that elephants spent several hours between 03.00 and 07.00 h in a *recumbent* position. The widely-held notion that elephants rarely or never lie down to sleep, and that they sleep while standing is apparently not true. Sleeping elephants reacted immediately to unfamiliar sounds but were little disturbed by their neighbours. Tobler investigated the sleeping behaviour of Asian female elephants (both circus and zoo elephants) for 294 nights. The animals were recorded continuously on time-lapse and vB ideotaped for 7–16 days consecutively. Sleep occurred both in recumbent and standing positions. Sleep onset occurred after 21.00 h, and increased progressively reaching a maximum between 01.00 and 04.00 h. Adult elephants slept for a total of 4.0–6.5 h.

6.2 Sleep in animals

The word 'sleep' is generally used only to describe the restful periods of higher animals, especially birds and mammals. It is now becoming increasingly clear that like the circadian organisation in the physiology and behaviour of organisms, the activity/rest cycles are also ancient evolutionary features. Kaiser (1988) reported the following behavioural and electromyographical sleep signs in the foragers of honeybees employing a variety of experimental methods: (1) reduced muscle tone; (2) decreased motility; (3) lowered body temperature; and (4) elevated reaction threshold accompanied prolonged rest in these diurnal insects. These phenomena strongly resemble the four characteristic features of sleep in humans, mammals and birds. It is thus very likely that the profound rest which forager bees experience at night is sleep. The antennae of sleeping bees show characteristic postures. High reaction thresholds are associated with particular antennal positions. The total sleep time, which Kaiser calculated from duration of antennal immobility plus duration of small antennal movements, in 24 h for two bees was 7.6 and 4.9 h. Just as sleep is an

active controlled process in mammals, the same appears to be the case for bees. Unlike mammals, bees experience deepest sleep towards the end of the sleep phase (Kaiser, 1988).

Two recent papers adduce compelling scientific evidence (Hendricks et al., 2000; Shaw et al., 2000) that the rest phase in *Drosophila melanogaster* is a mammalian sleep-like state and that the activity and rest in them correlates to sleep and waking in mammals. The results of these two laboratories have now given researchers powerful genetic tools to investigate sleep mechanisms and functions. In mammals, sleep is distinguished from inactivity both behaviourally and electrophysiologically, whereas, in invertebrates, the identification of sleep-like states depends primarily on the behavioural analysis of quiescence, increased arousal threshold and increased rest after prolonged waking. Shaw et al., (2000) could demonstrate that like mammalian sleep, an increasing arousal threshold, which is homeostatically controlled, characterises rest in *Drosophila*. Flies subjected to 12:12 h light/dark cycles exhibited sustained period of activity and rest with >90% of rest occurring during the dark period. If the flies were manually deprived of rest for 12 h, they recovered 50% of the rest that was lost in the first 24 h, a value comparable to sleep rebound seen in mammals after short-term sleep deprivation. As in mammals, rest is abundant in young flies, reduced in older flies, and is modulated by stimulants and narcotics. Caffeine increases waking and locomotor activity and antihistamines increases sleep latency in mammals. Flies given caffeine showed a dose-dependent decrease in rest. By contrast hydroxyzine, an antagonist of H1 histamine receptor, increased rest and reduced its latency. Thus two agents that modulate waking and sleep in mammals also modulate vigilance states in *Drosophila*. Results of behavioural, pharmacological, molecular and genetic investigations indicate that *Drosophila* rest shares many critical features with mammalian sleep. The identification of molecular correlates of sleep and waking that are conserved across evolution offers a new approach for studying the phylogeny of sleep. In an interesting book on "*Sleep*," in trying to emphasise the importance of a highly developed brain for sleep to occur, the author (Hobson, 1989) states "The central role of the brain in sleep can be conveyed by rephrasing Abraham Lincoln's famous declaration about government: sleep is of the brain, by the brain, and for the brain."

6.3 The electroencephalogram (EEG) and REM sleep

The first phase of modern sleep research lasted from 1928 to 1953 (Hobson, 1989) and saw the beginning of a quantum leap in our knowledge about sleep. This sudden spurt of knowledge occurred due to one major reason: scientists acquired the ability to study the brain as an intricate colony of neurons whose cooperative activity has measurable electrical and chemical properties. The modern concept of the brain as a colony is derived from the

work of Santiago Ramón y Cajal, a Spanish neuroanatomist who discovered in 1880 that the neuron was the structural and functional unit of the brain. Technical advances soon allowed scientists to measure the electrical activity of the brain and its constituent neurons. In 1928, the German psychiatrist Hans Berger successfully recorded the continuous electrical activity from the scalp of human subjects. It came as a great surprise that recurring periodically in sleep were phases of EEG activation with low voltage and fast waves that were as intense as those seen in waking. Eugene Aserinsky, a graduate student of Nathaniel Kleitman, first demonstrated (Aserinsky and Kleitman, 1953) that the so-called good night's sleep consists of two kinds of sleep: rapid eye movement or REM sleep, and non-REM sleep. This discovery dispelled the, until then, widely-held view (among others even by Ivan Pavlov) that the brain tuned down and lacked sensory activity during sleep.

Our nightly sleep begins with about 50–70 min of non-REM sleep during which the brain experiences four distinct stages, the fourth stage coinciding with deepest sleep. Then the first REM episode of the night begins. After sleep onset, the EEG changes progressively from a pattern of low voltage and fast frequency to one of high voltage and low frequency. The REM episode begins with rapid eye movements as though the subject were looking all around with the eyelids closed. The heart's rhythm flutters and the blood pressure fluctuates. Blood flow to the brain may increase up to 40%. The subject dreams vividly of running and strenuous exercise. Fortunately, these acts are prevented before the muscles translate them into real action. After about 20 min, the subjects lapse into non-REM sleep in a cycle that repeats throughout the night. The mechanism of REM generation, its physiological importance and functional significance are yet to be completely understood. Various studies conducted on rodents include stimulation, lesioning, chemical injection and REM deprivation to understand the function of REM sleep. Results of such studies reveal that REM sleep deprivation adversely affects several functions involving the brain, nervous system, mental faculty and general behaviour. The altered states return to normal with recovery of REM sleep. A combination of REM and non-REM sleep appears to be a necessity for alertness and a feeling of well-being during the subsequent phase of wakefulness. Dreams are associated with this stage of sleep and hence it is also known as dream sleep. Although the REM sleep has been identified across species, all the identifying characteristic signs may not be uniformly expressed in different species in the evolutionary ladder. Until recently, REM sleep was considered to have evolved recently because REM was absent in the primitive mammal monotremes (Allison et al., 1972). However, with the recent findings that the REM sleep-like state is present in the platypus, an egg-laying mammal, considered as one of the earliest mammals, it is likely that REM had evolved more than 100 million years ago (Siegel et al., 1997).

6.4 Sleep at birth

On the basis of the long-held assumption that ontogeny (development of the individual) recapitulates phylogeny, one would expect that slow-wave sleep (non-REM) arises earlier in human development than REM sleep. In reality, however, REM sleep in human development is the earliest recognisable sleep state, while non-REM sleep develops much later. This may be because the brain stem structures causing REM sleep occur early in intra-uterine life, whereas the systems necessary to non-REM sleep mature only after birth. One must remember that the human infant is not fully mature at birth in contrast to the newborn anthropoid ape. In this sense, the human infant is "altricial," needing maternal attention. The relative proportions of each 24 h day that are devoted to wakefulness, REM sleep, and non-REM sleep change dramatically over our lifetime. Newborn babies have more REM sleep than adults. Thus, we can witness in infants fast asleep, a surprising range of facial expressions – grimaces, vocalisations, stretching and clutching of the hands and reactions of the whole body resembling startle responses. Watching a sleeping infant is the easiest way to *see* REM sleep and appreciate the brain activation involved. Apparently such observations were what helped Eugene Aserinsky to discover REM sleep.

Much of our knowledge about the development of sleep in the later months of gestation comes from prematurely born human infants. Infants born at 24–26 weeks, who usually do not survive, writhe continuously. This early state of activation will later become REM sleep. The capacity to sleep through longer periods is developed in the weeks after birth. The entrainment of the sleep/wake rhythm becomes the major task for the mother and infant in the first three months of life. Kleitman and Engelmann (1953) stated on the basis of results of an early experiment on this subject that "this infant began its adjustment by developing a twenty-five hour periodicity. From the middle of the eighteenth to the end of the twenty-first week, the consolidated long sleep hours appear stationary, indicating that the diurnal periodicity is now down to the astronomically correct twenty-four hours."

6.5 Sleep in different stages of life

Early childhood (age 1–5) By age 5, the polycyclic pattern of early life is completely left behind for a diurnal one. The child sleeps for 10–12 h and may additionally have an early afternoon nap. A nap at this time prevents the irritability and hyperactivity into which children lapse from about 4 to 6 p.m. In response to demands from adults and the natural trauma of growing up, completely normal children may wet their bed, sleepwalk, talk in their sleep, or have nightmares.

Sleep in adolescence (age 12–18) Adolescence is the time of life when

both sex and social interactions become very important – sexual feelings and scenes begin to occur in dreams. Teenage males begin to experience so-called "wet dreams". This complex phenomenon may help us to understand the relationship of the mind and the brain. That sexual feelings which can be consciously controlled in the state of wakefulness, but which in sleep can produce erections and culminate in ejaculation in males without external stimulation, is compelling testimony to the powers of REM to mimic the sensorimotor aspects of waking life. In almost all cultures, adolescents tend to stay up later at night, sleep later in the morning and build up huge sleep deficits which they clear over weekends with prodigious bouts of sleep.

Sleep in early adulthood (age 18–30) Young adulthood is the time in life when individuals may first notice – and complain about – the way in which their own sleep differs from the norm. By the time a person is 30, he or she is usually aware of a personal style of sleep and feels more or less comfortable with it. But courtship, marriage, and childbearing bring a host of variables to the picture; not surprisingly many of the sleep problems that emerge at this stage arise from crying babies and snoring spouses.

Sleep at the age of 30 to 45 *At this stage in their life many people experience* a shallowing of sleep and a shortening of its duration. The episodes of waking up increases and it takes longer to fall asleep. This is also the stage in life when most people go in for a change in life style often becoming in the process more sedentary. Frequent intake of tea or coffee (stimulants) and intake of more alcohol (relaxant) has mixed, often adverse, effects on sleep.

Sleep in late middle age (age 45 to 60) Loss of hormones, a characteristic of this phase, may accelerate reversal of sleep trends seen in adolescence. Some people stoically accept the bothersome arousals that begin to occur generally after only three hours of sleep and turn to reading, writing or listening to music before going back to bed. With the decline of stage IV sleep, there is a corresponding increase in the lighter stages of non-REM sleep. We lie neither asleep nor fully awake. The nature of the work we do and the demands of our professions must surely impact on the quality of sleep in people of late middle age, but very little quantitative data is available on this subject.

Sleep in old age (age 60+) Sleep duration tends to decrease at this stage; it also tends to become shallower. But there are many old people who sleep well and enough. It is well to remember Wilse Webb's caution that the duration of sleep stages may be consistent from night to night for any given subject but differ from subject to subject. It is arbitrary to make generalisations about age groups in the matter of sleep. Very often the sleep mechanisms governing the kind and amount of sleep each person gets are determined by the person's constitution.

6.6 The functions of sleep

Most scientists are convinced that sleep helps both energy conservation and information processing, but we do not yet have an objective physiological measure of our subjective sense of restoration and improved mental alertness after a night of sound sleep. The function of human sleep has still remained an enigma. There are theories galore such as the heteroplastic theory (innate vs. learning), maintenance theories, learning theories and the "sleeping to forget" theory. Francis Crick and Graeme Mitchison have proposed that REM sleep serve to remove undesirable data from memory. They suggest that just as it is important to reinforce certain associations and the network of neurons encoding them – it is equally crucial to weaken others. The psychological fact that dreams are so difficult to remember suggests to Crick and Mitchison that the process might have been designed to erase rather than strengthen certain memories.

6.7 Circadian rhythms and shift work

Shift work, i.e., working at odd hours is becoming widespread for social, technological and economic reasons. Data on the physiological rhythms of shift workers who live on abnormal time routines have been the source of much information on human circadian rhythms. Most studies in the West on shift workers had focused on illnesses. The practical implications of circadian rhythms for night work first became the subject of intensive investigation during the 1914–1918 war. In Britain, a Health of Munition Workers Committee found that with fortnightly changes of shift, absenteeism was less and output higher during the first week than in the second week. In a final report, the same committee felt that there was no significant difference in output between men on alternate day and night shifts as compared with those on continuous night work (Conroy and Mills, 1970). Circadian rhythms are of obvious interest in trans-time-zone air travel, to submarine crew and astronauts. In spite of the fact that human night work has been prevalent since the Roman times it is only in the decades after the Industrial Revolution that it became common. Many industrialised countries introduced and adopted the *shift work* system to optimise utilisation of human resources and to ensure continuity in operation of industries (Pati et al., 2001). It is estimated that at present nearly 1/5 of the global work force works in shifts. Quality in the current day lifestyle demands round-the- clock service from various indispensable sectors such as public health, transport, security (both internal and external), communication and media. Thus shift work has become a routine feature of life in the 21st century and this scenario is unlikely to change if the present pace of growth and development in industries are to continue.

Several scientists have investigated the problems of shift workers in relation to three important contexts and modulatory factors, namely, circadian rhythms, sleep and social/psychosocial/domestic factors. These

factors are considered to be important in determining the coping ability of a person to shift work. Besides these, there may be many more factors yet to be identified. All these factors interact with each other and influence the worker's tolerance to shift work. There are many people who are intolerant to shift work. Some kinds of intolerance are job-specific while others depend upon the internal constitution of the individual.

6.8 Intolerance to shift work

The severity of clinical problems may vary among individuals. On the basis of the intensity of medical complications, Alain Reinberg classified workers having good tolerance (with neither complaints nor medical problems), poor tolerance (with medical complaints) and very poor tolerance (severe clinical problems). Some researchers believe that clinical intolerance to shift work is independent of the individual's age and duration of shift working experience. On the contrary, there are some authors who believe that aging is associated with a decreased tolerance to shift work, the critical age being on an average 40–45 years. When very poor tolerance to shift work is encountered by some individuals, the situation may lead to severe disruption of their circadian rhythms in their physiological processes. This disruption of circadian processes may result in a condition that Aschoff (1965) called "internal desynchronisation" in which the circadian organisation, in the sleep/wakefulness on the one hand and in the rectal temperature profile on the other, split and the two rhythms free-run with different periodicities. An example of internal desynchronisation in a very poor tolerant shift worker (male aged 31 y) who had shift work experience of 3 years is described here (Reinberg et al., 1984; 1988): both day-to-day peak time locations and power spectra of the shift worker show that oral temperature, right- and left-hand grip strength had non-24 h periods while the sleep-wake rhythm had a period of 24 h. In another study, internal desynchronisation appeared in a subject (male of age 39 y) who had good tolerance to shift work and had been shift working for 14 years. The rhythms in sleep-wake, oral temperature and left-hand grip strength had a period of 24 h while right-hand grip strength had a non-24 h period (Pati et al., 2001). These studies of Reinberg et al. (1988) clearly reveal that both internal desynchronisation of circadian rhythms and development of intolerance do not depend on duration of shift work experience. More intensive studies are needed to evolve a generalization. However, Motohashi (1999) as well as Pati and Saini (1991) and Chandravanshi and Pati (2000) confirmed the phenomenon of internal desynchronisation among intolerant shift workers dwelling in Japan and India, respectively. Night shift workers are more prone to sleep disorders like insomnia. This abnormality is characterised by difficulty in falling and staying asleep. It has been shown that a 72 h sleep-deprivation does not obliterate circadian rhythms. However, while parameters of physiological rhythms (adrenaline, body temperature) remained unchanged, there was a decreasing trend in performance (logical reasoning,

calculation skills) and an increasing trend of self-rated fatigue and sleepiness. In addition to performance decrements, chronic sleep deprivation, may lead to many other clinical complications. It has been reported that total sleep deprivation may lead to devastating consequences, such as death as reported in non-human primates (Rechtschaffen et al., 1983).

6.9 Results of the MKU experiments

Circadian rhythm research has understandably addressed itself to sleep/wake patterns in humans held in experimental social isolation without time cues for long durations. Data have accumulated on the sleeping times in humans living in caves or in experimental isolation bunkers. All researchers are in agreement that under cave and bunker conditions the sleep/wake rhythms free-run with periods longer than 24 h showing values in the range of 24.5–26.5 h. In rare cases, both in the cave and bunker situations, human's subjects showed sleep/wake cycles having values of 42–48 h. Such rhythms have been labeled *circabidian* (Wever, 1979). Some individual findings of early experiments conducted inside caves are worth recounting for they appear to be scientifically sound even today. A subject called Workman went into a cave wearing a wristwatch with the intention of following normal timings for eating, sleeping and waking. After a week or two of isolation, Workman found it increasingly difficult to do so, being unable to sleep at night, and sleeping late in the morning. After 3 weeks he abandoned his intention, and went to bed when he felt so inclined adopting a 24.7 h cycle. Knowledge of time was obviously inadequate and an endogenous periodicity of ca 25 h forced him to modify his habits. N. Kleitman and Bruce Richardson, two Americans, lived for 32 days inside the mammoth caves in Kentucky and clocked 28 h free-running days. The first well-maintained record of a prolonged stay in a cave without a watch is that of Siffre. He stayed 61 days in a cave and reported the time of his retiring for sleeping and waking up to experimenters at the surface by means of a telephone. His sleep-wake rhythm had a period of 24.5 h (Conroy and Mills, 1970). Estimates of the period of sleep/wake cycles for the cave dwellers studied by different authors are

Subject	Period of sleep/wake (h)
Siffre	24.5
Workman	24.7
Laures	24.6
Senni	24.8
Lafferty	25.0
Maretaet	24.6

Source: Conroy and Mills, 1970.

When humans are held in experimental social isolation they are known to consistently *underestimate* passage of time and therefore show sleep/wake rhythms of 24–26 h. Aschoff was the first scientist to have investigated circadian rhythms in humans held in a 'time-free environment' for extended periods. His first experiment was on himself when in 1961 he chose to live in a deep cellar of a University of Munich hospital, which had served as an air raid shelter during the war. He published the results of this experiment in 1962 (Ascoff and Wever, 1962) which clearly indicated that the circadian rhythms in sleep/wakefulness and meal timings free-ran with a period >24 hours. This encouraged him to have the first human isolation facility constructed for the proper study of circadian rhythms in humans, on a hillside at the Max-Planck-Institute for Behavioural Physiology in Erling. One of the most significant findings to emerge very early from his studies, was the so-called internal desynchronisation between the rhythm of sleep/wakefulness and the rhythm of the rectal temperature (Aschoff, 1965). This phenomenon argued that at least two different oscillators underlie the circadian organisation in humans.

Of the more than 150 human subjects investigated by Aschoff and Wever for properties of free-running circadian rhythms in humans only two subjects showed periods "that might be shorter than 24 h." Aschoff presided over and performed experiments in this, the most celebrated human isolation facility of its kind in the period 1964-1985. By 1976, he and Wever had already performed 205 experiments, 184 of these on singly isolated subjects, 18 experiments with two subjects and 3 experiments with four subjects (Wever, 1979). Aschoff continued to publish original papers on data obtained from the bunker and in the period between 1990 and 1999 published over 22 papers on the subject (Daan, 2001). The human isolation facility at Andechs is now not functional since the retirement of R Wever, ca. 1990.

Davy reported in a paper to the Royal Society, a daily rhythmic variation in body temperature in 1845, noting that his body temperature was higher during the day and lower at night. Baerensprung (1851; 1852) took records of his body temperature during the day and night and fixed the time of lowest body temperature as 04.00 h and of maximum as being between 18.00 and 19.00 h. Kleitman and Ramsaroop (1948) reported a daily periodicity in body temperature and heart rate. Aschoff (1955) described the diurnal time course of body temperature in humans. It was also Aschoff and his colleagues who firmly established that the body (rectal) temperature in humans in social isolation free-runs with a period longer than 24 h (Aschoff et al., 1967a and b; Aschoff and Wever, 1962). There are two states in which the two major rhythms (1) the sleep/wake rhythm and (2) the rectal temperature rhythm, express themselves during free-runs. In one state they are coupled or "internally synchronised" high peaks coinciding with middle of activity (wake) time and the low troughs coinciding with rest (sleep) midpoint. The other state occurs when the sleep/wake rhythm becomes nearly circabidian and the rectal temperature free-runs with conservative periods close to 25 h.

I have investigated human circadian rhythms in young subjects held under prolonged isolation for periods of 21–43 calendar days in an isolation facility, specially built for the purpose in the Department of Animal Behaviour and Physiology of the MKU. Experiments in this facility were carried out on 14 human subjects in 16 long-term experiments in the period 1987–1996. Most of the figures reproduced here illustrate data obtained by us in our decade-long experiments. The facility consists of a living room of 25'×25' dimensions with a false ceiling. There were two walls on all four sides with a 10" space which was filled with sand to make it a fortification impervious to noise/sound (bird songs etc) from outside. There was a chute attached to the facility, where food and other requirements were placed. When one of us entered the outer chute, a red light within would come on warning the subject not to open the door, to avoid social interactions. Even though there were intercom telephone facilities the subjects were encouraged to communicate by means of written messages. The outermost room was the instruments room with two one and a half ton air-conditioners which could cool the air to 18° to 19°C. This pre-cooled air was then sucked into the fortified living area by means of ducts fitted with fans and sound mufflers to cool it to a comfortable 25°C. In view of the vagaries of electricity supply in the MKU campus the isolation facility was additionally linked to dedicated diesel-operated generators. The facility was also well-ventilated for some of the subjects preferred to cook their own food. Air fresheners were available to the subjects at all times as also electric hotplates, utensils, a refrigerator, fresh fruits, toilet facilities, bathroom, twin beds, a writing table and chairs, an easy chair, bicycle ergometer and exercising dumbells. The subjects could listen to taped music and had plentiful reading and writing material on hand. A stop watch was available for short interval (2-minute) estimations.

We recorded several parameters such as sleep/wakefulness, rectal temperature, timing of toilet visits, taking bath, breakfast, lunch, supper, snacks, nap, presumed 2-hourly blood pressure measurements, exercise time etc. Each time the subject performed a task, he/she pressed the appropriate button on a panel which would make an electrical contact and deflect the assigned channel of a 20-channel E/A event recorder. All the data were entered in a notebook by G Marimuthu on a day-to-day basis. Body temperature and wrists movements were continuously picked up by a 'solicorder' machine weighing just 500 gm worn at the waist by the experimental subject. One probe had a movement sensor which the subject wore on the left wrist and the other had a temperature sensitive thermocouple which was inserted into the rectum. The data were impressed on an interface card which was replaced every week and printed out by an Apple II e computer.

It must be pointed out that working on human circadian rhythms is a scientifically strenuous and demanding undertaking. Experiments on humans can be carried out only after a duly constituted Medical Ethics Committee satisfies itself that the experiments are non-invasive and clears them. The subjects must be screened to ascertain that they are not averse to being in

social isolation. Endogenous depressives must be avoided. Since the experimenters cannot see the subjects in isolation they can only infer their activities as reported by them. These constraints may explain why so few laboratories in so few countries undertake experiments of this kind. Even so research on the circadian rhythms in humans under stringent conditions of social isolation are not only important and of theoretical interest but are also turning out to be of great practical importance.

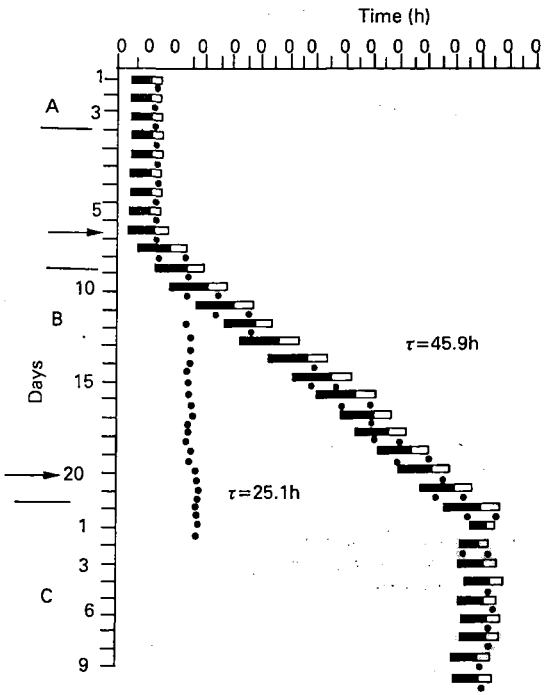

Fig. 6.1 Consecutive sleep (white bars) and wakefulness (black bars) rhythms and rectal temperature rhythms (solid circles indicating minima) of a 24-year-old female living through pre- and post-isolation days (A) and (C) and under socio-temporal isolation in self-selected LD conditions (B). The experiment lasted 47 (3 + 35 + 9) calendar days. The two rhythms began to desynchronise from subjective day 11 onwards. The values of the period of both rhythms are given in the text as means ±SD. The sleep–wake rhythms during isolation are plotted in subjective days, whereas the rectal temperature rhythms are plotted on both calendar and subjective day scales. The 00.00 to 00.00 h on the abscissa are measures of 24 h. The mean values of the periods 45.9 h and 25.1 h indicated periods for sleep-wake and temperature rhythms, respectively, and are measured from subjective days 11 to 22. (After Chandrashekaran et al., 1997).

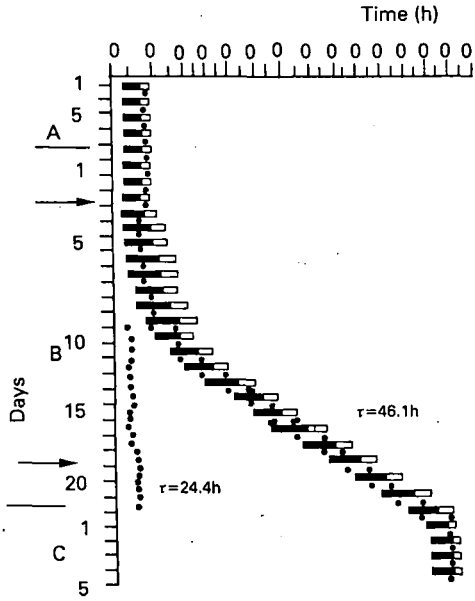

Fig. 6.2 Sleep and wakefulness and rectal temperature of the same female, as was employed in the first experiment (Fig. 6.1) at the age of 26 years. Rectal temperature during the pre-isolation days (A) could not be recorded due to a technical snag in the solicorder. The experiment lasted 44 (7 + 32 + 5) calendar days. The mean values of the periods 46.1 h and 24.4 h indicated periods for sleep–wakefulness and temperature rhythms, respectively, are measured from subjective days 9 to 21. Other details as in Fig. 6.1 (After Chandrashekaran et al., 1997).

The MKU-facility was manned day and night by research students and technicians giving the subject in isolation a sense of security. Subjects were requested to terminate experiments and walk out, any time of day or night, if any discomfort is felt. In fact before the start of the experiment, the subjects spend at least one night and a day to acclimatize themselves to their experimental surroundings. No experiment had to be prematurely terminated except in one case in which the subject developed fever and was asked to come out. In all our experiments subjects experienced self-selected LD cycles and vouched for the fact that they found their experience exciting and comfortable.

The sleep/wakefulness pattern of a 24-year female subject who spent a total of 35 days (4 May–8 June 1989) in social isolation is reproduced in Fig. 6.1 (Chandrashekaran et al., 1991).

Wakefulness time is represented by the black bars and sleep by the open

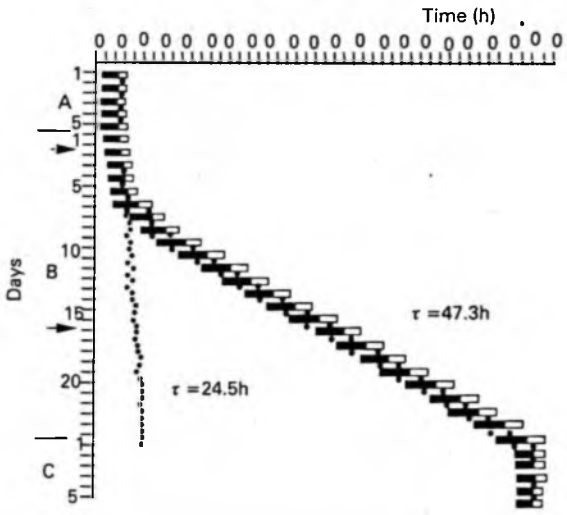

Fig. 6.3 Sleep–wakefulness and rectal temperature rhythms of the same female as was employed in the previous two experiments (Fig. 6.1 and 6.2) at age 28 years. Rectal temperature could not be recorded on the 5th day of pre-isolation (A) and on the first two days of isolation owing to a technical snag in the solicorder. The experiment lasted 53 (5 + 43 + 5) calendar days. The mean values of the periods 47.3 h and 24.5 h indicated periods for sleep–wakefulness and temperature rhythms, respectively, and are measured from subjective days 6 to 24. Other details as in Fig. 6.1. (After Chandrashekaran et al., 1997).

bars The abscissa gives time in days 0–0 h describing a 24 h day. The sleep/wake pattern of the subject was recorded for 3 days before (A) and 9 days after social isolation of the experiment (C). Using the solicorder, the activity and rest patterns and the rectal temperature profiles were also measured. The subject lived in the women's hostel before and after social isolation. The dots reproduced in the figure indicate time of minimum body temperature. It is clear that during the pre- and post-isolation periods, the sleep/wake and rectal temperature profiles of the subject had periods of 24.0 h each indicating that these rhythms are entrained to 24.0 h of the normal day. During the period of prolonged social isolation of this experiment, the subject underestimated time very severely and went to sleep 22 times and woke up 22 times in 35 calendar days. The subject entered the HIF on May 4, 1989 and kept a log book and entered the presumed date on waking up in every cycle. Since the subject had severely underestimated time and had as a result experienced only 22 subjective days in her data notebook, she had indicated the date of the termination of the experiment as being 26 May 1989 when it was in reality 8 June 1989. As may be seen from Fig. 6.1, the sleep-wake

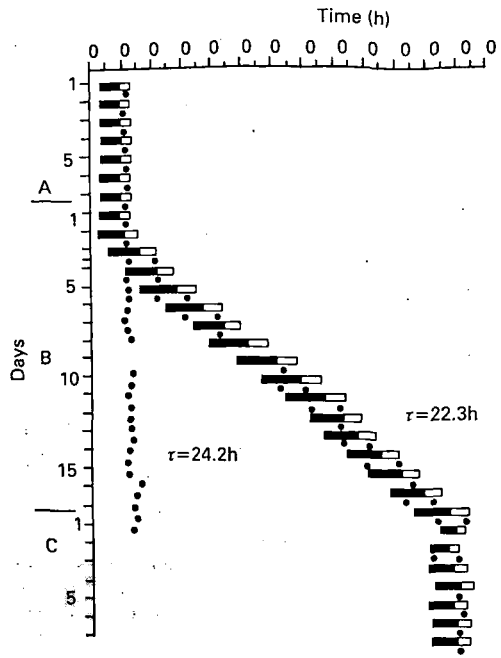

Fig. 6.4 Sleep–wakefulness and rectal temperature rhythms of a 25-year-old male. The experiment lasted 42 (7+28+7) calendar days. The mean values of the periods 42.3 h and 24.2 indicated periods for sleep-wake and temperature thythms, respectively, and are measured from subjective days 5 to 17. Other details as in Fig. 6.1. (After Chandrashekaran et al., 1997).

rhythm during the 35 days' social isolation (B) begins to free-run and on *subjective* day 11, the rather conservative rectal temperature rhythm and the sleep-wake rhythm become internally desynchronised. The average period of the sleep/wake rhythm in the free-running state was 45.9 h. On certain days the subject remained awake for 32 h and subsequently slept for 16 h. She therefore showed a sleep-wake period (16 + 32 = 48 h) of 48.0 h which is technically called a circabidian rhythm. Such a phenomenon has been demonstrated, besides in Aschoff's human subjects, only for the activity/rest pattern in a squirrel monkey. We obtained instances of free-runs in sleep-wake rhythms becoming circabidian, and therefore resulting in a state of internal desynchronisation, between the sleep/wake rhythm on the one hand, and the rectal temperature rhythm on the other, in *four* out of our 16 experiments. Figures 6.2–6.4 illustrate data from the other three experiments.

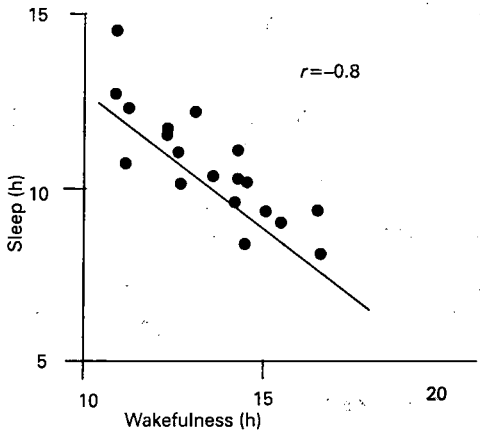

Fig. 6.5 Negative correlation between sleep and preceding wakefulness obtained from data on a 35-year-old female subject. Her sleep–wakefulness and rectal temperature rhythms, unlike in the earlier four instances, remained internally synchronised during the entire period of the isolation, which lasted 20 calendar days. (After Chandrashekaran et al., 1997).

The marathon experiments, data from which are contained in Figs. 6.1, 6.2 and 6.3, involved social isolation for periods 35, 32 and 43 days, were performed on the same female subject in 1989, 1991 and 1993, which, as we have pointed out in one of our publications (Chandrashekaran et al., 1997), is a record in human circadian rhythm research. In Fig. 6.4 are reproduced sleep/wake and rectal temperature rhythms obtained on a 25-year-old male in an experiment that lasted 42 (7+28+7) calendar days. In this experiment also, a big disparity in the period lengths of sleep/wake (42.3 h) and rectal temperature (24.2 h) developed resulting in the 'internal desynchronisation' of the two rhythms.

Normal human sleep/wakefulness has roughly a 1:2 ratio. Interestingly a similar relationship continues even when the sleep/wake rhythms free-run in experimental social isolation. Aschoff demonstrated (Aschoff et al., 1971) that the sleep and wakefulness in subjects in his bunker were *negatively* correlated (Chandrashekaran *et al.* 1997). We found such a correlation only in one instance, which is reproduced in Fig. 6.5.

The subject was a 35-year-old lady and unlike the other subjects was an outsider with no knowledge of chronobiology. It must be emphasised here, however, that neither knowledge of chronobiology nor of probable outcome of results appear to influence human circadian rhythms data obtained in any laboratory until now. Most of Aschoff's human subjects, although they showed circadian free-running rhythms in sleep/wake and rectal temperature

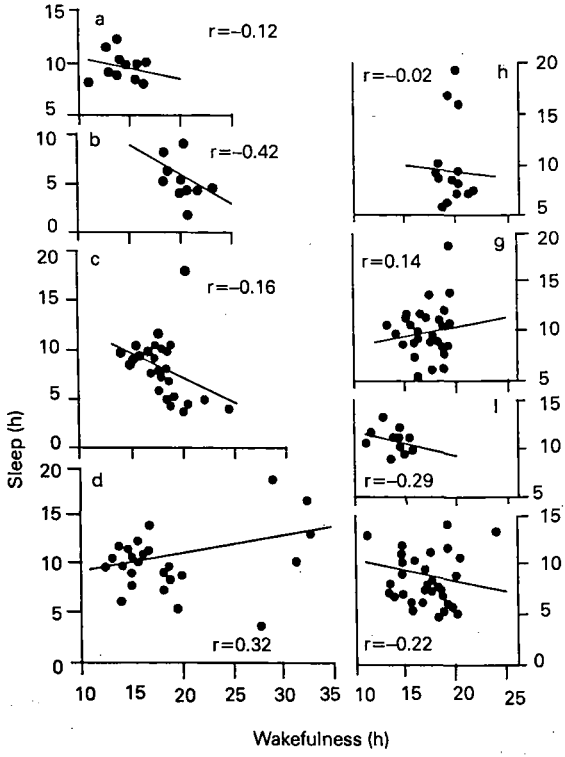

Fig. 6.6 Correlations that are *not* significant between sleep and preceding wakefulness data obtained from 8 human subjects (6 males and 2 females). The sleep–wakefulness and the rectal temperature rhythms , in all eight subjects, remained internally synchronised during the entire period of isolation of the experiments , which ranged from 14 to 35 calendar days. (After Chandrashekaran et al., 1997).

rhythm, remained internally synchronised. But this happened only in 8 out of our 16 experiments and no rigid correlation could be found between sleep and wakefulness. This is illustrated in Fig. 6.6.

The female subject whose sleep/wake correlations are presented in Fig. 6.5 was internally synchronised in respect to sleep/wake and rectal temperature rhythms. But something very interesting happened in the four experiments described here. As Figs. 6.7, 6.8, 6.9 and 6.10 show the sleep and wakefulness in the subjects in all four experiments showed a *positive* correlation.

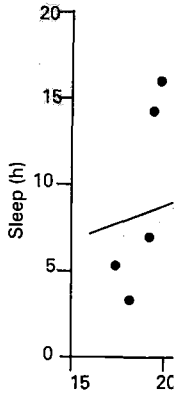

Fig. 6.7 Positive correlation between sleep and preceding wakefulness of a 24-year-old female subject during the period of isolation. Details from raw data illustrated in Fig. 6.1. The solid line shows the regression. *r* is the coefficient of correlation. (After Chandrashekaran et al., 1997).

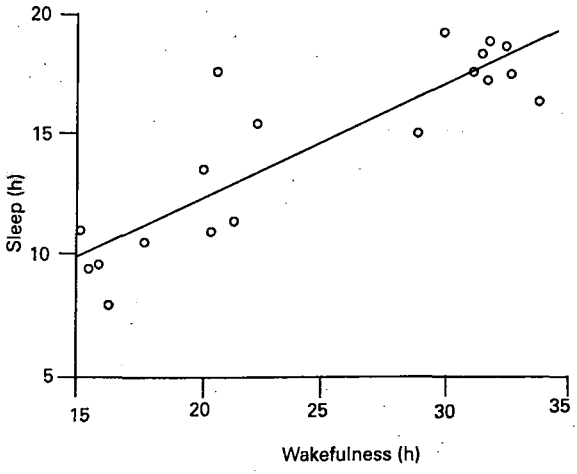

Fig. 6.8 Positive correlation between sleep and preceding wakefulness of a 26-year-old female subject during the period of isolation. Details from raw data illustrated in Fig. 6.2. Other details as in Fig. 6.7 (After Chandrashekaran et al., 1997).

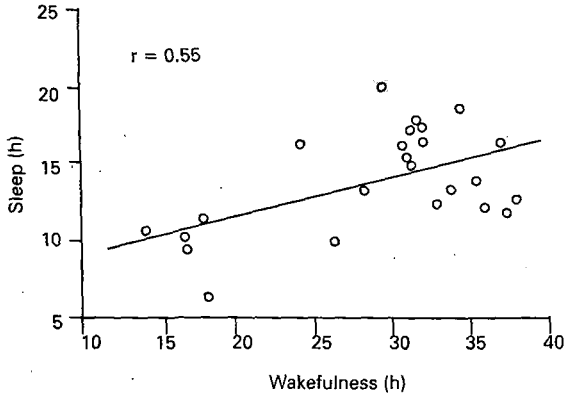

Fig. 6.9. Positive correlation between sleep and preceding wakefulness of a 28-year-old female subject during the period of isolation. Details from raw data illustrated in Fig. 6.3. Other details as in Fig. 6.7. (After Chandrashekaran et al., 1997).

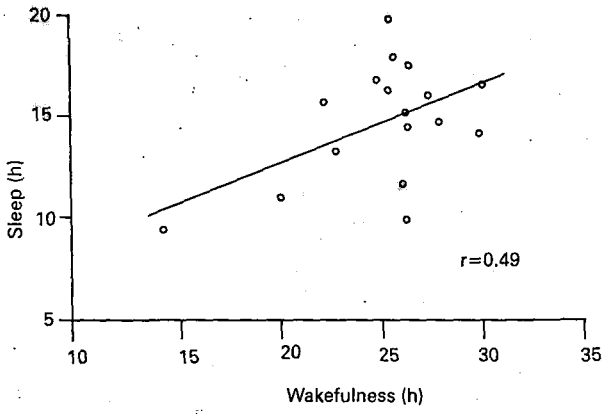

Fig. 6.10 Positive correlation between sleep and preceding wakefulness in a 25-year-old male subject during the period of isolation. Details from raw data illustrated in Fig. 6.4. Other details as in Fig. 6.7. (After Chandrashekaran et al., 1997).

Since in all four cases the sleep/wake rhythm and the rhythm in the rectal temperature had internally dissociated (unlike Aschoff's subjects) we have attributed this reversal in the quality of correlation to the phenomenon of "internal desynchronisation." It is also important to keep in mind that the data were obtained on two subjects, one a female and the other a male, which is inadequate in terms of sample size and statistics. But the phenomenon was interesting in itself and might have physiological implications and therefore clearly warrants further scientific study.

It must be pointed out that human rhythm researchers (Charles Czeisler and colleagues at the Brigham and Women's Hospital, Harvard Medical School in the USA, the most active team of medically-oriented researchers), also did not find consistent correlations between sleep and wakefulness in humans in isolation. The results of Czeisler and our own results also contradict the intuitive assumption that the duration of sleep is determined by prior wakefulness (Czeisler et al., 1980). Czeisler and colleagues also had wide variations in sleep duration in subjects in isolation, which they characterised as being "random." They reported variations in sleep duration, which depended on *when* (circadian phase at which) the subjects went to sleep, rather than how long they had been awake beforehand. The methodology Czeisler and colleagues use in their researches on human circadian rhythms is not the same as that followed by the team in India and in Germany. In our experiments and those of Aschoff there was absolutely no *social* contacts for the entire duration of the studies, whereas technicians and other personnel were allowed to be in brief contact with the experimental subjects in social isolation, in the researches of Czeisler et al. (1980).

6.10 Menstrual cycle not coupled to sleep/wake cycles

One important discovery we made in the course of our experiments was that the menstrual cycle in the human female is *not* coupled to the clock controlling sleep-wake cycles. It was widely believed until 1990 that a woman went to sleep 28 times and woke up 28 times between the two episodes of a menstrual cycle. The favoured hypothesis was that the 28 sleep/wake cycles and a gradual build up in the hormones involved in menstruation coincided, as in a "beat" phenomenon, once every 28 cycles to cause a menstrual episode. Thus, in the first experiment in which (Fig. 6.1) the female subject went to sleep and woke only 22 times in a 35-day calendar period, she had experienced 22 *subjective* sleep-wake cycles. Yet the two episodes of her menstrual cycle were exactly 28 calendar days (28 × 24 h) apart indicated by arrow heads in the figure. Similar results were obtained in the other two extended experiments on the same subject. The arrows in Figs. 6.2 and 6.3 indicate the two episodes of a menstrual cycle each which are exactly 28 *calendar* days apart in these experiments also (and not subjective days given in the ordinate). Our results demonstrate without any ambiguity that

the menstrual cycle in the human female under simultaneous social and temporal isolation is *not* coupled to the circadian/circabidian rhythm in sleep-wakefulness (Chandrashekaran et al., 1991). In rats it has been shown that the circadian rhythm in the rest/activity cycles are intimately *coupled* to the estrus cycle. In humans it may turn out that it is the absolute time for ovulation that determines the length of the menstrual cycle. A question which has remained unanswered is whether the rectal temperature clock, which free-runs with a period of *ca.* 24–25 h may not be involved in timing menstruation. Given the close to 24 h nature of the body temperature rhythm and the duration of menstrual cycles which can be variable in the range of 28 ± 1 day even in the same human female, it is difficult to decide in this matter with our limited data, especially in a situation where the signal may appear to be weaker than the noise.

6.11 Time estimation in social isolation

The word "time" holds different images for different people. For many of us it is always "now" that matters. The shortest duration for the perception of the present, which from a philosophical point of view may also be the perception of permanence, is about 0.11 s. The *distance* between two stimuli necessary to distinguish them as separate is even shorter: for touch it is 270 ms, for light 430 ms and so on. (One millisecond is one thousandth of a second.) If two stimuli occur closer than the specified interval, they will seem as one stimulus. These measurements are only for humans. Man becomes conscious of his "self," private self, around the beginning of the fourth year. Smaller children are not capable of comparing the duration of different events when they are not related to one another.

Another kind of time experience is the perception of *extended simultaneities* without the aid of any drug. Fischer (1967) tells the classic example of how Mozart was able to write down the famous *Miserere* of Gregorio Allegri after having listened to it only once. The ability to perceive extended simultaneities is not limited to past immortals, artistically gifted or even to the well-educated. Examples of such extended patterns have been recorded in our own time. AC Aitken, Professor of Mathematical Sciences at Edinburgh University, was able to experience the whole passage of music which would normally take half an hour, in the space of half a minute or less. Another example is that of Shakuntala Devi who, with no special training and no conscious deliberation, is able to quickly extract the 20th root of a 42 digit number or multiply figures yielding 39 digits, without hesitation and with no knowledge of how she did it.

A different brain function, which has been much studied by psychologists, is the ability of humans to estimate intervals of time. There are psychomimetic substances that are said to expand or contract one's sense of time, much like a time systole. It has often been suggested that

time sense depends on a clock which accelerates and slows with rise and fall of the body temperature. Our own (unpublished) results on this subject do not support this assumption. Short interval time estimation in humans in social isolation and in a state of free-run has been investigated by Aschoff and by my group. Aschoff reported for the first time (Aschoff et al., 1971), his entirely original observation, that 1 h intervals estimated by humans in his bunker were coupled to the duration of wakefulness (Aschoff, 1985a and b). He reported this finding in 1985 and around 1988, we observed this phenomenon ourselves. The ability in humans to measure time intervals may be assessed quantitatively in three ways: (1) estimation in which time spans are produced and the subject is asked to estimate the duration; (2) the subject is asked to indicate the passage of a certain interval; and (3) subject is asked to reproduce a presented time span.

In our experiments subjects were *not* asked to consciously measure time as such. They were asked to take "presumed" 2-hourly readings of their own blood pressure. We then noticed that subjects pressed the "time of 2-hourly blood pressure reading" button every 4–6 h on days they had remained awake for 30 h or more. When the wakefulness time was much closer to the normal wakefulness time in humans of ca. 16 h, they pressed the button (indicating timing of BP readings) every 116–124 min. The 2-h time estimations were coupled to duration of wakefulness. This is clearly to be seen in Fig. 6.11 (Chandrashekaran et al., 1991).

Aschoff found that this correlation broke down for very short intervals of 2-min estimation. We have limited data showing that there was a positive

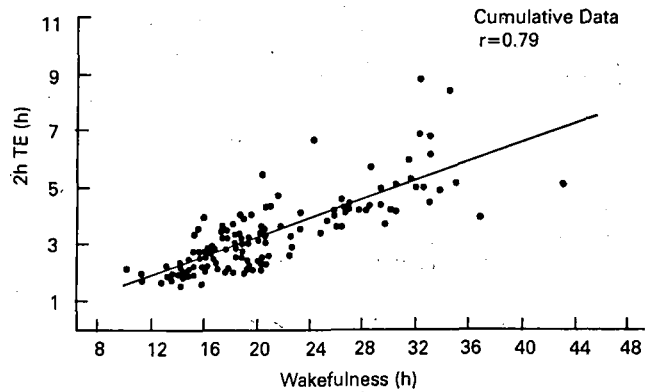

Fig. 6.11 The cumulative 2-h (time estimation) TE values (128 data points) of eight human subjects, whose sleep–wakefulness correlations are shown in Fig.6.6 to 6.10. There is a significant linear correlation between 2-h TE and duration of wakefulness. *r* = coefficient of correlation. (After Chandrashekaran 1994).

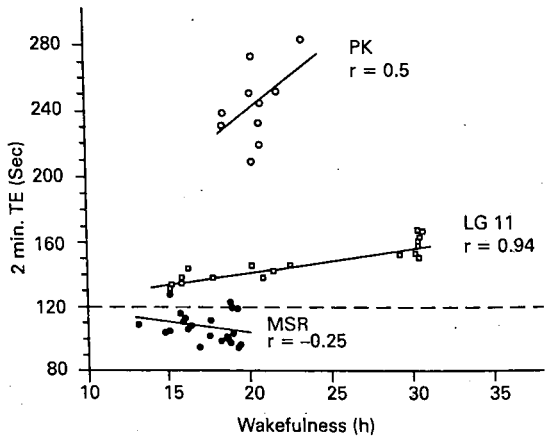

Fig. 6.12 Measurements of 2-min (120 sec) TE made by three human subjects, two males and one female, who lived in social isolation (PK for 15 days; MSR for 27 days; LG (female) for 32 days) plotted against duration of wakefulness. (After Chandrashekaran, 1994).

correlation between hours of wakefulness (alpha) and 2-min estimations. Very interestingly, this correlation between alpha and 2-min estimations did not happen in one case (PK) shown in Fig. 6.12.

It turned out that the subject had fever (Chandrashekaran, 1994). The experiment was terminated immediately. For short interval (2-min) estimations, the subjects had been asked to take the help of stopwatches. They start the stopwatch and stop it when they think 2 min have elapsed, without consciously trying to count the passage of seconds. PK in some trials waited until 4 min (240 s) for 2 min estimations. The question is if PKs' "head clock" was running faster in his fevered condition, or slower so that he perceives 2 min intervals as having lasted 4 min.? Obviously, a lot more data should be gathered on this point but for reasons which will be clear to anybody it is not easy (nor ethical) to perform such experiments in humans with fever! Hoagland (1933) first described the effects of fever on time estimation and implied that our "psychological clocks" depend on the relative speeds of our metabolic processes. However, one could speculate on the basis of the limited data obtained on the human subject PK (then a presumably healthy 26-year-old male) that clear-cut correlations between alpha and short interval time estimation may occur only in physically healthy persons (Chandrashekaran, 1994).

The 2 h estimation was so reliably correlated with alpha that even the first two or three readings for the day would give away how long the subject

was going to stay awake in that cycle. It is as though the body *knew* already at the time of waking, when it would be retiring for sleep that night. Ashoff (1990) formulated it thus "This means that the organism "knows" already at the time of waking up for how long "alpha" will last on that particular "day." Our findings and those of Aschoff go against the grain of many intuitive models that describe onset of sleep as being the function of duration of wakefulness or as a consequence of fatigue. It also repudiates the other models which made provisions for the gradual accumulation of a hypothetical *sleep substance* that slowly accumulates during hours of wakefulness to trigger sleep. Another important formulation of Aschoff (1990) was that in humans under social isolation and free-running rhythms in sleep/wakefulness the hourly means of activity were negatively correlated to alpha to such a degree that the total amount of activity per "day" remained constant irrespective of large variations in "alpha."

6.12 Meal timing in social isolation

A widely-held impression is that hunger in humans can signal meal timings. A given number of hours after breakfast, people believe they would know on their own whether it was time to have lunch and so on. As Aschoff had demonstrated, this is contrary to his findings (Aschoff, 1985b). Thus our subjects, who voluntarily preferred to have only breakfast, lunch and dinner (though snacks were allowed but had to be indicated), on occasions when they remained awake for long durations such as 30 h or so, had breakfast 1 or 2 h after waking up and then waited for ca. 14–16 h before having lunch. Translated into our workday it is as if one had breakfast at 8 a.m. and lunch at 10 p.m.! Then the subjects would wait for another 14 h before supper. In other words meal timings also *free-ran* in keeping with the sleep/wake cycle (and not the rectal temperature rhythm). So it is not really, as is widely believed, that 5–6 h after a square breakfast, our "tummy clock" will alert us to head for lunch. Neither the quality nor even the quantity of breakfast or lunch seem to directly influence meal timings (Chandrashekaran, unpublished).

6.13 Social cues and entrainment

The nature of the actual social zeitgebers, apart from the ubiquitous LD cycles, that entrain rhythms, is still not known. But the idea that circadian clocks can be reset/entrained by stimuli other than light and temperature has had early origins (Halberg et al., 1954). Halberg et al. (1954) demonstrated that blinded mice if kept in isolation in DD had free-running rhythms which entrained to LD cycles if normal sighted mice were placed in the same room. The authors postulated olfactory and auditory

mechanisms to mediate "social synchronisation" of the circadian rhythm in these mice (*Mus musculus)*. These experiments are among the earliest to be carried out in the modern experimental tradition. In mammals there have been reports both of social entrainment (Crowley and Bovet, 1980; Marimuthu et al., 1981; Mrosovsky, 1988; Erkert and Schardt, 1991) and failure to obtain social entrainment (Sulzman et al., 1977; Refinetti et al., 1992). Among the earliest experimental investigations of the problem of social cues and entrainment of human circadian rhythms was Aschoff et al.'s paper (1971) in which they had reported that human circadian rhythms entrained to social cues under conditions of DD. In this context it is interesting to recall how in the case of the colony of hipposiderid bats, social synchronisation of the circadian rhythms in the flight/rest activity of captive bats was possible only in the DD of the natural cave, and how this social entrainment was abolished and a free-run of the rhythms was caused by artificially created LL. Similarly, the mother mouse in *Mus booduga* could effect entrainment by her presence/absence cycles of the locomotor activity rhythms of her pups only in DD, and this maternal entrainment promptly broke down in LL of just 10–20 lux. The effect of light on social entrainment of circadian rhythms in animals and humans is an area open for continued research. R.Wever (1979) performed an experiment with humans inside the Andechs bunker inside which he gave 12:12 h LD cycles. The circadian rhythms of the human subjects free-ran and did not entrain. Wever then worked into the light half-cycle, one hourly gong signals. Now, the rhythms in the sleep/wake and rectal temperature of the human subjects entrained. Wever (1979) writes, "Nobody considered that the signals would contribute to the zeitgeber effect. Only when the gong failed to operate shortly after the beginning of one of the experiments, the importance of the signals was discovered. The rhythm started to free-run in spite of the persisting light-dark cycle... it is suggested that the signals operated as social contacts and that this social zeitgeber is more effective than the light-dark zeitgeber." Wever even felt that "The pure physical zeitgebers are less effective than those which include a component *perceived* (emphasis added) as social contacts. This means that the range of entrainment of the physical (zeitgebers) is smaller than those of the social zeitgebers." Wever himself did not investigate his very interesting postulate nor has anyone else repeated the "gong signal experiments." Subsequent findings on human circadian rhythms have clearly established that LD cycles entrain circadian rhythms. It is often and routinely (but unwarrantedly) held that *social cues* entrain circadian rhythms in humans. In fact, referees of papers on human circadian rhythms even object to the use of the expression *societal* day. In this context it is of interest to recall the words of Derk-Jan Dijk (1994), "In many blind subjects free-running rhythms in various variables including plasma melatonin and sleep propensity have been observed - even though these blind subjects were living in a 24 h social environment and tried to adhere

to a 24 h sleep-wake cycle. The entrainment of some blind subjects may be mediated by residual light input to the suprachiasmatic nucleus (SCN), which can be inferred from the observed suppression of melatonin after exposure to bright light.... Alternately, some blind subjects may be entrained by an as yet *unidentified social zeitgeber.* Unfortunately, the term social zeitgeber is vague and it is unclear what constitutes a social zeitgeber... in humans... no solid evidence for direct zeitgeber properties of social cues or non-photic stimuli have accumulated." A re-reading of the Aschoff et al., (1971) paper confirms that their statement, "In our experiments, social cues were apparently sufficient to entrain human circadian rhythms..." was only a postulate. The remark "Our results indicate that knowledge of the time of day, living routine, and social communication are powerful zeitgebers for the human circadian system" is also made in this paper. Interestingly, J Aschoff himself never commented in his later work on human circadian rhythms of the role of social cues in entrainment.

In one of my experiments I presented a subject whose sleep/wake rhythm was impressively free-running when her (functional) wristwatch indicated that it was noon. In a note I assured her that it was indeed noon and that her watch had not been tampered with to run faster or slower. She was encouraged to consult her watch but not live by it. Interestingly it did not make any difference and her bodily rhythms kept free running (Chandrashekaran, unpublished). The knowledge of time does *not* act as a zeitgeber and this was known since 1964 as a consequence of Workman's cave experiment on himself referred to earlier (Conroy and Mills, 1970). It may be relevant to add in this context, that our finding means that restored information of time of day from a clock/wrist watch given to a human in a state of free-run, is not a social cue. It has not been, however, experimentally tested if such information given as a 24 h recurrent input can entrain free-running human circadian rhythms.

6.14 Light and human circadian rhythms

It would be difficult for the reader to believe today that until 1980 it was erroneously held that LD cycles did not entrain human circadian rhythms. This impression was caused by the results of early experiments of R Wever in which the light intensity of his LD cycles was insufficient to entrain human circadian rhythms. We had then philosophised that since modern man does not wake up with the rising sun nor retire for sleep with the setting sun it was understandable that he was the only animal to defy entraining his circadian organisation by LD cycles. Now it is abundantly clear that LD cycles do entrain the biological clocks in humans. For a while it was recognised that bright light of 7000–13,000 lux is an effective circadian synchroniser in humans (Lewy et al., 1980; Czeisler et al., 1980; 1986; 1989) and it was widely believed that the human circadian pacemaker is

insensitive to ordinary indoor illumination of 50–300 lux. Information has come from Czeisler's laboratory (Bolvin et al., 1996) that light of even relatively low intensity (ca. 180 lux) significantly phase shifts the human circadian oscillator. This is good news for people living in temperate countries who have to live for extended periods of time in indoor lighting conditions.

Extraoptic photoreception had been demonstrated for the entrainment of the egg-laying rhythm of a grasshopper (Loher and Chandrashekaran, 1970), in amphibians. (Adler, 1969; Scharrer, 1928) birds, where the pineal has been implicated in perception of light (Menaker, 1968; Menaker and Keatts, 1968; Menaker et al., 1970), in both entrainment of circadian rhythms and in photoperiodic induction (Binkley 1971). When it comes to mammals it is almost textbook wisdom that there is no extra-retinal photoreception of any kind. Therefore it came as a surprise to chronobiologists when, in a paper published in *Science,* Campbell and Murphy (1998) claimed that the endogenous clocks of humans can be entrained by light application to an unexpected spot – the back of the knees (the popliteal region)! The magnitude of the phase shifts was dose-dependent – the product of intensity × duration. These findings were very important in the context of light therapy for adjustment of aberrant sleep–wake cycles and alleviation of winter depression. Campbell and Murphy posited a potential advantage of popliteal illumination over illumination of the eyes for therapy: It can be administered while patients are asleep. None of the available evidence suggests, however, that the circadian rhythm of adult mammals received information about environmental lighting from photoreceptors located outside of the eye. Navaneethakannan and Kumarasamy (1986) reported that the locomotor activity rhythms of the sighted diurnal palm squirrel *Funambulus palmarum* and the nocturnal field mouse *Mus booduga entrained* to natural LD conditions obtaineding close to a window in the laboratory. Daylight was on some days as bright as 15,000 lux. But after enucleation both the squirrel and mouse rhythms free-ran with period lengths shorter than 24 h and even the fluctuations in temperature and the uncontrolled noises of the laboratory and bird songs all around the laboratory failed to entrain their rhythms. Lockley et al., (1998) reported that bright light exposure (14,000–67,500 lux for 3 h) to the area behind the knee did not suppress plasma melatonin in humans. As this is being written, a paper is published categorically demonstrating that there was "no evidence for extraocular photoreceptors in the circadian system of the Syrian hamster." The authors conclude that in the absence of published experiments that suggest extraocular photoreception in adult mammals other than humans, and since there is now at least one published report of a failed attempt to confirm the results of Campbell and Murphy (1998) using human subjects they would like to believe that neither humans nor rodents have extraocular circadian photoreceptors but human circadian rhythms can be phase shifted by some uncontrolled aspect of the paradigm used by Campbell and Murphy (Yamasaki et al., 1999). It is interesting that chronobiology should still have unsolved

problems relating to even basic phenomena such as the exact mechanism of LD entrainment of human circadian rhythms.

6.15 The head clock

The ability of some people to wake up at will at an appointed hour is imputed to the influence of a hypothetical *head clock*. There are anecdotes dating back to the days of mesmerism that under hypnosis, people could count odd intervals such as 51 min. G.Clauser (1954), a physician, has written a fascinating little book on this subject. He has chronicled a few interesting eccentricities associated with waking up at will. The Bavarians knocked on their bedstead the number of times as the hour at which they wished to wake up. The inhabitants of Rhineland Westphalia stomped on their mattresses thrice if they wished to wake up at 3 a.m. In Schlesien and Thüringen one knocked on the walls of the bedroom. Farmers made as many knots in their handkerchief as the hour at which they wished to wake up and kept the knotted kerchief in their sleeping suit or under their pillow. It is still an open question if the head clock is of a quantitative or qualitative nature. Do good and bad timekeepers differ only in degree of performance or are there other features, characteristics and consequences to the phenomenon? R Weber, a psychiatrist, did the first critical experiments on the subject and published his findings in 1917. Unfortunately Weber's subjects were not reliable and often left him in the lurch, with the result that he began experimenting on himself. He could wake up at will at an appointed hour with an accuracy of ±8 min. He could do this 4–6 times in a night. Even late night parties and consumption of alcohol did not impair his performance. The precision of his head clock was the same in summer as in winter. The performance at the end of his researches showed the same degree of precision as when he had started his experiments. Although, he rightly concluded that he had not really learnt anything from the exercise. It soon dawned on Weber that since animals and plants are also known to measure time, the subject of the head *clock* might not even be a uniquely human attribute.

6.16 Circadian rhythms in human health, medicine and psychiatry

It is now well-established that over a hundred physiological functions in the human organism show a 24 h variation and that these rhythms are in synchrony with sleep and wakefulness, on the one hand, as well as the alternation of day and night, on the other. The following list of activities or physiological processes show 24 h (and by implication) circadian periodicity, which clearly indicates how circadian we humans, are:

Activity/rest cycle,
Activity of the adrenaline,
Aldosterone production,
Alertness// motor and memory skills,
Blood pressure,
Brain activity,
Ca/K/Na in urine,
Cardiovascular performance,
Catecholamines,
Cortisol,
EEG,
Endocrine activity,
Eosinophil content,
Ethanol metabolism/tolerance,
Estradiol,
Gastrointestinal rhythms,
Gonadal hormones,
Growth hormones,
Heart rate,
Insulin levels,
Noradrenalin,
Parasites,
Pineal secretion,
Pituitary activity,
Prolactin,
Pulse rate,
Saliva secretion,
Testosterone levels and
Thyroxine.

The list is not exhaustive but sufficient to hint that the human body is much like a clock shop. The applications of the results of circadian rhythm research were until recently only marginal in human medicine and welfare measures. Of the various reasons for this, the most serious was the ignorance of medical doctors of developments in the field. Medicines are routinely prescribed for intake three times a day (the tyranny of three times a day, as Franz Halberg puts it). A large body of information has already accumulated on circadian rhythms in pharmacology and in therapeutics necessitating a reappraisal of traditional habits in prescribing and attention to the *timing* of drug administration.

It is widely recognised by medical scientists, now, that the end of fever can be better gauged if temperature is measured in the morning when cutaneous heat loss is minimal. There is a disease called Cushing's disease, which results when there is a disorder of the adrenocortical function. The disorder expresses itself in the changes in plasma concentration of cortisol. Just before retiring to bed is the best time to take a sample of blood for the diagnosis of Cushing's disease. Similarly, it is well known that blood must be drawn from patients during the night to obtain microfilarial larvae, which cause filariasis. The larvae swim in the blood at night so that they can be sucked by nocturnally-biting mosquitoes and transmitted to other humans.

Chronobiology and findings in this field are increasingly gaining in importance in the treatment of diseases such as allergy, arthritis, asthma, cardiovascular diseases, endogenous depression and even cancer. The symptoms and characteristics of most diseases themselves are organised in a circadian pattern. The treatment of such diseases within the framework of circadian rhythmicity is commonly called "chronotherapy." It must, however, be pointed out that circadian rhythms still have not been demonstrated to be reliable markers of illness. Here an important law must be borne in mind: correlation must not prove causality.

It is now well-known that the world-wide number one killers are cardiovascular complications which are heavily influenced by circadian rhythms in blood pressure, pulse rate, blood clotting etc. Myocardial infarctions, heart attacks, strike twice as often in the mornings as they do during the rest of the day. Cancer is second only to heart diseases in frequency and lethality. Intensive research is going on the chronobiology of cancer therapy in the USA, Canada and France. It is now believed that certain cancer drugs in the fluoropyrimidine family, widely used in chemotherapy, are safest when most of the daily dose is given at night.

In northern latitudes, such as in Scandinavian countries, people often suffer from "winter depression" which often leads to suicide. It is suspected that the causative factor may be the extremely short days of winter. For a long time now the idea that circadian rhythm disturbances may account for endogenous depression in humans has been gaining ground. Historically, the following four clinical features of depression have encouraged the causal linking of circadian rhythms with depression:

1. Early awaking.
2. Diurnal variations in the sensitivity of symptoms.
3. Seasonality of attacks.
4. Cyclicity of illness.

Depressive hospital admissions, electroconvulsive treatments and suicides occur more frequently in winter and spring. Seasonal patterns of depression (and manic seizures) may have animal photoperiodic origins that continue as vestiges in humans.

Engelmann (1972) first reported that lithium ions slowed down the biological rhythms in the petal-opening rhythm of *Kalanchoe blossfeldiana* and the rhythm in the running activity of the mouse. It is speculated that endogenous depressives may be humans whose circadian rhythm is *out of phase* with the 24 h day outside. Thus, for example, a subject whose endogenous rhythm is 23.5 h would gradually be out of synchronisation and may slide into a state of depression. Endogenous depressives have been traditionally treated with lithium salts. If the lithium succeeded in slowing down the clock of the hypothetical depressive by 0.1 h (6 min) per day, he should be able to regain social synchrony after *five* days of lithium treatment. In clinical situations, patients are known to snap out of depression very suddenly and dramatically. In the Department of Psychiatry at the University of Tübingen in Germany, Burkhardt Pflug and colleagues discovered that endogenous depression could be alleviated in some instances by *sleep deprivation* for just one night. The patient feels as a rule much better the next day. In case the positive effect sleep deprivation has on the state of the mind of the patient wears off, renewed sleep deprivation might be undertaken a couple of days later. To speculate from the angle of biological clocks, sleep deprivation may reset the patients' clock to the 24 h temporal order of the environment much like a LD inversion does. There are many laboratories in the world where these matters are topics of intense research.

7. Looking Back

❄

As you set out for Ithaka
Hope your road is a long one
Full of adventure, full of discovery.

C.P. Cavafy

❄

This book is a collection of most, but not all, of my research. I have worked additionally with circadian rhythms in palm squirrels, on the courtship behaviour of grasshoppers and even on sleep movement rhythms in the cotton plant. By sheer chance, I was fortunate to have found at the very beginning of my career, as explained in Chapter 2, the kind of science that would hold my fascination for the rest of my working life. My friends at home and abroad persuaded me to write down my experiences in the pursuit of science – they said that my life and times would fascinate young researchers, especially those working in so-called Third World countries. I am therefore including in this book, a brief sketch of the events that paved the way to where I am now – not to attract attention but as a desire to inspire young students who find it hard to go on when circumstances are not conducive to research.

Many fortuitous events helped me along. I was fortunate enough to begin my college career in St Aloysius' College in Mangalore, a good Jesuit institution and complete Bachelor's and Master's degrees in Presidency College, Madras, a college thirty yeas older than the University of Madras. In 1960 I registered for the Ph D degree under the supervision of one of the greatest Indian zoologists of the last century, Dr CP Gnanamuthu . That was the year in which the Cold Spring Harbor Symposium met to deliberate on *Biological Clocks*. I met Ron Konopka, a student of Seymour Benzer, at Stanford University within weeks of their discovery of the first clock gene and had the singular honour of being a postdoc of Erwin Bünning, the biologist who first experimentally demonstrated the genetic basis of circadian rhythms, assigned for them an adaptive role in the context of photoperiodism, and wrote the first monograph on the physiological clock in 1958. I was also lucky enough to personally know Jürgen Aschoff (he unravelled the physiological mechanisms that regulate circadian rhythms in birds, mammals and humans, and defined the behaviour of circadian rhythm parameters in

light-entrained and free-running states in what has been called the Aschoff's Rule) and Colin Pittendrigh who brought to chronobiology a degree of experimental elegance and rigour which remain unsurpassed.

Dr. Chinnadurai Pittendrigh Gnanamuthu (1900–1969) was a very versatile zoologist who, at the time (1946) Dr AL Mudaliar the Vice-Chancellor of the University of Madras discovered him and offered him the Chair in Zoology at the University of Madras, was the only Indian head of a department of the American College at Madurai. He was also concurrently the Director of the Zoology Research Laboratory, situated in a (then) beautiful location close to the Cooum estuary and the Arabian Sea. He was a self-taught man and had obtained the DSc. working on flank movements in frogs and reptiles during respiration. He had also published over 150 single-author papers on the architecture of the fossil Foraminiferan shells (protozoa), all illustrated with exquisite pencil drawings. He was sought after as an external examiner in all the major universities of the country and routinely published his papers in the *Proc Royal Soc (London), Nature (London), J Exp Biol.* and *J Exp Zool.* He was a fellow of the Zoological Society of London and the Indian Academy of Sciences. A rather shy and private person, he was a devoted teacher, a genuine scientist and a conscientious mentor. He worked long hours very hard, and expected his students to follow suit. He was widely feared for his short temper but I knew him as a kind teacher and he appreciated the fact that I knew what was expected of me (my working day and night in the aquarium must have been an added factor!). Early in our acquaintance, he gifted me about thirty publications of Frank Brown Jr., and his students. These abstruse and wordy papers were my introduction to chronobiology. CPG was skeptical about the existence of any kind of endogenous rhythms – tidal, lunar, circadian or circannual. It is a tribute to his open-mindedness that he allowed me to interpret my work my way in the PhD thesis (Chapter 2).

Yet in 1964 when I obtained my doctorate from the University of Madras, I was diffident and unsure if my thesis contained any novelty – enough at least to attract sufficient attention so that I could continue to work on something which was becoming a burning interest to me. Dr G Neuweiler, a postdoc from the University of Tübingen who had come to us in 1963 to continue his work on flying foxes, advised me to get in touch with E Bünning. I had until then believed that he was a botanist but Neuweiler reassured me that Bünning also frequently worked with animals. It was then that I made, what I considered, the smartest move of my career and wrote to Bünning with a copy of my work describing tidal and diurnal rhythms and of the kymograph traces of the swimming activity of my crab. It cost me, what was then, a fortune. I also ruefully mentioned in my letter that no one in my university appeared to believe my story or that of the *Emerita asiatica*. By coincidence, Bünning himself was working with the Mediterranean green crab *Carcinus maenas* to study the tidal rhythms in their swimming activity. In three weeks I got a letter from Bünning, asking

me to "proceed to Tübingen"! End of June 1964 I did 'proceed' to Germany as a DAAD scholar. I put in four months at a Goethe Institute in Brilon, Westphalia, learning German.

7.1 Arriving in Germany

My first impressions of Germany are still fresh in my memory. It struck me that the sun was still up in the horizon at 8 pm, though no one walked on the street and I did not hear the voices of children. Very few birds sang that evening even though spring was giving way to summer. The meadows looked like neatly mowed lawns. The pavements and the streets were spotlessy clean. There were no odours outdoors, neither good nor bad. I had never seen anything like this before. There were curtains in all windows and all the windows had glass panes though not one was open. The walls of the buildings were in different shades of grey. People wore woollen clothes, also of different shades of gray. My room smelt of wall paper and the toilet was squeaking clean.

The people were curious when they saw us nine Indians move around as a team. Sushma Bhatnagar's (the only woman candidate among us)'s saree was another object of curiosity. When the German teacher Herr Laabs asked her how her name was pronounced, she said "with an *umlaut*".

Bünning sent me a nice letter of welcome and a copy of the first English edition of *The Physiological Clock* (1963). He reminded me that it might be a waste of time to study German grammar for four months instead of which I could go to Tübingen straightaway if I so desired. I wrote back thanking Bünning for the book and said that I wished to learn the language properly. In the end of October 1964, our language training over, I proceeded to the famous University of Tübingen to continue my research on crabs.

7.2 Tübingen

Neuweiler, now back in the Zoologisches Institut, Tübingen as a lecturer, was in the station to receive me. It was he who got me a room to stay in and saw to it that I was comfortable (he was impressed to see that I ate German food without any complaint). He wanted to hear what my 'first' impressions of Germany were. His first impressions of India were that people were inquisitive – spoke to you in railway trains, buses, asked if you were married, had a father, mother, brother and sister and so on. I said that I was very disappointed that people in Germany were *not* inquisitive, did *not* look at me and speak to me in railway trains, did *not* ask if I was married, had father, mother, brother, sister and so on!

Seriously, though, the landscape around Tübingen was like in a fairy tale, with the thousand-year-old Bebenhausen Chapel with Gothic

architecture, and the Würmlinger Kapelle perched on a little hill. Walking over long stretches and hiking (*wanderungen*) is a favourite German pastime. They even have route maps for the purpose. The students took full advantage of the sylvan surroundings and went on treks during weekends. In later years, while attending the Gordon Research Conferences on Chronobiology, I saw the beautiful New England countryside and the White Mountains in USA and was surprised to notice that the only walking that most Americans seemed to do was to and from their cars.

It also impressed me how German students could identify the plants, butterflies, birds and animals; even students of theology could do this. What a far cry from the scene here in India where students of biology are unlikely to identify the flora and fauna of their own places. I soon became integrated with German student life which seemed to be much richer, socially as well as culturally, than student life in India perhaps even today.

Students were in constant movement apparently going from one department to another. The departments and lecture halls were strewn all over the town much as in Cambridge and Oxford and there were students coming from the USA, UK, Australia, S.Africa, Asia and Africa.

I met many interesting people; one of them was M Ilmas from Lahore. Ilmas had come in 1962 as a DAAD student intending to do the Ph.D. with the mathematician Gnäser, which he never did. He was a prominent victim of the academic freedom granted to grown up students to shape their own lives at their own pace.

German universities followed a peculiar educational system at the heart of which is the philosophy of academic freedom (*akademische Freiheit*). This "freedom" has been the undoing of many students from India and Pakistan who came to do higher studies in Germany, and who had been raised on syllabi, tedious lectures and market notes in the colleges. The freedom of German universities refers to the "freedom of the teacher to offer courses of his expertise or choice" (*venia legendi*) and the freedom of the student to attend courses and lectures of his choice. But freedom can be misused too, and is misused even in German universities. There are students who study into their 40s and teachers, especially if they lack ambition and idealism, who get away with minimal teaching. German universities also did not have fixed periods of years of study. It was a common phenomenon for students to "take it easy" in the first year of university study, after the rigours of the years at the school (Gymnasium), a phenomenon which may indeed be universal.

The State hardly ever interfered in the internal affairs of universities. There are no written examinations, no September/March/September, therefore no examination time, no cramming, no question paper leaks and answer paper corrections! Each student decided for himself when he was ready to be examined. The onus for the path a student's studies would take lay entirely with him. This system of academic freedom was the nemesis of the weaker section of the students but vastly promoted the committed and the

gifted. But the rewards, checks and balances prevailing there generally restore a healthy equilibrium. It was a testing ground and each scholar achieved to the extent he strove and found the appropriate rung in the ladder.

In the 1990s the German universities began changing and are becamimg more like Anglo-Saxon universities, in order to attract students from Asia, Africa and elsewhere. They have now even introduced English language BA, MA and MBA programmes.

The academic ladder in German universities had very few rungs and each position was very difficult to obtain. After the student revolts of 1969, the universities have created many professorships in each subject area. Until then, many of the earlier Ordinarius Professors, given their powers, tended to be tyrannical and ruled the roost according to their own likes and dislikes. But it is amazing how many of the professors remained good, created excellence around them with their freedom and were also very decent human beings. Erwin Bünning was one such.

7.3 Bünning's laboratory

The Botanisches Institut (BI) in Wilhelmstrasse, where I was to work, was fortunately not destroyed in the war. It looked much as it might have looked in the days of Hugo von Mohl who had it built in 1846. The research scholars in the Botanisches Institut impressed me as being a purposeful lot, working late hours in the evening and attending various colloquia, seminars, lectures and practicals

The laboratories in Germany turned out good work because there was good teaching and because there were good teachers and scientists. In the BI there were eight climate controlled rooms (I would later call them chronocubicles in Madurai) and I had access to two of them. All my *Drosophila* work from 1967 to 1969 was done manually, tapping out eclosing flies into soap water in petridishes, killing them and counting them at leisure with a camel hair brush. As I counted flies I reminded myself that I was in an institute made famous by the work of stalwarts. That for me was, and still is, "atmosphere of excellence". The most sophisticated equipment I used in the BI was time-lapse photography, but then at Madras I had used the more sophisticated audio-oscillator. Bünning and his students still used hand-wound thermohygrograph smoked drums for recording leaf movement rhythms of bean plants, the method used by Wilhelm Pfeffer (1845–1920).

Even though I knew before leaving India, that Bünning was a great man in a vague way, I got to know of his pre-eminence among European biologists only in the course of the first three years I spent in his laboratory. My ignorance was pardonable on two counts. I was a zoologist by training; further he began publishing his papers in English only after 1959–1960 in journals which were available in the library of the University of Madras. In the beginning I was so much in awe of him that I never even attempted to

talk to him. Later when I was doing my experiments on *Drosophila,* especially the ones on the significance of transients, he would drop in every morning to ask me what the day's, in my case the night's, labour had brought.

In a year Bünning had me "transferred" to an Alexander von Humboldt fellowship. It was he who suggested in 1966 that since I was getting very interesting results, I should extend my Alexander von Humboldt stipend by a year. In 1967 he encouraged me to write up my findings for publication, which I did in two papers, both of which he sent to H. Autrum for consideration for publication in the *Zeitschrift fur Vergleichende Physiologie* which later became *J Comp Physiol A.* In a week's time I got the letter of acceptance. I am especially proud of the fact that Bünning never wrote a paper in English after 1964 in which he has not thanked me for help with his English.

Erwin Bünning was born in 1906 in Hamburg, the son of the school teacher Hinrich Bünning and Hermine Bünning (nee Winkler). He studied in Hamburg (1912 –1925) and in the Universities of Berlin and Göttingen (1925–1928). He had high praise for the German school and the university system and the freedom it gave to teachers to teach Darwin's theories which at that time was not allowed in many countries. He received the Dr.Phil. degree in 1929 under the supervision of Hans Kniep. Bünning complained that there was too much philosophy and speculation and too little of scientific experimentation in the earlier epochs of the German universities. The movement called *'romantische Naturphilosophie'* held sway over German biology for far too long. In France and Great Britain, experimental work in the nineteenth century very nearly replaced pure speculation in astronomy, physics and biology, while in Germany, the influence of certain philosophers such as Schelling (1775–1854), Hegel (1770–1831) and Oken (1779–1851) was still very strong. Only a *proper world of ideas* was considered to lead to any progress in learning about the world. Schelling called Bacon, Newton and Boyle destroyers of astronomy and physics and the great Goethe characterised Newton's optics as being plain nonsense. In the prevailing antagonistic atmosphere, some biologists like Hans Driesch (1867–1941) gave up laboratory research and took to philosophy.

Bünning went to Indonesia (1938–1939) for a year which was a respite from the Nazi atrocities. Mrs Eleanore Bünning often recalled that year with bitterness for she had stayed back with two small children. Once back, he was conscripted into the army as a soldier in 1939. He was then made an associate professor at the University of Strasburg which enabled his hapless family to leave Königsberg before the terrible end of that part of history. Only after the end of the war did Bünning at 39 become a full professor in 1945 at the University of Cologne and in 1946 at Tübingen. In 1953, the University of Munich and in 1957, the University of Göttingen offered him a chair, both of which he declined. Bünning found that Tübingen with its rich biological heritage was ideal for him and he remained there for the rest of his career and life.

Bünning had a fine sense of humour and his anecdotes were full of history, insights and content. He never repeated himself although he talked on many topics. I have heard it said in the 1960s that he was the Pope of botany on the continent and the Swiss microbiologist Zaehner stated in a public lecture that Frey-Wissling in Zürich and Bünning were the two greatest living biologists.

In hindsight, 1964–1967, were my best post-doctoral research years – I worked hard and also vastly enjoyed myself. I did not then know that based on my experimental data amassed in those years I would continue to do further valuable work later at the University of California, Berkeley. In 1967, bowing to pressure from my family to come home for my brother's marriage, I left Germany two months before the Humboldt fellowship would expire.

7.4 The re-entry crisis

Going back to India was not easy for me. After the cool, clean air of Germany, my country's blazing heat struck me like a force from hell. Things all around me looked grimy and run down. The man, who received me, asked why I had ever cared to return and within hours I got to thinking the same. Looking back, I can understand that my state of unhappiness was an immediate reaction to the many real problems that India was then facing – in its standard of living as well as its state of higher education. Furthermore that was the first time I was venturing out into the big, bad world outside the confines and coordinates of a life in a university. I still did not know if I disliked life back in India so much that I wanted to leave for the USA straightaway, which was common among Indians who used Germany as a transit *en route* to the USA.

Sometime in June 1967 I took up my job as a Scientific Pool Officer, a cadre created by the CSIR for scientists who returned from abroad and were jobless. The better pool officers got a job while the others returned to the West, disappeared or dropped out of doing science. The Director of the National Institute of Oceanography, Dr NK Panikkar was a good zoologist. He saw that I was unhappy and restless marking time in the Head Office at Haus Khaz, Delhi and had me sent to the field unit of the NIO at Miramar, Goa. Dr PV Dehadrai, a fish physiologist, and the geologist Dr NK Srivastava were my colleagues. Soon I was promoted as Scientist B. I was there ostentatiously to do further research on tidal and circadian rhythms and I began to work on fiddler crabs captured from the banks of the Mandovi River. Although, I still felt that I was going nowhere, socially I was very happy. I loved the place – the Goan food and the cashew fenny.

Working conditions in CSIR laboratories were still bad. I remember "placing an order" for a set of student kymograph drums made in Ambala. The drums arrived some eight months later. Everything was in short supply: fresh milk and eggs included. According to the natives all problems were 'thanks

to the Government of India'. They all said that life had been so much better when the Portuguese were around. There were no workshops anywhere in Goa where I could get the actographs done. I improvised and caught a few fiddler crabs and started my first DD experiments in the toilet downstairs. There were beautiful tidal rhythms in the locomotor activity of this fiddler crab *Uca annulipes*.

I began writing popular articles for *The Hindu*. Feeling rather disgruntled and full with scientific discontent I wrote an angry letter titled, 'That hapless being, the Indian scientist'. To my surprise *The Hindu* published it as an article in their Sunday edition (25 December 1967) and reproduced it in their international edition. The theme was how politicians asked the scientists to work for the betterment of living conditions for the people but paid the scientists themselves pitiful salaries and offered them shoddy working conditions. I pointed out that in India, people did not mind being second or third rate in sports, performing arts, painting, music, politics, living conditions, but when it came to science, the society wanted scientists to be as good as their counterparts in the USA, U.K., Germany and so on.

My letter had its repercussions. On his next visit to Goa, Dr Panikkar gently informed me that as a government employee, I was expected not to run down the office of the Prime Minister. After that, I refrained from writing to any newspaper. But, for the record, Dr Panikkar was a very understanding boss. He once asked me if I'd care to take up a Rockefeller Fellowship to work in any research centre of excellence within India – the newly constructed All India Institute of Medical Sciences in New Delhi, for example. The remuneration would be Rs.1000 (then called a four-figure salary) and the fellowship would be for five years. I asked him what my future would be after five years. Dr Panikker vaguely said that by then some university or the other would offer me a chair. It is interesting to speculate what would indeed have happened if I had agreed to this suggestion, by no means bad at all. But by then I was thinking of the Miller Fellowship in USA and thinking of leaving India for good.

Werner Loher, to whom I had communicated my dissatisfaction of the working conditions in India, had nominated me for a Miller Invitation Fellowship to be held at Berkeley, UC for two years. If I had known about the lofty ideals, the stalwarts who were prominent Miller Fellows and the limited number of seats, I would have backed out of sheer fright. But at that time, all I wanted to do was quit India and find the happiness I had left behind in Germany, or better still, Tübingen.

My March/April 1968 though, I had still not got the nomination form. Just a few days before the last date, I got an envelope which looked much travelled. It had travelled all the way to Italy and back because a helpful hand had decided that the Miramar on the address must be the one in Italy and the confirming factor was that Maroli (my first name) was also an Italian name! It was my nomination form.

I rushed to the office, filled up the form and typed out a half-page research proposal on my "Olympia", in which I had written that I wanted to experimentally verify if a "dawn and dusk" model I had proposed for the *Drosophila* eclosion rhythm would stand experimental verification. I posted it with "late fee". I knew that my proposal was brief, but it was genuine and precisely formulated. The Miller Institute got the proposal a day *after* it had made the selections. But my research proposal was appreciated and they placed me number one in the waiting list. I knew that the chances of anyone declining a Miller Fellowship were very slim and I was disappointed. But it so happened that an English candidate who had been selected as Miller Fellow, had been offered a job in a reputed English University and therefore did not take the fellowship. I was selected from the alternate list and became a Miller Invitation Fellow 1968–70 at Berkeley, University of California.

It would seem from my above account that working in a CSIR laboratory was the pits for a scientist. But the fact was that my heart was set to work in a university anywhere and not in a CSIR laboratory. This is not a criticism of this great institution which gave me a senior fellowship to be a postdoc, made me a pool officer and scientist and when I was at Madurai Kamaraj University (MKU) gave me research grants and the grand prize. CSIR has offered jobs to thousands of scientists, who had nowhere else to go in those days except the USA.

7.5 Berkeley .1968–1970

I flew, with my newly-married wife Shashikala, to the USA via Europe in mid-August 1968. En route we stopped over in Tübingen and met old friends and the kindly Bünnings. The day we met coincided with the Russian troups marching into Prague. Bünning seemed to be pre-occupied – he had seen two wars and was worried that the world was being nudged towards a third war.

He appeared to be glad I was going to the USA and wanted me to take up a permanent job there. Neuweiler, by then happily married to Edda , told me he would never have believed that I would leave India for a second time. I, according to him, was too much of a classical Indian to do this. I just was not the type. Neuweiler's *affair* with India was still on. And he believed that people like me belonged in India.

Loher, who I had to work with, had a small laboratory and I was given a table and a chair in it. Even important scientists like the entomologist John Cassida had small cubby hole-like laboratories in the Wellman Hall, Division of Entomology. This was the world's most affluent university department, made famous for its contributions on biological control of parasites. Besides Wellman, there were the Gianini Hall and the Hilgard Hall in the Division of Entomology. Their experimental stations were in the Oxford Tract, some distance away.

After Germany everything about California was expansive, big and generous; even the ice cream cones were big. We liked the bohemian character of Berkeley in 1968. Everywhere you looked there were hippies, happeners, 'flower power' people, beatniks, peaceniks, vietniks. It was funny how the Berkeley dons reacted to all this. In their heart of hearts, most of them were peaceable people and progressive enough in their outlook. But that was not enough - one also had to assert, externally in talk or clothes, their message and participate in the various 'sit ins'. People like Loher and me were ambivalent and went only to the extent of not wearing a necktie and a white shirt. I had never in my life worn jeans and was not going to wear them now.

Berkeley was also rife with political and social unrest. The students openly condemned the Vietnam War and protested against it and the establishment. A familiar figure in Berkeley joining the protesting students, was Linus Pauling. Others who joined in were Owen Chamberlain (NL), and Victor Schwarz, the physicist rumoured to be in the running for a Nobel Prize.

The Berkeley campus was beautiful beyond words - I guess it compensated for all the unrest there. There was a big eucalyptus grove and a nature reservation area with a wild gushing stream. There were two gymnasiums, a swimming pool, the spacious Pauly Ball Room, the Sproul Plaza, the Computer Centre, the celebrated library and various departments.

I settled down to work and decided that I would study for some time *Drosophila* eclosion rhythms. I had a lot of data from Germany which till now I did not have the time to examine. I showed some of them to Colin Pittendrigh and he asked why I had not published these beautiful data yet. This was a great compliment coming from Pittendrigh and I was duly gratified. I wrote three papers out of them in the course of the next year and published two in *Z vergl Physiol* and one in *J Exp Zool.* I performed a few experiments, using a walk-in environmental chamber in the Oxford Tract on *Drosophila* eclosion rhythms. The experiments probed phase shifts in the rhythm in response to light pulses of varying intensities. The results are reproduced in Fig. 1.11. Michael Land, a fellow Miller Fellow, told me he loved it but it took Bünning's endorsement to pronounce the significance of my findings for daylength measurement in organisms.

Sometime in 1970 I got a very excited telephone call from Ebo Gwinner. Gwinner was doing some post-doctoral work with Colin Pittendrigh. He asked me to rush to Stanford if I wanted to meet the man who discovered the clock gene in *Drosophila*. A year earlier, Colin Pittendrigh had come to Berkeley to deliver a lecture. At that time he told me that if someone were to discover *a clock gene* then "we could lick the problem of circadian rhythms". I was not sure what Pitt meant. The way he said it made it look as though the eventuality lay in the distant future. The young man I met, Ron Konopka, a graduate student of Seymour Benzer at Caltech, was doing recordings of the locomotor activity rhythms of fruit flies with *per* locus mutations. For one who had just made such a breakthrough in chronobiology,

he was quite a modest guy. Konopka and Benzer published their findings ir PNAS in 1971.

It was in Berkeley that I discovered that in doing science I an essentially a loner and I came to resent the holding-hands kind o collaboration that was prevalent there. Werner wanted me to do fo grasshoppers what I had done to *Drosophila*. I was tiring of working ir Berkeley but did not want to work elsewhere in the USA either. Afte Berkeley any other university in the USA would have been disappointing Germany was still my "land of milk and honey" and I wrote to Bünning Engelmann and Jürgen Aschoff. Aschoff wrote back saying that he ha very much liked my papers but was unable to add a *Drosophila* laborator to his Max-Planck-Institut. He had therefore taken the liberty of telling a DFG adminstrator how important it was for Engelmann to hire me a Research Associate in the BI at Tübingen. Klaus Brinkmann, who wa then working in Berkeley, was not happy to hear this. He thought that i was time I was on my own and not working under or with anyone. Bu once again in 1970 I was as desperate to leave as in 1968.

7.6 Tübingen revisited

Tübingen and Germany had changed vastly in the three years I was awa All science departments and clinics were now up in the hills. They looke like Berkeley buildings or could have been in any other city in Germany The old-world coziness was gone.

The word "stress" had entered the vocabulary of Germans and the pac of life was changing fast too. That was the first time I felt that one mus avoid re-visiting a place dearly cherished and frozen in memory and time Tübingen re-visited was not the same place I knew. Most student friends had known had moved on to other places. My mood also swung from a hig on leaving Berkeley to the autumnal gloom of Germany. In Berkeley it ha been sunny practically *every* day and the sky always blue. The lack c seasons in California and the perpetual sunshine had struck me as bein monotonous. But here in Germany the days were becoming shorter an shorter and the temperature dropped.

I was thirtythree had chucked a job in India and was again driftin Furthermore I had always held named post-doctoral fellowships but now was working with, actually for, Engelmann. It was soon indeed the wint of my discontent.

I met Bünning, Rätze and Kautt. They were happy to see me personall and maybe a bit sad too. Introducing me to his American biologist son-i law Dr Franklin, Bünning said that I was "one of those sad Indians wh travel all over the world in search of jobs". Bünning had decided to retire i 1971 and he warned me that as a zoologist my future in a botany institu was precarious. Neuweiler offered me a wissenschaftlicher Assistent pos

I was confused. I was recovering from Berkeley and needed some time t think about what I really wanted to do with my life.

Setting all my confusion aside, I lost no time starting work on th *Drosophila* cultures. Engelmann now had an elegant automatic recordin device, so I could get huge cultures going. I had some forty channels all t myself and was given two spacious climate- controlled laboratories built b Siemens. One could get light intensities of up to 80,000 lux and bring down to 0.3 lux by turning a nob. There was even reliable humidity contro At Engelmann's behest expensive dawn-simulating and dusk-simulatin devices had been fitted in. In short, the facilities for research in chronobiolog were state- of- the art and world class. This was indeed a far cry from m facilities in the NIO at Goa and the walk-in chamber of the Wellman Hall i the Oxford Tract at Berkeley. Although, I must frankly admit that I did nc have the faintest idea for what purpose I would need all these fancy facilitie working as I did with *Drosophila* eclosion rhythms. Engelmann, as usua performed experiments on leaf movements of the telegraph plants, flowerin in *Chenopodium rubrum,* petal movement rhythms of *Kalancho blossfeldiana* flowers, phototactic rhythms in the green alga *Euglena viridi* all going simultaneously or successively. He had one or two technician working for him but could be often seen carrying trays of flower pots an other experimental gadgets himself at all hours of the day and night. H needed most of these for his teaching the Grosspraktika to senior semeste students and the summer and winter classes on chronobiology he offered His inputs in terms of teaching and manpower training were tremendous.

After Bünning retired things got murky. That is the first time I experience 'politics' in German university life. Bünning was a great believer i integrated biology and said subject divisions like zoology and botany wer meaningless. Hence the name of his institute: Institut für Biologie I. Afte his retirement, zoologists started calling themselves Institut für Biologie I and the microbiologists Institut für Biologie III. But the old names wer reinstated within weeks of Bünning's death in 1990.

The finest thing that happened to us, in this period, was the birth of ou first daughter Sujata on 12 March 1972. Never in my life had I felt s personally responsible and sure of my ability to see through all life' difficulties. Life now was imbued with purpose.

I wrote ecstatic letters, began thinking about the future, and had somethin to look forward to in life. As it turned out I published some of my fines papers during this period. The Engelmann Arbeitsgruppe swelled and a one time we were 19 to 20 strong and were the biggest team in biology Students thronged to us. I began teaching in Grosspraktika offered b Engelmann to senior semester students, though I found it tough and becam tense at the approach of a new semester. I had six students working wit me for their projects. Two projects resulted in published papers.

By 1975 though, I had this unwarranted sense that I was aimlessl drifting. I was academically active and I had a further two years of securit

in Tübingen but I was not sure anymore if I should stay on that long. Engelmann, even though very soft-spoken, looked and acted unsure and insecure. Further it was difficult to know if Engelmann was happy or unhappy about my presence. He, for that matter I too, little realised to what extent I was playing a pivotal role in keeping the cohesion of Engelmann's team. I had a feeling that Bünning's retirement understandably brought in intra-departmental tensions.

For the first time in my career, I wanted to be on my own and not play second fiddle. I was tiring of the West and wanted to go home. I wrote to S Krishnaswamy (SK) at MKU, who had been persuading me to join his department ever since my NIO days. A Reader in a university in India has a lot of freedom especially if the head of the department was a non-interfering man. SK immediately went to work and got the syndicate and the enlightened Vice-Chancellor to agree to "create" a post of Reader in Animal Behaviour. This hand-written letter of invitation arrived, written by him and not the Registrar:

"I am very happy to inform you that our project has been included in the Indo-German Cultural pact, between Germany and India. This would be a project for many many years and you will be able to build one of the finest schools in India and the World. I am sure you will be able to attract students from far and wide, I have taken one Subbaraj, First Class 3rd Rank, a very brilliant fellow and put him to work on bats. He will be transferred to you as soon as you can join us".

Later the appointment order would come through the proper channels. By the time I left Germany in 1975, Engelmann's group of co-workers began dwindling in size and by the mid-eighties he was literally all by himself. Once Neuweiler was sure that I was going to the MKU on the Indo-German Project, he did not want to leave anything to chance. He made me write to the Alexander von Humboldt-Stiftung for a 'Gerätespende', money for equipment. Neuweiler added DM. 3, 00,000 and we called our collaborative project the Indo-German Project on Animal Behaviour (IGPAB). This would become the most affluent of all UGC/DAAD binational programmes. I left Germany at end of May 1975.

7.7 The School of Biological Sciences at MKU

S. Krishnaswamy had his training as a zoologist at the University of Madras and the University of Southampton, UK and the British Museum at London. He was sent to Madurai to head the post-graduate zoology research laboratory of the Madras University in 1962. Similarly, physicists, chemists and mathematicians had also been sent to the postgraduate extension centre of the University of Madras at Madurai. These men would all become "men of the zero hour", when the Madurai Kamaraj University came into being on 1 February 1966. The university was fortunate to have the great Tamil scholar

and linguist TP Meenakshi Sundaran (TPM) as the first Vice-Chancellor. TPM had great faith in the quality of his colleagues and faculty. He was especially fond of SK whom he gave full freedom in the matter of recruiting faculty-by-invitation.

At a practical level, however, the integration of botany, zoology and other areas of modern biology into one, as we had done in the School of Biological Sciences (SBS), was fraught with difficulties. Our M.Sc. students could not find placement as teachers in colleges as they were neither botanists nor zoologists although they were highly prized as Ph.D. entrants at IISc, TIFR, CCMB, NII, etc. The teaching programme at SBS/MKU was good because, except in core subjects like biochemistry and molecular biology, the rest of us had to compete for students to teach. I appreciated being able to attract a class size of six students! In 1979 I taught a class of nine college teachers studying for the M.Phil. degree chronobiology and had to give out nine different projects. They were faculty improvement programme (FIP) candidates supported by the UGC. All this was back-breaking work for me because I did all the teaching in my subject – 32 lectures a semester. My colleagues and I took our teaching very seriously and of course our research as well.

Recognition of my efforts and scientific contributions came soon too. S Krishnaswamy rejoiced at any recognition that came our way. He always gave credit to his colleagues, where it was due; jealousy and rancour were emotions alien to him.

Even though Madurai was the back of beyond in terms of accessibility we had a stream of distinguished scientist visitors, especially come to the SBS. We had collaborations with CNRS France, Oxford UK and Berkeley UC and the Berlin University. The Indo-German Project on Animal Behaviour (IGPAB) flourished because S Krishnaswamy welcomed the German teaching faculty: Neuweiler and his colleagues warmly and was kind to visiting German students (some nine of them) who did field work in Madurai on bats under my supervision. I played at 'still being in Germany' at MKU and it worked. When I left MKU, I left behind all my equipment and begun to worry even then whether we could sustain the excellence of the earlier days – "the ephemeral nature of excellence" as Britton Chance once wrote.

7.8 Setting up a laboratory in a university

This account is being given as it may interest those who plan to set up a university laboratory in an India of the future which, one fondly hopes, would become a more enabling environment.

I first want to admit candidly that I did not really know what I would do subsequently at MKU. I only knew that my years and life in Berkeley and in a re-visited Tübingen only left in me a great but unarticulated desire to be on my own. After all, all the science I had done and all the papers I had

written until 1975 reflected my independent ideas and effort, with no intellectual input besides published literature, from any quarters. I wanted success on my own terms, which could be mine, only if I continued my work on circadian rhythms. As he told me often, Neuweiler knew that the Indo-German Project on Animal Behaviour, depended on me and how successful I was. But the IGPAB for me was only a mechanism that enabled me to train manpower in neurophysiology and ethology besides taking care of my own interests.

In binational collaborations, especially with laboratories in the USA and Europe, I had noticed that scientists in universities in India reduced the undertaking to a few visits abroad and setting up a laboratory in India which would serve the interests of the foreign hosts. Interestingly, this had happened in the School of Biological Sciences itself. An Indo-US (Smithsonian Institution) 5-year collaboration for the study of fauna in fresh- water ponds, had left behind a lux meter, a few pipettes and thermometers. Neuweiler was from the very beginning keen on making the IGPAB a truly collaborative venture where both partners benefitted materially and intellectually.

R.Subbaraj had already begun his researches on the tomb bat *Taphozous melanopogon* and was waiting for me to join him. I found the heat of Madurai, with the mercury often touching 41°C, unbearable, oppressive and debilitating. The first three years were the most difficult for Subbaraj and me. I had all the time in the world (my family was in Bangalore) to go to bat caves and even stay up in the hot steamy nights in the laboratory (two rooms 25′ × 25′). There were plastic buckets, bamboo poles to erect the mist nets and practically nothing else. All our equipment under the IGPAB, to the tune of half a million DM., had been acquired and were stored in a basement by Neuweiler in Siesmayerstrasse in Frankfurt but due to various bureaucratic problems, we could not avail them. A list of the equipment to be imported had to be sent to the Directorate General for Trade and Development (DGTD), because if the equipment was manufactured in India it would hamper the indigenous manufacturer's prospects. The list then had to be sent to the Defense Ministry to check the "security" angle. They even objected to walkie-talkies (toys used by European children), noctovision glasses and field oscilloscopes! I often thought of JBS.Haldane's words that scientists were quitting India not because of poor salaries, but because of the bureaucracy. Two people, who were of genuine help to the IGPAB, besides those mentioned, were Mr Christian Reiser, Director of DAAD at New Delhi and Mr LR Mal (now retired) of the U.G.C. When the UGC formally declared that the IGPAB may begin, Christian Reiser sent a telegram "Let the bats fly!" We finally overcame every hurdle and in September 1978 we launched the project. The Governor of Tamil Nadu and the Education Minister participated in the inauguration of the IGPAB.

Günther and Gerta Fleissner, of Frankfurt University conceived and conducted (the first) the neurophysiology courses, along the lines of the

'Grosspraktika' of German Universities. Pedagogically both the Fleissners were very gifted people and I personally learnt a tremendous amount about teaching and neurophysiology from them. We had a perfect "meteorological station" erected in the garden with a thermo-hygrograph, windspeed, a wind-direction recorder, a precipitation meter and a sunshine recorder. I had small "chronocubicles" set up and had it inaugurated by Bünning in December 1978. I installed my ten Esterline Angus 20-Channel Event Recorders in place. Anil Wason from Jodhpur, PFL Selvanayagam from JNU/NEHU and Satpal Singh from TIFR joined me as post-doctoral researchers. In the same period some four students joined me as research scholars. The group size had to be bigger to enable field ethology work on the foraging behaviour of bats. The equipment for the field-work on bats included a big military style tent. We acquired petromax lights and set up sticky drum insect traps.

I physically participated in and enjoyed the all-night "foraging by bats" adventures along with Neuweiler, Gerd Schuller, Subbaraj, Marimuthu and the others.

It was around this time that our second daughter Sonali was born on 12 March 1978, Sonali added a new dimension to our domestic happiness. We moved to a house (quarters) in the MKU campus and I would get away every year for six weeks to Germany with the help of the UGC and the DAAD in May/June.

We got fantastic results working with bats. G Marimuthu working deep inside a natural cave, observed that there was *social synchronization* of circadian rhythms in a hipposiderid bat. We had our first publications in the *Journal of Comparative PhysiologyA,. Oecologia, Naturwissenschaften,* and *Behavioral Ecology and Sociobiology.* We were important speakers at the annual meetings of the Ethological Society of India (ESI) and its newsletter edited until his death by GJ Phanuel, was full of news about our doings. I had offers of a Chair in Animal Behaviour from JNU, NEHU and the University of Hyderabad. It was a good feeling and I felt recognized. All our "neurophysiology" courses, three in 1978, 1979 and 1981 and the *Biological Oscillations* workshop I conducted, with Bünning, Engelmann, K Brinkmann, DS Saunders, V Nanjundiah, LR Ganesan (of Madura College) and myself as teachers in December 1978, were widely appreciated for their high *niveau.* It should be remembered that besides my Ph.D. research at the University of Madras, which was then one of the top three universities in India, I had no experience in setting up a laboratory. In the case of the IGPAB, the expectations were also high. If I had to maintain the high standards of excellence they wanted, I thought that I could not rely entirely on anyone, even in trivial matters. I, therefore, not only had to deal with my German partners, but also with local carpenters, PWD junior engineers and masons. Now, when I hear people talk glibly about bringing excellence into research and teaching in our universities, I ask myself, "how and at what cost to the individual?"

I must here digress a bit. In 1977, I had taught at the Mahabaleshwar seminar on modern areas of biology, organized by Vidyanand Nanjundiah, O

Siddiqi and John Barnabas and given three lectures on my work with *Drosophila*.. Anand Sarabhai, one of the teachers, heard my lectures on *Drosophila* rhythms and was impressed. He himself was raising cultures of *D. melanogaster* in a private laboratory in his house. Anand was a molecular biologist who had the distinction of getting his PhD under the joint supervision of Francis Crick and Sydney Brenner in the early 1960s working in the MRC Laboratory in Cambridge. He came to see my lab in Madurai in 1978 and we discussed our research and future plans. I was mildly embarrassed with the primitive equipment with which we were then managing. Student kymograph drums, tilting wooden cages for recording the ambulatory activity of bats and clumsy running wheels for recording the running/rest activity of squirrels and a small cubicle were all that we had. But I spoke to Anand about the 'kingdom to come' – half a million German DM worth of equipment, bought for us but lying idle in a cellar in Frankfurt. Anand Sarabhai was neither impressed nor interested in the German equipment and told me that he could get me all that and more, if I agreed to move to Ahmedabad and become part of a grandiose institute he had in mind for research in modern areas of biology – the Centre for Biological Sciences (CBS) – which was to be built in a two-acre plot the Sarabhais owned between the Physical Research Laboratory and the Space Applications Centre.

I started weighing my options. In terms of temperature and landscape, Ahmedabad was no improvement to Madurai. But the CBS would be air-conditioned and I would have a house built for me, also air-conditioned and with a swimming pool, in Navarangpura. Anand was offering me the highest starting salary permissible and I also took a liking for Anand Sarabhai. Further it sounded so much like an adventure and a new life away from the petty politics and self-limiting obsessions and inadequacies of university life. But after much mulling over the exciting prospects and for a variety of practical reasons (research students, university affiliation, the harsh summers) and on the sane advice of a good friend and well-wisher BR Seshachar, then at the Centre for Theoretical Studies at IISc, I decided many months later to stay on in MKU.

7.9 Teaching and the university

The biggest mental adjustment I had to make was the way faculty members are treated in universities in our country. In contrast to the prestige enjoyed by faculty members in Europe, teachers in resurgent India had a low, pitiable profile. They are called "teaching staff" as opposed to the "non-teaching staff" and while the latter in MKU were well-organised and gave themselves five university buses, in which they travelled in reasonable comfort, members of the faculty came by city buses. I envied the prestige IISc and TIFR colleagues enjoyed, in contrast, in their institutions. I was yet to visit as National Lecturer of UGC and the DST, even worse off universities in northern India. Another feature, which is unbelievably

medieval, is the process through which a university teacher has to go if he wanted to teach a new subject.

The best institution in the SBS was the "lunch club" to which all the faculty brought their food to a common table. The place was also filled with fun and games. The ritual of food-sharing mitigated the inter-individual aggression, much of it concealed.

With S Krishnaswamy (boss) becoming the vice-chancellor in 1985, MKU got a new breath of life. It was during his tenure that the Department of Computer Sciences came into being with initial assistance from the DRDO. Many other departments like Journalism and Science Communication, Art History, Drama and Aesthetics, the Institute of Spoken English owe their origin to the keen interest boss took in getting them going. In October 1988, just a few days into his second term as Vice-Chancellor, he was smitten by stroke and passed away after a few weeks. S.Krishnaswamy was unique in many ways, his kindness to all around him and his compassion for the oppressed were his strengths. He took in many students as research scholars often because they were first generation graduates. A grateful School of Biological Sciences and University have instituted an annual S. Krishnaswamy Memorial Endowment Lecture to perpetuate his memory and built a simple memorial in his name in the botanical garden.

For me MKU was not the same after November 1988. I had often contemplated moving to some place in Banglore but stayed on because the human circadian rhythms experiments I had started in 1987 were in full swing. Boss was thrilled with the human isolation facility and the work I had initiated. He wanted to be a subject of study himself but Prem, his wife would not hear of it, mainly because of the strict regimen of medicine which he had to follow.

Another reason I found fulfillment in the MKU years was because I taught "chronobiology" as an elective course for the first time in Asia. J.Feldman who (along with M.Hoyle) isolated circadian clock mutants in *Neurospora crassa* in 1973, after reading an article I wrote (Chandrashekaran M.K. 1980. "Teaching chronobiology in Indian universities". *Journal of Higher Education* (UGC) 5: 1–8.), wrote that he too planned to follow my example and teach the subject! I also got invited to the first ever Gordon Research Conference on Chronobiology in USA in 1978 and I would attend six more. Markl at Constance, Dietrich Neumann at Cologne and Ludger Rensing at Bremen, Rudolf Rübsamen at Bochhum (and later at Leipzig), Peter Miller at Oxford, David Lloyd at Cardiff and David Saunders at Edinburgh, Woody Hastings at Harvard and the Honmas at Hokkaido all got me to go to their Universities to lecture on our work. As far as I am concerned this was success and recognition on my own terms.

7.10 Rooms without windows - the human isolation facility

Broth Eternal

"Let us recall how quickly even a long period of time slips by when we are ill and in bed. It is forever the same day that repeats it; but because it is the same, it is not quite correct to speak of "repetition." Unvariedness, present at a standstill or Eternity would come closer to it.

They bring you the mid-day broth, as they brought it yesterday and will bring tomorrow. At that very instant, it comes over you – you know not whence or how; you feel dizzy, as you see the soup coming, the time-boundaries get blurred, merge into one another. And what is revealed to you, as a true aspect of Being is a frozen Present, in which they eternally bring you the broth. But it would be paradoxical to speak of time passing slowly in connection with Eternity."

THOMAS MANN *IN* "MAGIC MOUNTAIN"

All too often I am asked, referring to the experiments on human circadian rhythms, "given the opportunity, would you do all that in the same way, again?". To the saga of the construction of the human isolation facility (HIF) at SBS/MKU, and the subsequent work in it, I can honestly reply "never"!

I was completely alone during the whole process. Even though the scientific returns were handsome, novel and exciting, it meant a great deal of personal responsibility and investment of a lot of personal time and energy.

I first saw the famous "bunker" of Aschoff in 1965 or 1966 built into the hillside in Erling, consisting of two identical living quarters underground, thoroughly insulated from external stmuli. I was awe-struck with the ingenuity of the planning, the gadgetry and the instruments. The idea grew in me to build something like the "bunker" of Aschoff late in 1980 soon after the "Thrust Area Programmes" of the DST were discussed in Indian Petrochemicals Limited at Baroda. It is then that I realised that science and technology do not grow of their own accord or spring from university laboratories. There had to be enabling agents and men with dreams and vision, even though they are often forgotten later on, or are re-called only in documents buried in archives.

The atmosphere was heady indeed and all this within *three years* after the Tübingen interlude. For the first time in history, big money was coming to the aid of science in India in an organised manner. It was clearly the time to think big if I, for good or for bad, were really to continue at MKU, and I

wrote up an ambitious project with a five year support from the DST. The project was called "Unit of Neurobiology and Mecahnisms of Behaviour (UNMB)" and the support came through the "'Intensification of Research in High Priority Areas (IRHPA)" programme of the SERC/DST. This scheme came the closest to the philosophy of the Max-Planck-Institutes (Max Perutz's policy was "No politics, no committees, no reports, no referees, no interviews – just gifted, highly motivated people picked by a few men of good judgement") in which projects and Abteilungs and whole institutes were bound to a person. The DST claimed, with justifiable pride, that the MKU had the vision to make a university department of my UNMB, which I called the Department of Animal Behaviour and Physiology (DAB&P), the first chair for animal behaviour in a university in India established in 1985.

In the middle of 1981, when I made my presentation at the DST in New Delhi, MGK Menon was the Secretary, Government of India, and guiding the destinies of the DST. I spoke to him of my plans to have a separate "chronocubicles complex with a human isolation facility" built for me at MKU. He was literally startled and told me that "no brick and mortar facility" was permissible under the research project support by the DST. I told him but that was *all* I wanted from the DST, that no university would give me "rooms without windows" as infrastructure, that I had plenty of gadgets and equipment from Germany and all I needed was the space to install them in. As a special case he did sanction the construction of my unique, unique anywhere, building complex. The building had sixteen 8' × 8', windowless but well-ventilated, chronocubicles for work on bats, mice and squirrels and a human isolation facility, and this chronocubicle complex was inaugurated by Yash Pal. Later both S Varadarajan and S Ramachandran, the founder secretary of DBT, were a great source of strength and encouragement.

There were hurdles galore. The site for the building, which had to be some distance away from other buildings, had to be chosen. The blue-print was suggested personally by me and there were endless discussions with the Chief Engineer (called Estate Officer) and his uncomprehending colleagues. I first thought that I would have the HIF underground as in Erling. But I came across a major problem – the engineers did not know how to flush the toilet and expel waste water without special pumps and motors! I was truly 'alone, alone, all all alone' in this undertaking. Besides this project, there was also the back-breaking MSc. and MPhil. teaching chores and 'file-pushing' and research supervision of five research scholars.

I had consulted Jürgen Aschoff, the first scientist ever to experimentally study "human circadian rhythms" as early as in 1962, about how to go about the task of the construction of the human isolation facility (HIF). He was thrilled and enthusiastic about my new project and told me not to waste too much money on an underground construction for, he said "Humans are the most insensitive animals. Just close doors and windows and they slip into a state of free-run". Aschoff was absolutely right, as my later

Fig. 7.1 A view of the human isolation facility at HIF

results indicated. Therefore the HIF at the MKU (Fig. 7.1) was built at ground level with two walls on all sides with 8 inches space between them, which was filled with sand. I stood around in the hot sun (always 39–41°C) and supervised and advised the PWD engineers and the masons.

PN Tandon, who was the Chairman of the Management Advisory Committee constituted by the DST to overlook the activities of my UNMB, was a great source of encouragement. G Marimuthu, among my students was the one to bestow meticulous care in the actual performance of the difficult experiments and in making careful entries of all data in a log book during the period 1987 to 1996. The human experiments would not have been possible without Marimuthu's perseverance and dedication. Not surprisingly he was also the first human subject to volunteer. He also vastly enjoyed the media attention that followed this first historic experiment and his photograph was splashed all over in newspapers and magazines.

The HIF was in fact ready for use in the course of 1986 but I was reluctant to begin, feeling that maybe I was not qualified to undertake such an experiment. In 1987 Serge Daan of the University of Groningen visited me. Daan had first-hand knowledge of the bunker of Aschoff, and many other HIF all over the world. I asked Serge if he would be my consultant. He said that would mean that I would be doing all the work and appending his name to whatever papers that may be written. He said that would be unfair and he advised me to start and assured me that, in these matters, I knew as much as he did. Within days of Serge Daan leaving for Groningen, I began the first human circadian rhythm (HCR) experiment.

Looking back, the HCR experiments were not meant to be centrestage in my career although it did gain a lot of popularity. The only semi-popular account about the HCR and the HIF was written by L Geetha, who had participated in three marathon experiments establishing a record, in *Resonance* – a journal of science education. I performed the last of the human experiments in 1996 but G Marimuthu continued for a while to do further experiments. The scientific results obtained from my experiments were duly published in journals (described in Chapter 6) and I had lectured on our results at Harvard Medical School, University of Groningen, Oxford University, Gordon Research Conferences on Chronobiology and the International Congress for Physiological Sciences at Helsinki. This is the story behind the construction of the HIF at MKU.

Soon after, I left the MKU to join the JNCASR in Jakkur in June 1996.

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Chronobiology is the study of biological rhythms which are governed by endogenous pacemakers and entrained by geophysical correlates of the environment. Thus, there are circadian (daily), lunar-monthly and circannual rhythms characterising the biology and behaviour of organisms. Erwin Bünning, an eminent biologist, wrote in 1973, 'As recently as 15 to 20 years ago, to proclaim the existence of an endogenous diurnal rhythm was regarded, even by well-known biologists, as subscribing to a mystical or metaphysical notion'. Today it is recognised that biological time-keeping is a universal property of life on earth.

Chronobiology research is now at the cutting edge of fields of enquiry ranging from microbial genetics to ethology to the treatment of human psychiatric illnesses. It is emerging as the most interdisciplinary of all biological disciplines and is the subject of countless papers in well-reputed science journals.

The purpose of this book is not to compete with the speciality journals which provide the latest information in the field. The aim is to excite the curiosity of younger people and tempt them to enter the world of biological rhythm research and break new ground.

The book is unconventional as it is a monograph containing the author's long years of research on this fascinating subject. It contains case studies ranging from the tidal and circadian rhythms in the swimming activity of an intertidal crab, to how accurately humans living in social isolation for long periods can 'tell time'.

M K Chandrashekaran's work in chronobiology originated while he was working on his Ph.D. thesis in India and continued in Germany and the USA. After he returned to India, he joined the Madurai Kamaraj University in 1975 and established the first university chair in animal behaviour. He also studied the circadian rhythms in bats, mice and humans. Since 1996 he has been with the Evolutionary and Organismal Biology Unit, JNCASR.

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