J Comp Physiol (1982) 149:311–316

Journal of Comparative Physiology · A
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Isolation of an Insect Circadian Clock*

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Accepted August 9, 1982

Summary. Under constant conditions the compound eyes of the ground beetle *Anthia sexguttata* exhibit sensitivity changes in a very clear circadian rhythm. Usually the rhythms in both eyes in constant darkness are mutually coupled. After transection of the optic tract between the lobula and the supraesophageal ganglion the circadian rhythms of the two eyes continue without interruption, but coupling between them is abolished. Even if the entire supraesophageal ganglion is removed, leaving the optic ganglia intact, the circadian rhythms in the eyes continue without interruption independently. But the rhythm is abolished if the region of the lobula is damaged.

The experiments show that *Anthia* has circadian pacemakers in the left and right optic ganglia in or close to the lobula. These pacemakers can function independently from the rest of the brain and control circadian rhythms of physiological events.

Introduction

In recent years the search for the anatomical substrate of metazoan circadian clocks has been focussed on the nervous system (Lit. ref. to Aschoff 1981). As yet, however, there are only rough indications as to the parts of the nervous system in which it is likely to be found. In certain insects – giant silkmoths (Truman 1972), *Drosophila* (Handler and Konopka 1979) and recently crickets (Cymborowski 1981) – transplanted supraesophageal ganglia have been shown to impart circadian rhythmicity to the recipient. The first definite evidence of a possible site of insect circadian pacemakers was published by Nishiitsutsuji-Uwo and Pittendrigh (1968). They showed that the optic lobes of the cockroach are involved in the circadian pacemaker system responsible for generating the circadian pattern of locomotion. This finding was confirmed by several authors and has since been extended to other insects (cockroaches: Roberts 1974; Sokolove 1975; crickets: Loher 1972; Sokolove and Loher 1975; Wiedenmann, in prep.; beetles: Balkenohl and Weber 1980). Moreover, evidence was obtained that cockroaches have two independent circadian oscillators in a bilaterally symmetrical arrangement (Page et al. 1977; Page 1981), situated in or close to the lobula (Sokolove 1975; Page 1978). ERG recordings from the beetle *Blaps gigas* demonstrated unequivocally that two distinct pacemakers control the circadian sensitivity rhythms in the two compound eyes; in continuous darkness (DD) the oscillations of the two can be entirely independent of one another (Koehler and Fleissner 1978). Although evidence is accumulated in the meantime that each of the two optic lobes in insects contains a circadian pacemaker, no direct proof of this hypothesis has been stated so far. The most convincing results in this context were published by Page (1982) just before submission of the present paper. Accordingly transplantation experiments in the cockroach *Leucophaea maderae* "suggest that the transplanted optic lobe contains a circadian clock" (Page 1982).

The experiments described here prove explicitly this theory for the ground beetle *Anthia sexguttata*: each optic lobe controls the circadian sensitivity fluctuation in the corresponding compound eye even after isolation from the brain. It is thus shown that the optic lobe – or parts of it – can function

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* Supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 45 „Vergleichende Neurobiologie des Verhaltens" E1

as a self-sustained oscillator independent of connections with the midbrain.

Materials and Methods

The beetles (*Anthia sexguttata*, Carabidae) were caught in southern India and kept in the laboratory under natural light conditions, at temperatures above 25° C and at 50–60% relative humidity. Water was supplied ad lib. and mealworms were provided as food.

The electroretinogram rhythms were recorded with permanently implanted platinum electrodes, by means of a fully automated testing device (Fleissner, unpublished). This device presented a brief (30 ms) light flash of constant intensity to each eye at intervals of 30 min. Continuous darkness only interrupted by these test light flashes are called 'DD' in this paper. This stimulus was conducted via a monofilament of a fiberoptic, the end of which was glued to the eye by a wax-colophony mixture in such a way that light could reach the eye only by way of this light-guide. The animals were mounted in a light-tight, ventilated Faraday-cage (30 × 30 × 30 cm) at constant temperature (26 ± 0.5° C) and DD. The amplitude of the ERG was recorded directly; the values obtained from the records with a digitizer (MOP AM 02, Kontron) were presented in graphic form by a computer (HP 9845T, Hewlett Packard).

The experimental animal was fixed by the head above a glass plate in such a way that the rest of the body and the legs were free to move. All operations were done under CO_2 anesthesia. The thick, heavily sclerotized cuticle of the head capsule was opened dorsally with a miniature milling tool; after the operation the piece of cuticle removed was replaced and sealed in position with a wax-colophony mixture. Care was taken to keep the tracheae in the region of the operation as nearly intact as possible. The brain tissue was cut by means of microscissors. During surgery the tissues were occasionally rinsed with small amounts of insect Ringer solution. The microsurgical operations were done under visual control with an operation microscope (OPMI 6, Zeiss) in accordance with stereotactic guidelines obtained from several lightmicroscope brain reconstructions (Gerta Fleissner, unpublished).

Results

Circadian Sensitivity Rhythms

In DD the eyes of *Anthia sexguttata* exhibit prominent circadian fluctuations in sensitivity (Fig. 1). An insensitive day state, usually about 10–12 h long, is followed by a night state also lasting about 10–12 h; the transitions between both last about 2 h, altogether summing up to a circadian period mostly longer than 24 h. In the subjective night the eye is 10^3 times as sensitive as during the subjective day. The adaptation rhythm can be observed as soon as the animal is transferred from the natural day to the experimental DD conditions. However, sometimes the peak ERG values that occur during the subjective night increase in amplitude during the first few cycles in DD. From the third period on, the circadian pattern of the amplitudes usually persists more or less exactly for the duration of the experiment in DD (Fig. 1). Slow changes, and sometimes infradian rhythms, may be superimposed on the circadian oscillation (Figs. 1 and 2a). These can affect the high amplitudes during the subjective night, or the low amplitudes during the subjective day, or both. In the course of these fluctuations the ERG undergoes pronounced changes in shape, which will be described in detail elsewhere (Fleissner, in preparation).

Ablations in the CNS

To locate the circadian pacemakers of these ERG rhythms, the optic tract was transected at different

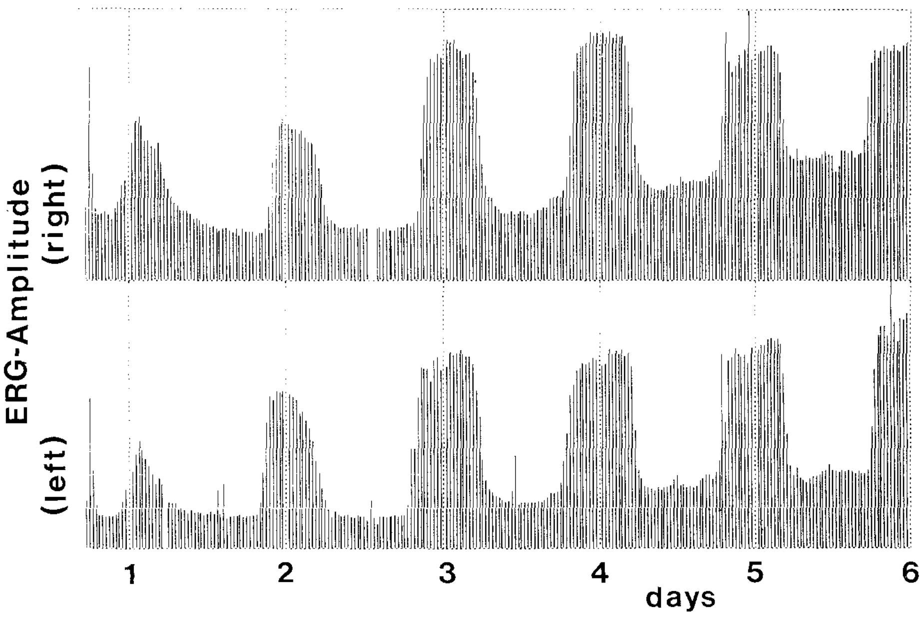

Fig. 1. Circadian changes of electroretinogram amplitude of the compound eye of *Anthia sexguttata* in constant darkness (DD). The ERG was elicited every 30 min by a light flash of 30 ms duration and constant intensity

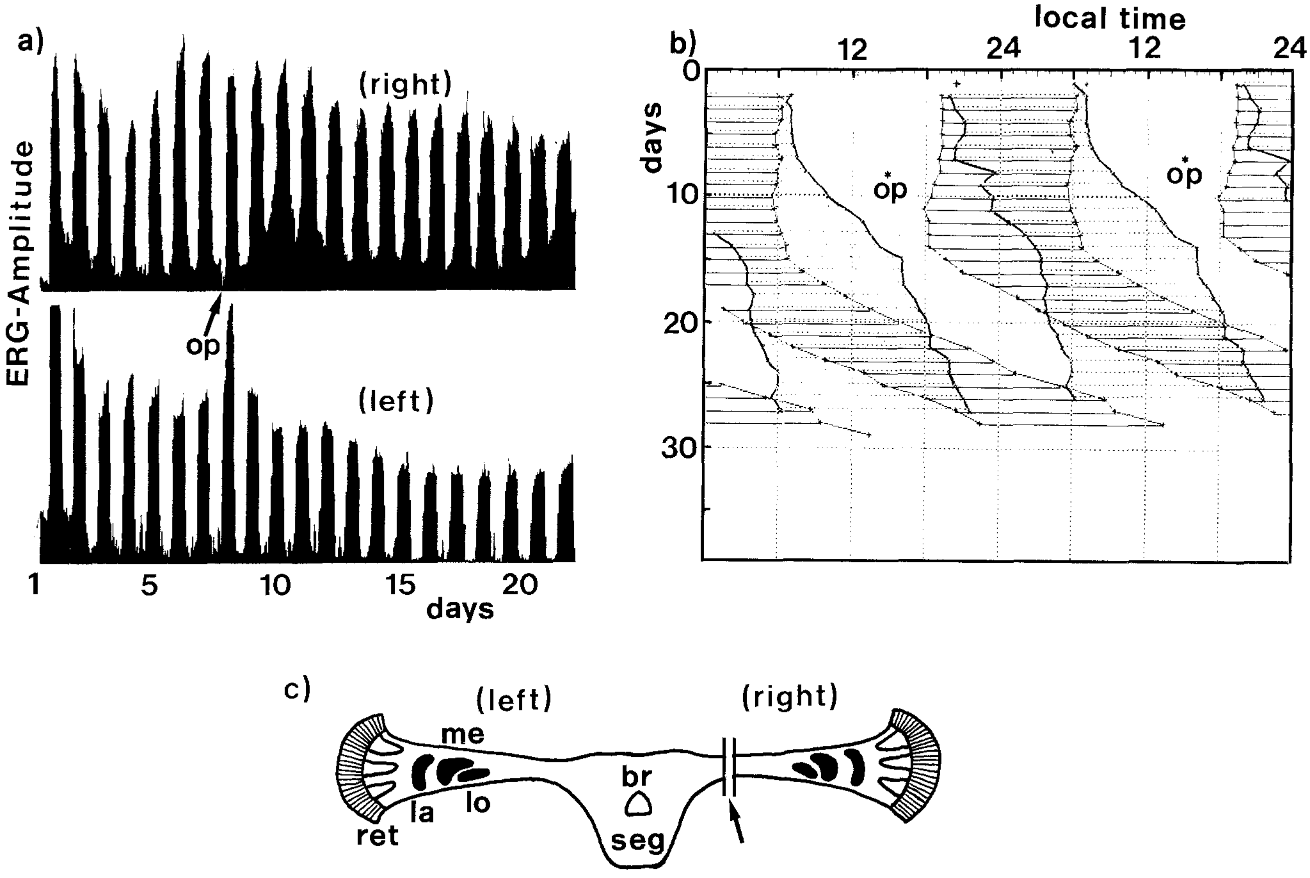

Fig. 2a–c. Effect of ablations in the brain of the beetle *Anthia* on the circadian sensitivity rhythm in the right and left compound eyes, as reflected in the ERG amplitudes. Conditions: DD, 26 °C. **a** Time courses of the ERG amplitude in right and left eye. On Day 8 the operation was done as shown in **c** (arrow). **b** Actogramlike presentation of the circadian states constructed from the data in **a**; the subjective circadian night states are drawn (.....) right eye and (——) left eye; asterisk: time of operation. **c** Schematic drawing showing the site of the operation: transection of the optic tract between the supraesophageal ganglion and the lobula. *ret* retina; *la* lamina; *me* medulla; *lo* lobula; *br* supraesophageal ganglion; *seg* subesophageal ganglion

positions. The results of 19 experiments (dissection between retina and optic lobe 3, between optic lobe and brain 12, extirpation of brain 4) can be summarized as follows:

The circadian sensitivity rhythm in a given eye always persists in DD as long as the transection sites are proximal to the ipsilateral neuropil of the optic lobula. Prerequisite for this, however, is that the tracheal supply to the eye and the optic lobe is not impaired distal to the transection. The circadian rhythm continues without interruption after the operation; the postoperative recovery time lasts only ca. 2–3 h. If, in addition, all the tracheae supplying an eye and its ganglia are cut, without a damage to the nervous tissue, the rhythm in the eye disappears, together with all ERG excitability. In the contralateral eye the circadian rhythm proceeds normally. The synchrony between the circadian rhythms of left and right eyes that is usually present in DD, in the pro- or antiphase, is abolished by transection of commissural connections proximal to the lobula or in the supraesophageal ganglion. If the region of the lobula is damaged one observes complex changes in the circadian rhythm of the ipsilateral eye, which in the extreme case may amount to complete extinction of the rhythm. Depending on the severity of the injury, the rhythms may return after one or more weeks. Partial transection of the optic nerve fibers between retina and lamina abolishes the rhythm in the corresponding part of the retina.

Two examples from this series of operations are illustrated in Figs. 2 and 3. The effect of unilateral transection of the optic tract, between lobula and subpraesophageal ganglion, is shown in Fig. 2a–c. "Transection" here means that a slice of brain tissue ca. 300 µm wide is cut out of this region, so that it is certain that no neuronal connection remained between the lobula and the supraesophageal ganglion. After this operation the

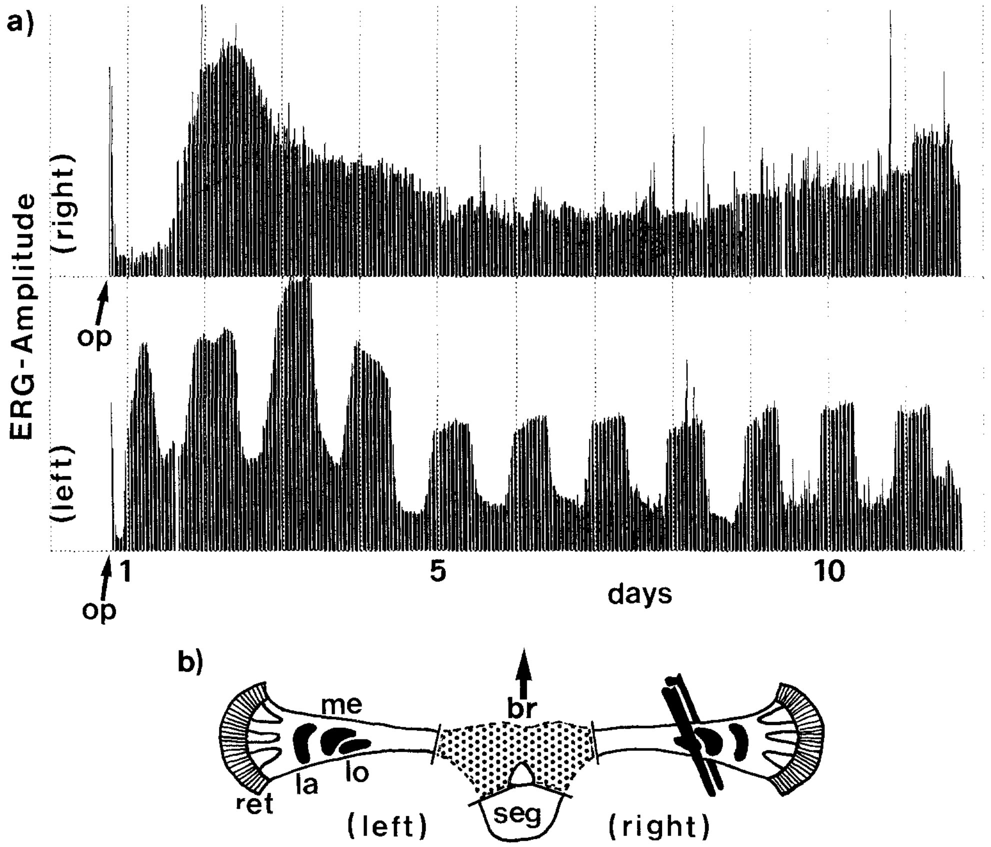

Fig. 3. a Graph of ERG sensitivity rhythm as in Fig. 2, for another animal and a different operation (on Day 1; arrow). Conditions as in Fig. 2. **b** Schematic drawing showing the operations for **a**. Supraesophageal ganglion completely removed except for the optic ganglia, right optic tract crushed with forceps in the region of the lobula. Abbreviations as in Fig. 2

ERG rhythm of the ipsilateral eye continued normally without interruption and oscillating as distinct as in the preoperative pattern (Fig. 2a). The only change was a transient increase in the level of the ERG-amplitudes in the day state; this, however, may be a progression in the original phase relation of a previously visible infradian oscillation, rather than an effect of the operation. In the contralateral eye the circadian rhythm continued as well. Only the first subjective night following the operation had an abnormally high ERG-amplitude.

Synchrony and desynchronisation of the rhythms in both eyes are illustrated in Fig. 2b: The sensitive states of the ERG are presented like activity in an actogram. During the first days in DD the two rhythms are coupled in prophase. On Day 7 the optic tract was cut between lobula and brain. The very day the onset of the night state on the operated side was delayed, and thereafter the rhythm as a whole moved away from that of the opposite side, revealing an individual period of $\tau = 25{,}2$ h. Its mean free-running period until the end of the experiment was then $\tau = 24.6$ h. The contralateral eye responded hardly at all to the operation, with only a slight tendency for the period to become shorter than 24 h. From Day 15 on, however, there was a spontaneous change to $\tau = 26$ h, which persisted until the end of the experiment. As a result, the phases of the two rhythms crossed each other around Day 20. In this process, neither of the two rhythms was affected by the other; the two were apparently independently free-running. Spontaneous changes to longer values of τ can often be observed after about two weeks in DD in nonoperated animals as well as in operated ones (Fleissner, in preparation).

Another example is presented in Fig. 3a, b. In this case the entire supraesophageal ganglion was removed except for both optic lobes that remained connected to their retinae. Despite of that, the circadian sensitivity rhythm continued immediately after the operation oscillating as normal. In such neurally isolated eyes the circadian ERG rhythm can persist normally for more than 4 weeks, even in the absence of the supraesophageal ganglion. In the case shown in Fig. 3 the region of the right lobula was crushed with forceps during the operation. This injury abolished the circadian rhythm of the ipsilateral eye completely. The ERG amplitude passed through one infradian oscillation and then remained at about the level typical of the day state.

Discussion

One central object of research analysing circadian clock systems is the localization of the circadian pacemakers. Physiological circadian parameters derived directly from the eyes such as ERG or compound action potentials have already proved in several species to be a powerful tool in this field (*Aplysia*: Lit. ref. to Rusak and Zucker 1979; *Limulus*: Barlow et al. 1980; Scorpion: Fleissner and Fleissner 1978; Crustacea: Lit. ref. to Larimer and Smith 1980). Astonishingly enough in insects such parameters have been used seldomly in the search for the clock (Koehler and Fleissner 1978, 1979). The ERG has the advantage of being a record from structures directly connected to the optic lobe, which has been one major target in localization experiments since Nishiitsutsuji-Uwo and Pittendrigh (1968). In this research mostly activity rhythms were used as the "hand of the clock". This behavioral rhythms are separated from the control mechanisms by long pathways and centers of neural information processing in thoracic, sub- and supraesophageal ganglia. The ERG-rhythm in *Anthia* has therefore substantial advantages in the neurobiological analysis of the circadian rhythm in an insect.

The results of the lesioning experiments described here are subject only to one interpretation: *Anthia sexguttata* has a completely functional circadian oscillator on each side of the brain, which controls the circadian sensitivity rhythm in the associated compound eye. Integrity of the neural tissue in or in the vicinity of the optic lobula seems to be essential for the appearance of the circadian ERG-rhythm. As mentioned above, transection of the eye-nerve between retina and lamina abolishes the rhythm in the corresponding part of the retina. This indicates that the control mechanism of the rhythm lies proximal to the lesion and not in the retina. Each oscillator in the optic lobe functions normally even if completely isolated from the CNS. It is then independent of the pacemaker on the contralateral side. Instead, in the intact animal both oscillators do communicate – obviously by a neural pathway. By mutual coupling they are usually synchronized to each other revealing a common mean period as it is infered from other data more clearly (Fleissner, in preparation). The mechanisms of coupling between the optic lobe pacemakers and the question of their complete independence from the brain will be subject of further experiments.

Nishiitsutsuji-Uwo and Pittendrigh (1968), working with the cockroach *Leucophaea*, were the first to propose the hypothesis that a circadian pacemaker exists within each optic lobe of this insect. Although this hypothesis was supported by a number of experiments on the same and other species (for citations refer to Introduction), conclusive proof was lacking. The lesioning experiments done by these authors were always designed to find treatments after which the circadian activity rhythm was extinguished. Thus the results could indicate as well the damage of crucial parts of the circadian clock but not of the circadian clock itself, which still could have been situated in the supraesophageal ganglion. Finally, disturbances of other factors such as the tracheal supply have also to be considered in the interpretation of postoperative deficits observed such as arrhythmicity in the activity pattern. Clearer indications that the optic lobes, do in fact house complete circadian oscillators were provided by Page and coworkers. They demonstrated that surgical procedures in one optic lobe of cockroaches cause circadian frequency changes (Page et al. 1977) and that the circadian activity rhythm of the cockroach can be affected by localized low temperature pulses at one optic lobe. This was explained by a circadian oscillator within the lobe (Page 1981). The transplantation experiments published recently by Page (1982) come close to a proof of the hypothesis that each optic lobe houses a complete circadian oscillator. Although there are still other possibilities of interpretation as Page himself prudently stated, the results add up to convincing evidence for this proposition.

The question remains whether the pacemakers isolated in the experiments presented here are the same as those that control circadian activity rhythms. Two arguments support this interpretation: 1. The search for both, the pacemakers responsible for activity rhythms as well as those controlling ERG-rhythms focus on the same possible locus inside the brain: The vicinity of the lobula. 2. The experiments on the beetle *Blaps gigas* (Koehler and Fleissner 1979) showed that the clarity of the circadian activity rhythm depends on the degree of internal synchronization between the rhythms of the two eyes. When internal right-left desynchronization occurred the circadian pattern of locomotor activity became so diffuse that it seemed to disappear.

Previously there have been three other direct demonstrations of circadian oscillators in the insect brain. In the giant silkmoth Truman (1972) transplanted the oscillator controlling the ecdysal rhythm by transferring the supraesophageal ganglion. Handler and Konopka (1979) transplanted *Drosophila* brains to genetically arrhythmic recipients, thereby transferring a circadian clock. It made the recipient's walking activity rhythmic, with the period of the donor animal. In the cricket *Acheta domesticus* Cymborowski (1981) also transplanted a circadian clock with the brain, imposing rhythmicity on a recipient previously made arrhythmic by surgical means. As in all three cases the donor brains did not connect to the nervous system of the recipient the control was evidently exerted by a signal substance, probably a neurosecrete, released into the hemolymph of the recipient by the donor brain. When compared with these cases, the circadian oscillators of *Anthia* reveal certain differences. It is at least distinct from that of the giant silkmoths studied by Truman. The *Anthia* oscillators continue to operate in the optic lobes after removal of the supraesophageal ganglion, whereas the oscillator of the silkmoth operates in the supraesophageal ganglion without the optic lobes. It is difficult to compare *Anthia* with *Drosophila* and the cricket, because in neither case it is reported whether the optic lobes were included in the transplanted brains. However, Cymborowski (1981) suggested that the circadian oscillator he transplanted was located in the neurosecretory cells of the pars intercerebralis. The circadian system of insects must therefore be assumed to comprise at least two different types of pacemakers. One of these, the "*Anthia*"-type in the optic lobes, is paired. It is completely functional and can oscillate independently from the brain.

Acknowledgements. I thank my wife, Dr. Gerta Fleissner, for neuroanatomical information about the brain of *Anthia*, and Dipl. Biol. Wilfried K. Koehler for the programs for the computer graphics. The discussions with both of them and with Dr. Gottfried Wiedenmann were much appreciated. I also thank Mr. Mani Manuel (Madurai, southern India) for providing the experimental animals.

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