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Coupled Biological Oscillators in a Cave Insect

G. A. ODA*†, I. L. CALDAS*, J. R. C. PIQUEIRA‡, J. M. WATERHOUSE§ AND M. D. MARQUES¶

*Institute of Physics, University of São Paulo, SP, Brazil, ‡Department of Electronic Engineering of Politechnic School, University of São Paulo, SP, Brazil, §Liverpool John Moores University, Liverpool, U.K. and ¶Museum of Zoology, University of São Paulo, SP, Brazil

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Insects that live in the interior of caves show the basic internal temporal organization of coupled oscillators. An analysis is made of the coupled moulting and oviposition cycles of *Folsomia candida*, a cave-dwelling Collembolan, with regard to their oscillatory nature, their phase dependent responses to external perturbations, the effect of coupling on these responses, and conjecture about the link of these cycles with circadian clocks in other organisms.

Introduction

INTERNAL TEMPORAL ORGANIZATION

In a uniform steady-state regime, there are no clues as to the passage of time; once in a periodic regime, however, time is "discovered" in the phase of the periodic motion and in fact that the peaks of each rhythm, in an organized motion, follow each other in a prescribed order (Nicolis & Prigogine, 1989).

Internal temporal organization of living systems is, in this sense, based on the rhythmic physiological processes, which maintain a constant phase relationship with each other. This relationship is attained by the mutual coupling between constituent oscillators, resulting in the integrity and temporal order within the organism. The internal temporal order is dependent, to a greater or lesser extent, on control by external entraining cycles, as that of light/dark. In this sense, the circadian system, comprising the 24 hr period rhythms, and functioning as a clock, plays an important role in the integration of the many different rhythms, in the majority of organisms. But still without this control, the pure coupling between some oscillations can be maintained in organisms that live in aperiodic or poorly periodic environments, stressing their primary importance for species survival (Pittendrigh, 1961).

We have studied the moulting and oviposition cycles of the springtail, an unpigmented and blind, tiny (approximately 1 mm) insect that lives in the interior of caves. Moulting, cuticle renewal and the shedding (ecdysis) of the old cuticle, in all Collembola species, occurs throughout life in a rhythmic pattern. In addition to continuous moultings, Collembola present other primitive characteristics: they are very small (usually less than 5 mm) wingless insects, that lack malpighian tubules and a tracheal system. They inhabit humid environments and are also found in the soil, crevices and caves. Reproduction in *Folsomia candida* is by oviposition and is accomplished via parthenogenesis. Ecdysis and oviposition rhythms show a remarkably precise phase relationship and their mean periods are, respectively, 3.9 ± 0.4 and 7.7 ± 0.7 days, thus showing no obvious environmental counterpart. Assuming the peculiarities of the cave

†Author to whom correspondence should be addressed.

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environment it is reasonable to suppose that the frequency spectrum of cave insects is not centered on the circadian frequency.

We pursued a formal characterization of these infradian rhythms (those with periods greater than 24 hr) and compared their properties with those of the better characterized circadian rhythms; as part of this, phase-resetting experiments were performed with temperature pulses. The results showed a lack of many of those features of the circadian phase response curves (PRCs) which have functional meaning relative to their clock-like nature (Pittendrigh & Daan, 1976). On the other hand, the rhythms showed smooth phase-dependent responses to external drives, which help their formal characterization as oscillators with a cyclic nature, and the visualization of the dynamics of shifting two coupled oscillators.

CYCLES COMPRISING SEQUENCES OF STAGES

Developmental and reproductive cycles have long been modeled as sequences of triggered events, each one leading to the next in a definite order. They can either stop in the last stage, so that an additional impulse is needed to restart them again, resembling an "hourglass" dynamics, or be self-sustained cycles, with the last event leading automatically to the first (Winfree, 1980).

The difference between a rhythmic event generated by a cycle like this and a rhythm that results from a limit cycle is stressed in Rossetti *et al.* (1989). In a cycle comprising a sequence of stages, separate events play a functional role in generating the periodicity, each one taking an amount of time to complete and then leading to the next (Mrosovsky, 1970; Joy & Mrosovsky, 1985).

The physiological basis of the moulting and oviposition suggests that the underlying hormonal events can be represented by cycles of triggered stages. The endocrine system of insects is mainly composed of the brain, the prothoracic glands and the *corpora allata* which release, respectively, the prothoracicotropic hormone (PTTH), the ecdysone and the juvenile hormone (JH). These hormones are released and stay in circulation in a definite temporal order. Apparently, there is a triggering effect of one hormone over other glands in the system (Williams, 1952).

These infradian cycles probably belong to a class of oscillators different from those associated with the limit cycles. Infradian cycles have been usually difficult to analyse, in view of their usual link with, and interference from, circadian gates. The use of cave insects as biological model opens the opportunity to study "pure" cycles, apparently devoid of control by circadian clocks, and to investigate their pure oscillatory nature.

The importance of analysing relationships between many different oscillators that comprise the temporal organization of the organism has long been stated (Pittendrigh, 1961, 1974). Here we show the dynamics of two pure cycles that are coupled to each other and their responses to external perturbations, and we conjecture about a mechanism that could generate such cycles and the manner of linking these with circadian clocks in other organisms.

Materials and Methods

A very brief report of the materials and methods of the experiments is presented here. More details about methodology and statistical analysis used will be published elsewhere (Marques *et al.*, unpublished data).

Two hundred and forty adult insects of the same generation were individually kept under constant conditions of temperature ($20 \pm 1^{\circ}C$), darkness, moisture and food availability. A drop of an emulsion of biological yeast was provided as food.

For each insect, we registered each 8 hr whether or not ecdysis or oviposition episodes had occurred. The observations were made under a microscope and red light.

Six free-running moulting cycles, corresponding to three oviposition cycles, were registered for each individual, for the determination of their average periods.

The insects were divided into N groups: $N = 15$ for moulting and $N = 12$ for oviposition, for the 24 hr temperature pulse experiment, treated in this paper. These numbers are different because in the former we subdivided some groups. Each group received a single temperature pulse at a different phase of each moulting cycle—cycles before and after an oviposition—in order to cover all the phases. The temperature pulse consisted of placing the groups of insects in an

incubator (25°C) for 3, 8, or 24 hr. After the pulse, the insects were returned to the previous constant conditions, under which all subsequent observations were made.

The data obtained were of the episodic type: the moulting cycle is completed with the deposition of the old cuticle, the ecdysis event; the oviposition cycle is completed with the deposition of the eggs. Both episodes were, thus, marker events that provided information about the period and the phase relationship between different cycles. This information on period enabled the determination of the phase change due to the temperature to be calculated: considering, for each insect, T_f as the period of the cycle when there was exposure to the pulse and T_m the average period of all the preceding free-running cycles, delays can be represented as $T_f/T_m > 1$, advances as $T_f/T_m < 1$ and no changes as $T_f/T_m = 1$. Accordingly, we plotted, for each group the relationship between the phase when the temperature pulse was applied and the average of the insects' T_f/T_m values.

Experimental results

FREE-RUNNING CHARACTERISTICS

The free-running recurrences of the moulting and oviposition events showed their self-sustained periodic nature, with periods of 3.9 ± 0.4 and 7.7 ± 0.7 days, respectively, as shown in Fig. 1. Moreover, circadian gates were not observed either in the timing of episodes of moulting or oviposition. When 50 individual insect data chosen at random were inspected separately, ecdysis was shown to occur with approximately uniform probability in any of the 8 hr observation windows. Often it took more than 8 hr for an oviposition episode to finish. Therefore, eggs from the same insect were observed in two or three consecutive inspections. The first window was used as phase reference for oviposition. Activities such as moulting and oviposition make the insect more vulnerable and one clear function of a circadian gate would be to enable the organism to accomplish them in the most favorable and safe time of the day.

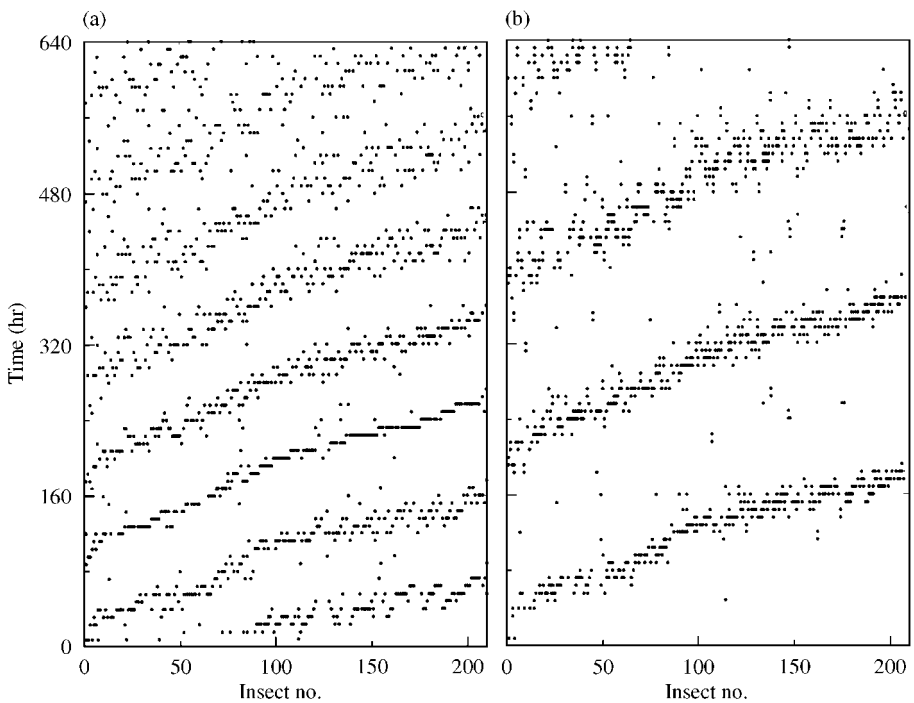

FIG. 1. Free running ecdysis and oviposition events. Insects were individually assigned a number, which is shown in the abscissa. Insects were not synchronized. The numbers were assigned in an order that permitted clear visualization of the rhythmic events. Each insect was inspected at 8 hr intervals for ecdysis or oviposition occurrence. Oviposition was often registered at more than one observational window, at each event. When (a) ecdysis or (b) oviposition was detected, it was registered as a dot at the corresponding (insect number) and (time) coordinate.

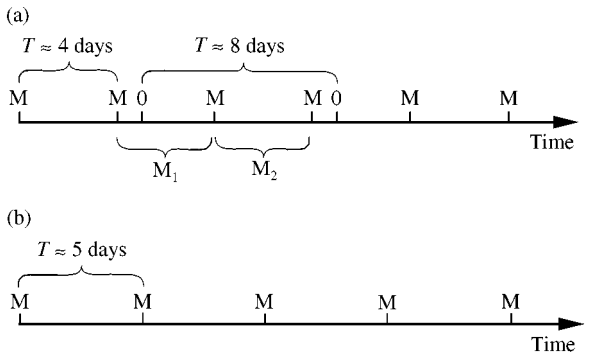

FIG. 2. Schematic view of the time sequence of ecdysis and oviposition events. (a) Periods and phase relationship between the two cycles for a normal insect. Each ecdysis event is indicated by M and each beginning of oviposition by O. Distinction is made between the first (M_1) and the second (M_2) moultings. (b) Period of moulting cycle for an insect without oviposition.

Each oviposition occurred after two moulting episodes, with a mean 18 ± 6 hr phase difference between the ecdysis event and the beginning of oviposition across insects, as shown in Fig. 2(a). This phase relationship was remarkably constant throughout the observations under free-running conditions.

Some insects ($\approx 1\%$) never laid eggs, and the moulting cycles had their periods extended to about 5 days, as can be seen in Fig. 2(b). These two observations—the constant phase relationship between the cycles and the lengthening of one cycle in the absence of the other—formed the basis for our view that the cycles were coupled. To investigate this further, the cycle comprising successive ecdysis events that included oviposition was called "first moulting" (M_1) (period $= 3.7 \pm 0.3$ days), and the other, "second moulting" (M_2) (period $= 4.0 \pm 0.7$ days), as shown in Fig. 2(a); periods of M_1 and M_2 show significant difference (t-test, $p < 0.001$); as a result, the effect of the temperature pulse could be analysed separately for each cycle.

THE PHASE-DEPENDENT RESPONSES

Statistically significant phase-dependent responses were obtained only in experiments using temperature pulses of 24 hr duration ($p < 0.0005$ for moulting and $p < 0.025$ for oviposition, ANOVA), and not for 3 and 8 hr durations. The 24 hr duration data are shown in Fig. 3. The resulting curves presented phase-dependent delays ($T_f/T_m > 1$), for both M_1 and M_2 and for oviposition. The absolute phase change values

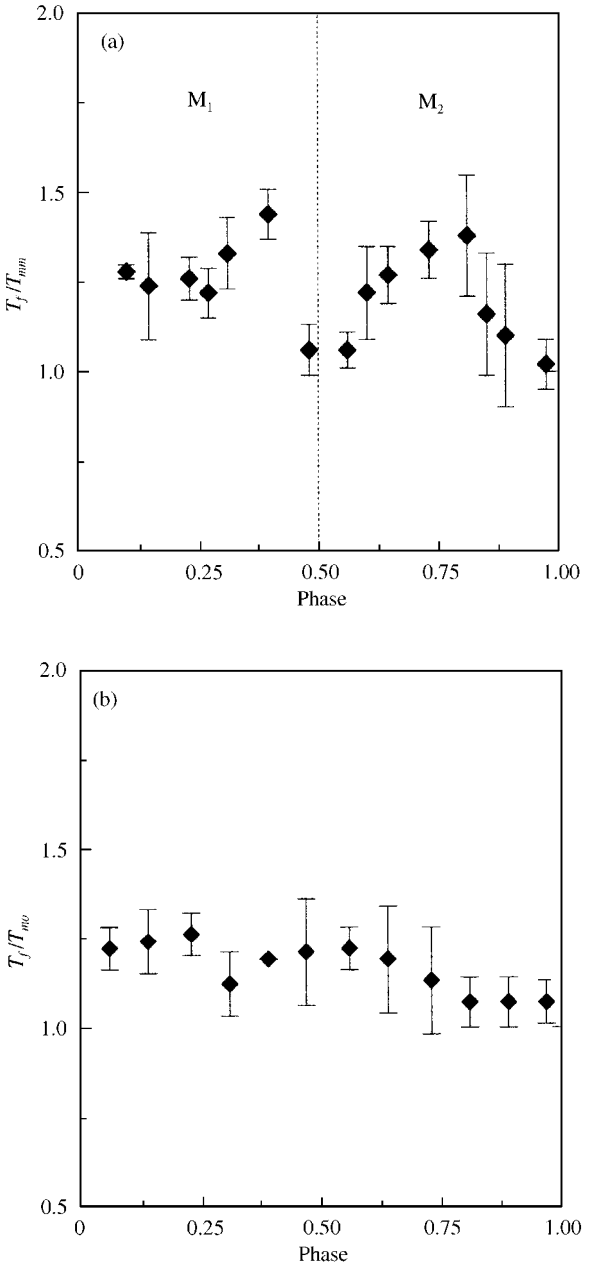

FIG. 3. Phase responses of (a) moulting and (b) oviposition cycles, for 24 hr temperature pulses ($20 \rightarrow 25 \rightarrow 20^{\circ}$C). Phases were assigned at the middle of the pulse durations. Phases are normalized to the average periods. Separate curves of M_1 and M_2 are shown together, in their temporal order of occurrence, corresponding to phases ($0 \rightarrow 0.5$) and ($0.5 \rightarrow 1$), respectively. T_f/T_{mm} = normalized phase shifts for moulting, T_f/T_{mo} = for oviposition. When we had sufficient data, we subdivided some moulting phase groups so that we have 15 phase groups for moulting graph and 12 groups for oviposition graph.

were nearly of the same order, for moulting and oviposition, and therefore values of T_f/T_m differed least from unity in the oviposition curve, due to normalization. A statistical test showed a significant difference between M_1 and M_2 curves ($0.005 < p < 0.01$, ANOVA).

Possible changes in the phase relationship between moulting and oviposition (phase difference between ecdysis episode and beginning of oviposition) due to the pulse were inspected in the following way: for each phase group, the average value for each insect phase relationship in all oviposition cycles before the pulse (Δ), the phase relationship in the first oviposition cycle after the pulse (Δ_1) and the phase relationship in the second cycle after the pulse (Δ_2) were measured. Considering that M_1 and M_2 were each subdivided into six phases [this time, we combined the subdivided phases shown in Fig. 3(a)], significant differences occurred between Δ and Δ_1 in the second phase point of M_1 ($p = 0.005$, t-test) (recovered at the next cycle) and the first phase point of M_2 ($p = 0.008$, t-test). The latter corresponds to a small moulting phase shift and a large oviposition phase shift [Fig. 3(a) and (b)], that led to this change in phase relationship, and which is not recovered by the second cycle after the pulse. Whether this recovery occurred in the next cycles could not be observed, since the experiment was stopped by this stage, due to the diminished number of insects alive. *F. candida* life cycle is around 5 months and survival falls to 50% when the insects are 100–110 days old (Snider, 1972). Two other phases that, conversely, had not shown significant changes 1 cycle after the pulse but present them in the second cycle after the pulse are the fourth phase point of M_1 and the fourth phase point of M_2 ($p = 0.03$ for both phases, t-test).

THE COUPLING EFFECTS

As previously stated, the T_f/T_m values assigned to each phase of the cycles were the result of averaging the values presented by a group of insects pulsed at the same phase. In all experiments—with 3, 8 or 24 hr pulses—all the phases showed an average $T_f/T_m > 1$. In each phase group, however, there were some insects that showed values $T_f/T_m < 1$. Interestingly, the ratio of N_{adv}/N_{del} (number of insects that presented $T_f/T_m < 1$/number of insects that presented $T_f/T_m > 1$, in each group) was dependent on phase, as can be seen in the graphs shown in Fig. 4. In each curve, maximum N_{adv}/N_{del} values tended to be at the later phases. This phase dependency was statistically significant for 24 hr pulses ($F = 0.035$, exact Fisher test for the extreme value phases), even for the 3 hr ($F = 0.027$, exact Fisher test), and 8 hr ($F = 0.044$, exact Fisher test) pulse curves, whose average values for T_f/T_m had not shown significant phase dependencies. Therefore, these graphs can be used as an alternative way of showing phase-dependent responses of the cycles to the short temperature pulses.

Comparing the general shape of the curves shown in Fig. 4 for the 3, 8 and 24 hr pulse experiments, a tendency for decreasing amplitudes produced by the longer pulses curves can be seen. Due to normal fluctuations of inter-individual differences, if the effect of the temperature pulse were zero, an equal number of N_{adv}/N_{del}, for each phase group would be expected. According to the observation that the net effect of the temperature pulse on these cycles is to delay the angular velocity, the longer the pulse, the more efficient had it been in delaying the majority of insects in each group. In this sense, the N_{adv}/N_{del} amplitudes might have diminished with longer temperature pulses due to this effect. Related to this, in some way the effectiveness of temperature pulses in causing a delay was greater in the earlier phases of each moulting and oviposition cycle, since the maximum N_{adv}/N_{del} values are always at the later phases.

Further, differences of N_{adv}/N_{del} between M_1 and M_2 can be seen; in all curves, there is a tendency for higher values in M_2. With 24 hr temperature pulses, this difference can be investigated in a systematic way. There are peaks of N_{adv}/N_{del} in the later phases of both M_1 and M_2, but the M_2 peak is higher than that of M_1. Further, the ($N_{adv}/N_{del} \times$ phase) curve of the oviposition, for the 24 hr pulse experiment, also shows higher values in the later phases of this cycle. Finally, the phase relationship between oviposition and moulting, (Fig. 2), shows the asymmetric influence of oviposition upon the two moulting cycles. If there were no coupling

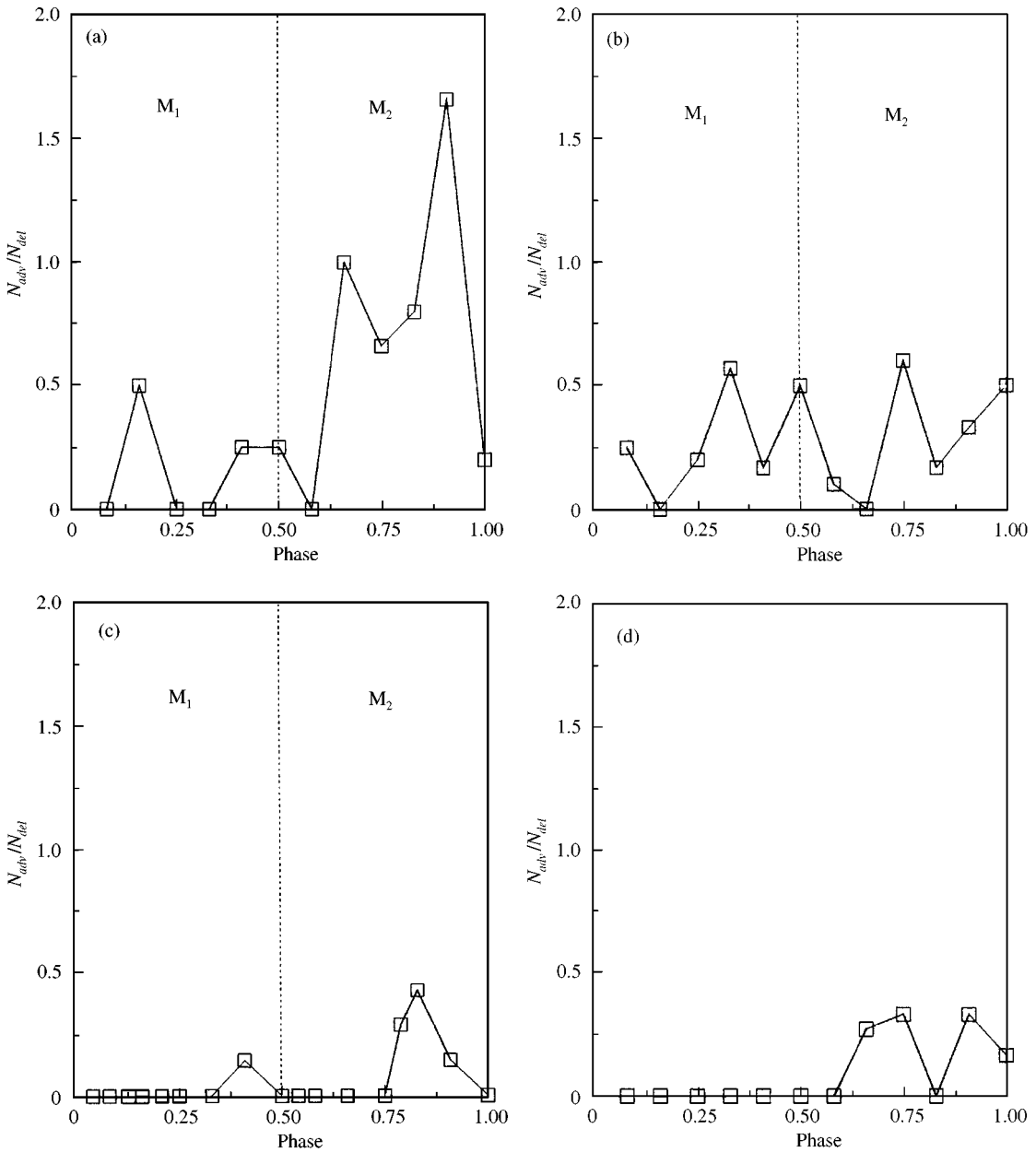

FIG. 4. For each group of insects pulsed at the same phases, the ratio of N_{adv}/N_{del} (no. of phase advances/no. of phase delays) is shown. Phases are normalized to the average periods. (a) 3 hr pulse on moulting, (b) 8 hr pulse on moulting, (c) 24 hr pulse on moulting, (d) 24 hr pulse on oviposition. M_1 = first moulting; M_2 = second moulting. Pulse (20 $\rightarrow$ 25 $\rightarrow$ 20°C).

between oviposition and moulting, both the M_1 and M_2 peaks would have been the same; the M_2 peak phase coincides with the oviposition peak phase [Fig. 4(c) and 4(d)], showing the coupling effect. Comparison between these two figures should consider, nevertheless, an 18 hr phase difference [$\approx 1/10$ of oviposition and $M_1 + M_2$ periods, in Fig. 4(c) and (d)] between moulting and oviposition cycles.

Discussions

The discrimination between a rhythm controlled by a limit cycle and one which is the result of a sequence of stages has not been easy (Tyson & Sachsenmaier, 1978; Gwinner, 1986; Goldbeter, 1996). Verification of the topological characteristics of the PRC has been proposed as the main criterion for the discrimination between limit-cycle oscillators and a one-dimensional

sequence of stages, since only the former can present a strong phase resetting Type 0 curve and a singularity in the phase space (Winfree, 1975; Lakin-Thomas, 1995). In the current experiment, perturbations by temperature pulses induced only weak phase resetting Type 1 curves and these with only delays, for both cycles of moulting and oviposition.

Much is known about the mechanisms generating limit-cycle circadian oscillators (Pavlidis, 1967; Winfree, 1980; Glass & Mackey, 1988), which already arise at the molecular level (Goldbeter, 1995). This implies a clock protein that exerts negative feedback on its own transcription genes and the feedback chain comprises mRNA as well as protein production and degradation. Minimal requirements for this structure to generate sustained, periodic oscillations for a wide range of parameter values are: (1) time delay between the beginning of the reaction chain and the negative feedback exerted by the final protein product; (2) nonlinearity in the protein production and in the cooperativity in the negative feedback (Friesen & Block, 1984; Olde Scheper *et al*., 1999). As a result, we have a rhythmic variation of the concentrations of the proteins and mRNAs involved in the loop, each of them maintaining a constant phase relationship with one another. These concentrations are the state variables of the resulting oscillator. State variable amplitudes change only transiently when externally perturbed, and the original amplitudes are recovered, as this is one of the main characteristics of limit-cycles.

On the other hand, it is known that an ordered sequence of hormones underlies many infradian cycles in insects. Prothoracicotropic hormone (PTTH) and ecdysone are widespread among different insect species. Each hormone was shown to stay in circulation for many days, with the PTTH releasing cells being inactive while the ecdysone releasing cells are active and vice versa, in the silkworm (Williams, 1952). They control developmental sequences both in insects that present metamorphosis and those that do not. They control different developmental sequences at different ages, such as pupation and adult development in the same insect. They also control both sequences that stop once it comes to an end, as in hourglasses, and sequences that cycle, like moulting and oviposition (Williams, 1952). What could be, then, the mechanism that generates the self-sustained oscillation, in a sequence of hormonal releases, as occur with such regularity as in *Folsomia*?

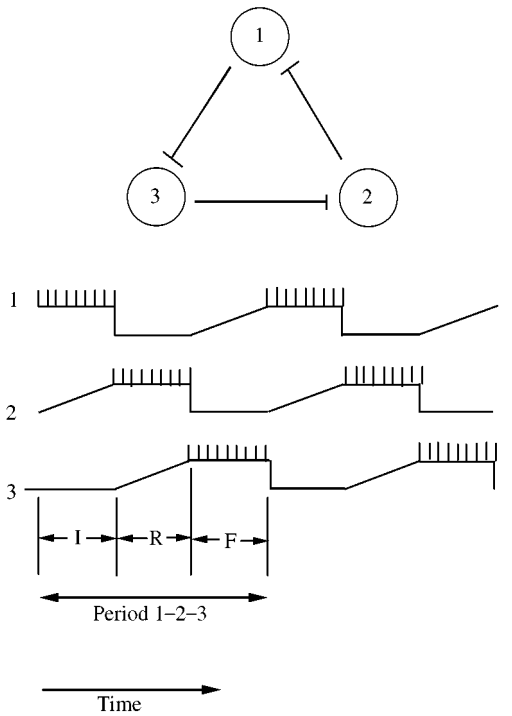

FIG. 5. Simple three-element neural network presenting recurrent cyclic inhibition, redrawn from Kling and Székely (1968). Inhibitory connections between tonically excited neurons are indicated by a T termination. The traces indicate the membrane potential and impulse burst activity in individual cells. Firing phase (F), Inhibited phase (I) and Recovery phase (R) are indicated.

We speculate about the possibility of a mechanism that could generate an oscillation at the hormone–gland interaction level, based on the more well-known neuronal network oscillators. "Recurrent cyclic inhibition" is a neuronal network model that has successfully simulated a ring of neurons firing in a strict sequential order. Neurons fire when their action potentials are above a threshold level and can have excitatory or inhibitory connections with others in a network. In the simple case of three neurons in such a chain (Fig. 5), for example, each tonically excited neuron inhibits the preceding one when it fires so that when neuron 1 fires, it inhibits neuron 3, while neuron 2 recovers from the past inhibition

exerted by 3. When neuron 2 reaches its firing threshold upon recovery it inhibits and "turns-off" neuron 1, allowing neuron 3 to recover from the past inhibition exerted by 1. When 3 in turn recovers and starts firing, it inhibits 2, allowing 1 to recover and beginning the cycle again. In Fig. 5, a schematic diagram of this simplest version of recurrent cyclic inhibition network is shown, together with the firing output of the neurons comprising the net, each one maintaining 120° with each other, a large phase relationship characteristic of the recurrent cyclic inhibition network outputs (Kling & Székely, 1968; Friesen & Stent, 1977; Glass & Mackey, 1988). Analytical study of these kind of networks, containing more elements or with more complex inhibitory connection patterns has shown that they can produce stable oscillations for a wide range of parameters and configurations (Kling & Székely, 1968).

In the case of the simple network described above, the resulting global oscillation can be represented by only one variable, the phase of the cycle, or which element is "on". Pulses applied to these systems only generate Type 1 resetting curves, as characteristic of one-dimensional oscillators, comprising delays and advances.

It is premature to assign a specific mechanism like the recurrent cyclic inhibition to the *Folsomia* hormonal cycle, but it does not seem unrealistic to construct some analogies. The time sequence of hormone concentrations in circulation and the time sequence of firing phases of inhibitory network outputs have some common features, making the necessary adjustments between each specific system: (1) hormones are released by different cells in a definite order, each one staying in circulation for a definite duration; (2) high concentration of each hormone present large phase relationships between each other (Williams, 1952; Truman, 1972); (3) hormones might exert inhibitory actions on other glands and (4) glands present refractory periods when they become inactive, needing a recovery time. In this sense, the hormonal cycle could be viewed as a self-sustained oscillator, where each hormone in circulation might be promoting the disinhibition of the glands that release the next one, in such a way as to generate a stable oscillation with large phase relationships between stages. Some glands would be "off" inhibited by the hormones released by glands that are "on". The transitions between these states would correspond to the recovery process, when the next hormone in the chain started increasing its concentration.

Hormonal sequences actually present more complicated profiles than the simple sequence shown in Fig. 5. Coupling of moulting and oviposition cycles adds complexity to the system and this might explain the different time durations and overlap between some hormones in circulation. Moulting associated with oviposition has another hormone underlying the cyclic process, the JH. The action of JH adds a lot of complexity on the PTTH-ecdysone sequence. In some insects, ecdysone can only be effective when JH concentration falls to zero. In others, it is sufficient that this concentration shows a decrease and still in others, there is a minimum JH concentration necessary for the effectiveness of ecdysone. In *Folsomia*, ecdysone associated with JH results in ecdysis followed by oviposition (M_1) and ecdysone not associated with JH results in ecdysis alone (M_2). Another complicating factor in *Folsomia* moulting cycle is the link with alimentation cycles, a very common link in many insect species. Feeding triggers neuronal terminations at the stomach tissues that send direct impulses to the PTTH releasing glands in the brain (Palévody & Grimal, 1976). It is, in this way, very difficult to explicitly identify these cycles, their interactions and the coupling pathways. According to the recurrent cyclic inhibition model, coupled cycles could correspond to more complex networks presenting extra inhibitory connections. A series of different recurrent cyclic inhibition network structures are presented in Kling & Székely (1968).

In the circadian system model proposed by Pittendrigh & Bruce (1959), a master clock, composed of one or more oscillators, controls the timing of slave rhythms that are lower in the hierarchy (Pittendrigh, 1981). Circadian gates have been observed on ecdysis or oviposition events in some insects (Page, 1985). In these cases, the full cycle comprising a sequence of stages takes a definite time to be completed, but the ecdysis or oviposition events occur only at strictly limited favorable times of the day, when the gate opens. It has also been shown that this gating

process may be linked to one specific hormonal event in a hormonal sequence (Truman, 1972).

Pittendrigh & Skopik (1970) showed explicitly that a phase shift of the circadian clock due to a light pulse changed the timing of the gate in eclosion of *Drosophila*, leaving the developmental sequence of stages leading to eclosion itself unaltered, while different temperatures changed the rate of the developmental sequence, leaving the gate time unaltered. This temperature effect on development alone was also shown by maintaining the insects in LL, and thus damping the circadian oscillator. However, the whole developmental sequence of eclosion happens only once per insect and thus is not a cycle. In our work, we have two coupled developmental sequences that cycle throughout the insect life and the clock is apparently absent due to the cave environment. These cycles are self-sustained oscillators possibly arising at the level of hormone productions and we showed their smooth phase-dependent responses to temperature pulses.

We propose that moulting and oviposition cycles, both for the insects that present circadian gates and for those that do not, have the same oscillatory nature, possibly of recurrent cyclic inhibition, as shown in Fig. 6. A limit-cycle clock may be linked to one of the cycle stages in a master–slave manner. In this case, the stage can be temporarily inhibited, until a favorable time of day is reached, after which the cycle proceeds with its normal sequence. This clock may have lost its adaptive meaning for the cave insects due to a relaxation of selective pressure in the cave environment, and "pure" cycles for moulting and oviposition without interruption are revealed.

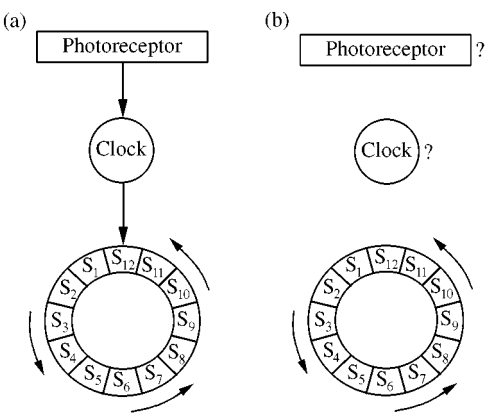

FIG. 6. Schematic comparison of sequences of hormonal stages cycles. Each stage represented by S is viewed as the time of high concentration of each hormone in circulation: (a) for an insect that presents circadian gates, (b) for the cave insect that apparently has lost the circadian component.

Conclusions

Most of the endocrine and other physiological processes of insects are based upon sequence of hormonal stages cycles. These cycles probably have a very complex structure but we propose that they can be viewed as one-dimensional oscillators arising at the hormone–gland interaction level. Moulting and oviposition cycles present regular periods, phase relationships, smooth phase-dependent responses to temperature pulses and coupling effects. These cycles are thus competent oscillators by themselves, but some of them need the environmental phase information from the circadian clock, which opens a gate for final episodic events such as oviposition or ecdysis to occur at the most favorable time of the day. The circadian gate often regulates some intermediate stages, in the process of internal organization, but in this case also ultimately transmitting the phase information from the environment. This environmental phase information is apparently not important for many species that never leave the cave. For such cave species the circadian clock, as well as vision, have lost adaptive meaning in this environment. On the other hand, proper timing of different cycles among themselves—the internal temporal organization—is always of vital importance and this is achieved by the maintenance of rhythmic processes and coupling of their underlying oscillators in the cave organisms.

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