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Self-organization of circadian rhythms in groups of honeybees (*Apis mellifera* L.)

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Abstract. Workers in social groups of honeybees (*Apis mellifera* L.) synchronize their individual free-running circadian rhythms to an overall group rhythm. By monitoring the activity of bees by recording the oxygen consumption and intragroup temperature, it is shown that the rhythm coordination is in part achieved by temperature fluctuations as an intragroup *Zeitgeber*. Trophallaxis was shown to have only a minor (if any) effect on circadian rhythm synchronization. A model incorporating a feed back loop between temperature and activity can plausibly explain the observed synchronization of individual rhythms in social groups as a self-organization phenomenon.

Key words: *Apis mellifera* – Circadian rhythm – Synchronization – Temperature – *Zeitgeber*

Introduction

Alternating phases of rest and activity of organisms are governed by exogenous *Zeitgebers* and an endogenous biological clock. Endogenous circadian rhythms of individual organisms in the absence of environmental time cues are a well-established phenomenon throughout the animal kingdom (Aschoff 1989). In the honeybee (*Apis mellifera*) free-running circadian rhythms of individual bees under constant environmental conditions have been reported. Like many other insects, individual worker bees, though clearly diurnal organisms, do not follow Aschoff's rule (Aschoff 1960) and express a longer period under constant light (LL) than under constant dark (DD) conditions (Moore and Rankin 1985; Spangler 1972; Frisch and Aschoff 1987; B. Frisch and N. Koeniger unpublished manuscript). The endogenous rhythm keys in on a variety of external *Zeitgebers*. In honeybees, the light-dark cycle has been shown to have a most significant effect on the expression of activity cycles of both individual workers and colonies of the honeybee (Renner 1959; Beier 1968). Further cues have been suggested as *Zeitgebers*, including the time of food availability (Beier 1968) and fluctuations in the earth's magnetic field (Martin et al. 1983; Korall and Martin 1987). Neumann (1988), however, strongly questioned that the earth's magnetic field had any effect on activity rhythms and could not support the findings of Martin et al. (1983). Irrespective of this contention, it is clear that the endogenous circadian rhythms of individual workers isolated from the colony can be very different. B. Frisch and N. Koeniger (unpubl. manuscript) report that the period of free-running rhythms of isolated individual workers of a single colony can vary by more than 45 min. However, to function as a whole, the colony is clearly in need of some *Zeitgeber* to synchronize these individual clocks. Medugorac and Lindauer (1967) found that foraging activity is affected by social parameters. Workers entrained to a specific foraging time in their mother colony foraged at two times when transferred to another host colony: their original time, and the foraging time the host colony was entrained to. Moritz and Sakofski (1991) showed that the queen interferes with the circadian rhythms of social groups of honeybees; however, the queen alone cannot function as the "clock of the colony" since queenless worker groups have perfectly synchronized free-running rhythms (Southwick and Moritz 1987). These authors found that the bees required close physical contact to establish a synchronized group rhythm. In the present paper we study the mode of group synchronization in groups of bees with different phase entrainment, and the possible synchronization mechanisms through a behavioural (trophallaxis) and a physiological parameter (temperature) that could potentially serve as clock control systems in social groups of honeybees.

Materials and methods

Phase entrainment and experimental groups. We analyzed the synchronization mechanisms by mixing bees that were entrained to

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two different light-dark (LD) regimes. Three colonies were kept in the field (outdoors) and three were kept in an indoor flight room (conditions as given in van Praagh 1972) that was programmed to an LD phase, 8 h shifted from the outside LD cycle (the flight-room lighting was switched on 8 h after sunrise and switched off 8 h after sunset). The onset of light and dark was readjusted during the experiments, corresponding to the change in outside conditions. After 4 weeks most bees in the flight room had never encountered a natural LD cycle and they were completely entrained to the 8-h phase shift. During the time when indoor and outdoor light phase overlapped, workers were collected from the honey stores of in- and outdoor colonies and placed in screened cages in groups of 100. Groups with various ratios of indoor and outdoor bees, and unmixed groups, were tested. The groups were transferred to constant dark conditions (DD) in an incubator (20° C, 60% rel.hum.) with pollen and honey supplies for 7 days. Circadian activity rhythms were monitored over 7 days by recording the oxygen consumption of the groups using a technique similar to that of Moritz and Sakofski (1991). Oxygen levels were determined with a zirconia cell oxygen analyzer (Applied Electrochemistry S3-A) in a computer controlled flow-through system (30 ml/min) and recorded on line (for details of the oxygen measuring system, see Moritz and Sakofski 1991).

The raw data were smoothed with the moving average technique (mean over ± 1.6 h, STATGRAPHICS package) and the periods were determined by calculating a regression of peak activity on time in the periodograms (Enright 1981). Phase shifts were computed from the differences between the times of peak activity of the smoothed data.

Trophallaxis as Zeitgeber. In order to reveal the effect of trophallaxis on the synchronization of the group activity cycle, 50 indoor and outdoor bees each were kept separately in two sub-compartments of the cages. The subgroups were separated by a plexiglas sheet (1.2 mm) perforated with 20, 30 or 35 holes (diameter 2.5 mm) in order to prevent mixing of the subgroups and restrict, but not completely obstruct the food exchange between both groups. Groups were also tested with solid plexiglas divisions to entirely preclude trophallaxis.

Instead of measuring the overall oxygen consumption of the group, temperatures were measured in each subgroup as an indication of the metabolic activity. Two thermistors monitored the temperature next to the centre of each side of the Plexiglas division and the readings were stored on-line through a 3-day experimental period, after which synchronization had usually occurred. To quantify the trophallactic food flow from one compartment to the other, one subgroup was fed with methylene-blue-dyed sugar syrup (1% dye, max. absorption at 661 nm) the other one with sugar dyed with new coccine red (1% dye, max. absorption at 509 nm). At the end of the experiment each subgroup was killed by freezing, homogenized in ethanol, and centrifuged at 2000 g_{av}. The optical density was determined over a spectral range from 450–700 nm in order to quantify the amounts of dye (and transferred food) in each subgroup.

Temperature as Zeitgeber. Unmixed groups of 100 in- and outdoor bees (5 groups each) were tested throughout a 7-day period. The groups were initially kept under DD at 21° C. After 10 h, during the period of low activity of the outdoor bees, the temperature was decreased to 13° C and reset to 21° C after 8 h for the rest of the experiment. The oxygen concentration in each group was monitored as described above.

To reveal the sensitivity of the free-running period to temperature changes we exposed outdoor bee groups to a cooling pulse of 2°, 3°, 6°, and 8° C (7–10 groups of 100 bees each) for 8 h during the activity phase. Activity was measured via the intragroup temperature by thermistors as described above.

Results

Mixed groups

The free-running period of the in- and outdoor groups was not significantly different (20.95 ± 0.34 h, $n=10$, and 21.5 ± 0.27 h, $n=10$ respectively). Overall, an average period of 21.0 ± 0.17 h ($n=50$ groups) was determined through a periodogram regression analysis (Enright 1981). There was a significant phase shift of 4.73 ± 0.56 h in the free-running cycle of unmixed indoor groups compared to the outdoor bees. The mixed groups with unrestricted trophallaxis had synchronized free-running rhythms with intermediate phase shifts corresponding to the group composition (Fig. 1). The data fit a linear regression model ($b=0.0284$; $r^2=0.55$, $P<0.001$) in which the group rhythm reflects the arithmetic mean of all individual group members.

Restricted trophallaxis

Although the bees were theoretically able to perform trophallaxis in the cages with the perforated plexiglas divisions, we did not find any traces of transferred food in either subgroups. Rhythm synchronization, however, was possible (Fig. 2), though it was not as efficient as in groups with unrestricted contact. There was no significant correlation between the number of holes in the dividing sheet and the group phase shift between two subgroups divided by a perforated division. The phase shift averaged 1.3 ± 0.19 h ($n=120$).

In cages with a solid plexiglas plane, synchronization was also observed, and the phase shift between the two groups was significantly smaller (2.84 ± 0.36 h, $n=120$) than between completely separated in- and outdoor groups in separate cages ($t=2.1$ $P<0.05$). On the other hand, the phase shift remained significantly larger than in cages with perforated divisions ($t=3.78$, $P<0.001$, Fig. 2).

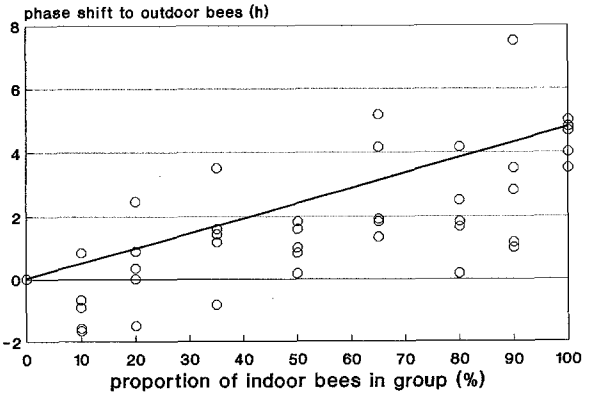

Fig. 1. Phase shift of circadian rhythm in mixed groups composed of in- and outdoor groups in relation to the phase of pure outdoor groups. The *straight line* represents the expected phase shift if the group rhythm equals the arithmetic mean of the individual rhythms of all group members

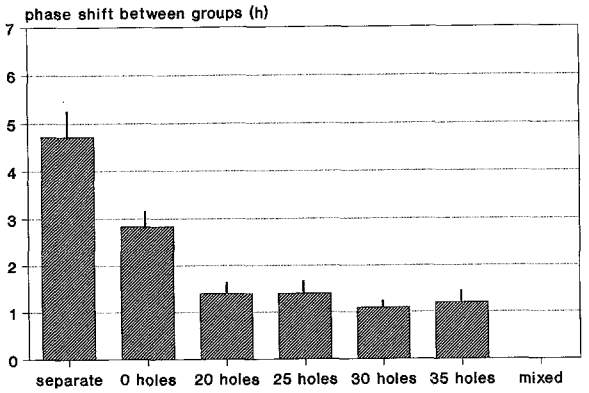

Fig. 2. Phase shift between two groups of in- and outdoor worker bees. *Separate*, groups in completely separated cages; *0 holes*, two groups in one cage but separated by a 1.2-mm plexiglas division; *20*, *25*, *30* and *35 holes*, division is perforated with the corresponding number of 2.5-mm diameter holes; *Mixed*=both groups in the same cage with no division

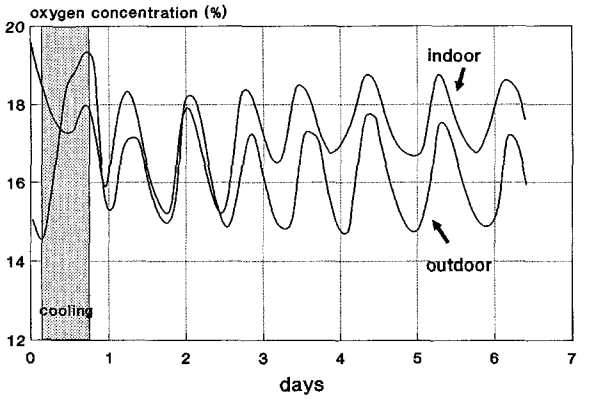

Fig. 3. Synchronization of the metabolic activity (measured via oxygen concentration) in an initially out-of-phase indoor and an outdoor group of 100 workers after a cooling pulse of 8° C. The effect is most conspicuous in the indoor group where the activity abruptly rises when the environmental temperature is again set to the old value of 21 °C

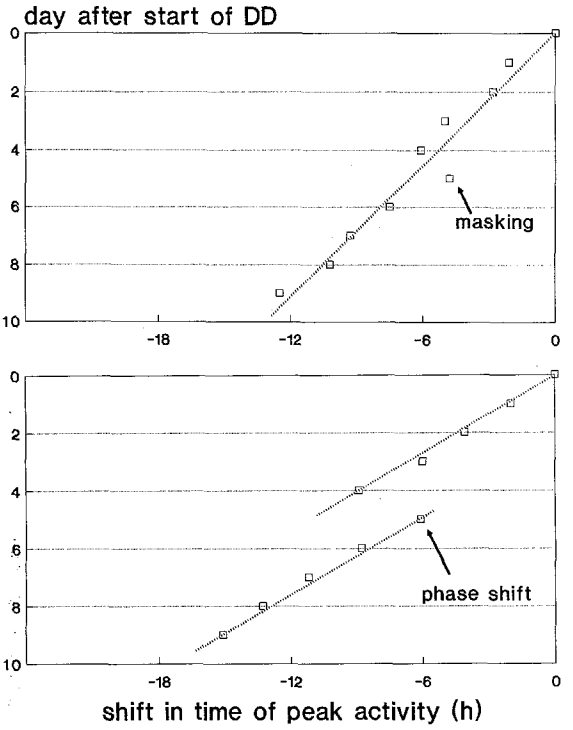

Fig. 4. Shifts in the time of peak activity during subsequent days after onset of constant dark (*DD*). The groups were treated with a cooling pulse of 6 °C for 8 h on day 5. Three out of seven tested groups responded with a masking of the free running rhythm and returned to the original oscillation (*top*). Five groups responded with a permanent phase shift (*bottom*)

Cooling pulse

Figure 3 shows a typical time plot of an indoor and an outdoor group of bees that were treated with an 8-h cooling pulse of 8° C 10 h after the experiment started. The initially out-of-phase groups key in on a common free running rhythm. Although the absolute levels of metabolic activity appear to be different, there is a conspicuous phase synchronization after 18 h. In ten such experiments, the average phase difference between in- and outdoor groups after the temperature treatment decreased to 2.4 ± 0.38 h compared to 4.73 h under constant temperature ($t = 4.45$, $P < 0.001$).

The thermal insulation provided by the plexiglas division appeared to be insignificant for the biological phenomena found in this study. A control experiment, in which one compartment was left empty, and the other one filled with 50 workers, showed that the temperature in the empty compartment followed all temperature fluctuations of the cluster within the range of the thermistor accuracy. The difference between the two temperature readings on either side of the plexiglas division averaged at 0.0 ± 0.05° C ($n = 45$).

The effect of temperature as a *Zeitgeber* was confirmed in the experiments using different temperatures in the cooling pulse. A reduction of 6° C seems to be a critical temperature in the setting we choose. Four groups responded with a permanent phase shift ranging from 2.7 h to 7.9 h, but three other groups masked their circadian rhythm with a single extended rest phase and returned to the original free-running activity oscillation (Fig. 4). An 8-h decrease of the temperature b 3° C had no long-term effect on the free-running rhythm. Only the cycle directly exposed to the cooling pulse was extended, masking the circadian rhythm. A decrease of 2° C had no effect at all, and the circadian activity cycle remained undisturbed. Even a 24-h oscillation of the ambient temperature by ± 2° C between objective day and night, did not interfere with the free-running rhythm of outdoor groups. The 8° C cooling pulse caused a significant permanent phase shift of 2.7 ± 0.65 h (ranging from 1.6 to 6.4 h) in the activity rhythm in all nine groups tested. In no case was the period affected by the cooling pulse.

Discussion

Obviously, groups of honeybee workers were able to evolve a joint group cycle within a very short time even

if composed of subgroups which were entrained to very different LD cycles (8-h phase shift). Although trophallaxis has been suggested to play a major role in communication (Korst and Velthuis 1982), the mode of synchronization of circadian rhythms seems to be, if at all, only weakly affected by social food exchange. Groups composed of differently phase-entrained bees did show synchronization even if food exchange was completely prevented by the plexiglas division. Synchronization was more clearly expressed in the groups if the division was perforated, yet the amount of food exchanged was below the detection limits of our analytical instruments. Furthermore, even in the case of solid divisions the bees were able to synchronize, though to a lower extent.

At the first glance this seems to contrast with the results of Southwick and Moritz (1987), who found that bees separated by a double wire mesh screen were unable to synchronize their free running activity rhythms. However, their experimental conditions were very different to some critical features of our present study. The test containers in Southwick and Moritz (1987) were flushed with high air-flow rates (200 ml/min) and the subgroups were separated by more than 10 mm. Given that temperature is the key to synchronization, we cannot expect the bees to evolve a synchronized rhythm in such a setting. It is unlikely that the bees in one compartment can perceive and respond to temperature fluctuations in the bee cluster in the other compartment of the test cage. Also, any poorly volatile or contact chemical cues would not be readily transmitted because of the wide space that separated the subgroups. Such chemical cues were suggested by Moritz and Sakofski (1991) when they observed that the queen played a significant *Zeitgeber* role in the synchronization of the group rhythm. The thin perforated plexiglas divisions used in our study would certainly allow the transmission of such chemical cues. Moreover, as the control experiment revealed, temperature fluctuations in a cluster of bees on one side could easily be detected on the other.

Intragroup temperature fluctuations provide a particularly attractive model for group rhythm synchronization, in that it needs no hierarchical control system to allow the development of one common group rhythm. If two sinusoidal waves with a phase shift are averaged, a new, angle-shifted sinusoidal wave will result. Thus even if all the workers maintain their own individual activity rhythm, at the group level we would expect to see a perfect average activity cycle. This reflects exactly what we find in our experiments with unrestricted mixed worker groups. Immediately after mixing, the groups reveal a new "average" cyclic activity pattern (Southwick and Moritz 1987; this study). Since activity relates directly to metabolic excess temperature, a thermal group cycle develops. In due time the individual bees will key in to this new temperature cycle and the phase shift is completed at the individual level. This scenario has experimental support from our experiment in which we decreased the temperature by an 8° C cooling pulse and reset the clock by almost 2.5 h. This single cooling pulse caused, in some cases, perfect synchronization of completely separated groups. The experiments in which we modified the magnitude of the cooling pulse clearly demonstrate that temperature differences greater than 6° C can form a *Zeitgeber* and cause permanent phase shifts in the free-running cycle. The oscillating system is compensated for temperature fluctuations of 2° C and less. The 3° C cooling pulse caused only a single cycle shift and had no long-term effects on the activity rhythm in our bioassay. In the colony, however, such temperature changes may have an effect on the colony activity rhythm, if the low temperature phases are repeated at circadian intervals.

In the mixed-group situation, the bees were exposed to continuous temperature fluctuations corresponding to the individual rhythm patterns of the group members. It is well known that temperature determines the activity of the bee. In the temperature range between 30° and 20° C an increase in temperature directly relates to an increase in metabolic activity in both individual bees and groups (for recent reviews see Heinrich 1993; Moritz and Southwick 1992). A feedback loop between temperature and metabolic activity, based on an individual endogenous circadian rhythm, can therefore plausibly explain the synchronization of individual activity rhythms to the group rhythm.

Although temperature is an attractive candidate to explain group synchronization, it certainly operates in concert with other, more complex social *Zeitgebers*. The role of the queen as a social *Zeitgeber* has been addressed above (Moritz and Sakofski 1991). Our results with the groups divided by the perforated plexiglas sheets also indicate such effects. We found no significant difference between the thermal insulation value of perforated and solid plexiglas divisions. Nevertheless, the groups separated by perforated divisions were significantly better at synchronization than those with the solid divisions. Because there was no detectable food exchange, flow of some other cues must have affected the circadian rhythms of the groups on either side of the perforated divisions. The phase shift accomplished by these cues should not be underestimated, since they were of the same order of magnitude as that of the temperature effects. Experiments in which both groups are thermally isolated, but still can exchange these other as yet unknown cues, might further illuminate the mode of synchronization of individual circadian rhythms in social groups.

Circadian rhythms of social groups seem to present another example of self organization in colonies of the honey bee (Camazine et al. 1990; Seeley et al. 1991). Although queens have partial control over colony activity cycles, the very localized thermal conditions of a worker also determine its metabolic activity. This very simple feedback system alone can plausibly explain the regulation of colony activity cycles in the absence of external *Zeitgebers*. Such a mechanism may also have implications for colonies of social insects other than the honeybee, as temperature is a major determinant of insect activity in general (Wigglesworth 1984).

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