J Comp Physiol B (1989) 159:569–575

Journal of Comparative Physiology B Biochemical, Systemic, and Environmental Physiology

Endogenous 24-hour cycle of core temperature and oxygen consumption in week-old Zucker rat pups

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Accepted May 11, 1989

Summary. Experiments were designed to test whether or not the 24-h core temperature fluctuations in week-old rat pups are of endogenous origin. Lean (Fa/−) Zucker rat pups born on the same day to mothers maintained in two different colonies with light/dark cycles 12 h out of phase with each other were mother-reared through the first 3–4 days of life and then artificially reared simultaneously in constant dim light. Continuous, automatic measurement of core temperature and oxygen consumption during artificial rearing showed clear 24-h rhythms in 5- to 8-day-old pups. Each rhythm reached a daily minimum at a time corresponding to the beginning of the light period in the colony of origin. The amplitude of these rhythms did not diminish during artificial rearing, nor did the phase difference between the rhythms of pups originating in the two colonies systematically change. The persistent 12-h phase differences between these two groups of pups prove that the observed rhythms are not caused by exogenous stimuli. We conclude that the rat pup possesses an endogenous time-keeping mechanism that permits the expression of overt rhythmicity at the age of 1 week.

Key words: Artificial rearing – Circadian – Development – Newborn – Thermoregulation

Newborn rats display a surprising ability to maintain homeostasis in the face of physiological challenges, despite the incomplete differentiation of central nervous structures which seem to be important for normal autonomic functions in mature animals. Body temperature, for example, is regulated long before maturation of CNS areas usually considered essential for thermoregulation in the adult (Schmidt et al. 1987). Overt circadian rhythms of physiological variables, on the other hand, have been believed to appear rather late in development (Davis 1981), even though the metabolic activity of the suprachiasmatic nucleus (SCN) shows a daily rhythm before birth (Reppert and Schwartz 1983). Although circadian variations of physiological variables in week-old rat pups have been reported (Henning and Gisel 1980; Infurna 1981; Schmidt et al. 1986), a pup's susceptibility to the strongly circadian patterns of maternal care (Ader and Grota 1970; Leon 1986) make it impossible to infer an endogenous origin in these studies.

Artificial rearing of isolated rat pups under controlled ambient conditions and feeding schedules allows them to be studied in the absence of 24-h variations of external factors. Smith and Anderson (1984) used this technique to study the development of locomotor rhythmicity, and in the work reported here we employed it to learn whether the 24-h fluctuations of core temperature observed in week-old rat pups (Schmidt et al. 1986) are of exogenous or endogenous origin. This was done by continuously and automatically measuring thermoregulatory responses in two groups of 5- to 8-day-old pups artificially reared simultaneously in continuous light from 4 days of age but stemming from mothers living in light/dark cycles that were 12 h out of phase.

Methods

Animals

Lean (Fa/−) Zucker rats were derived from breeding stock maintained in two different rooms at 22 °C and 55% relative

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Abbreviations: Tc core temperature; *LD-pups* born to a mother entrained to a 7:00 to 19:00 light cycle, then artificially reared in constant dim light; *DL-pups* born to a mother entrained to a 19:00 to 7:00 light cycle, then artificially reared in constant dim light; *SCN* suprachiasmatic nucleus

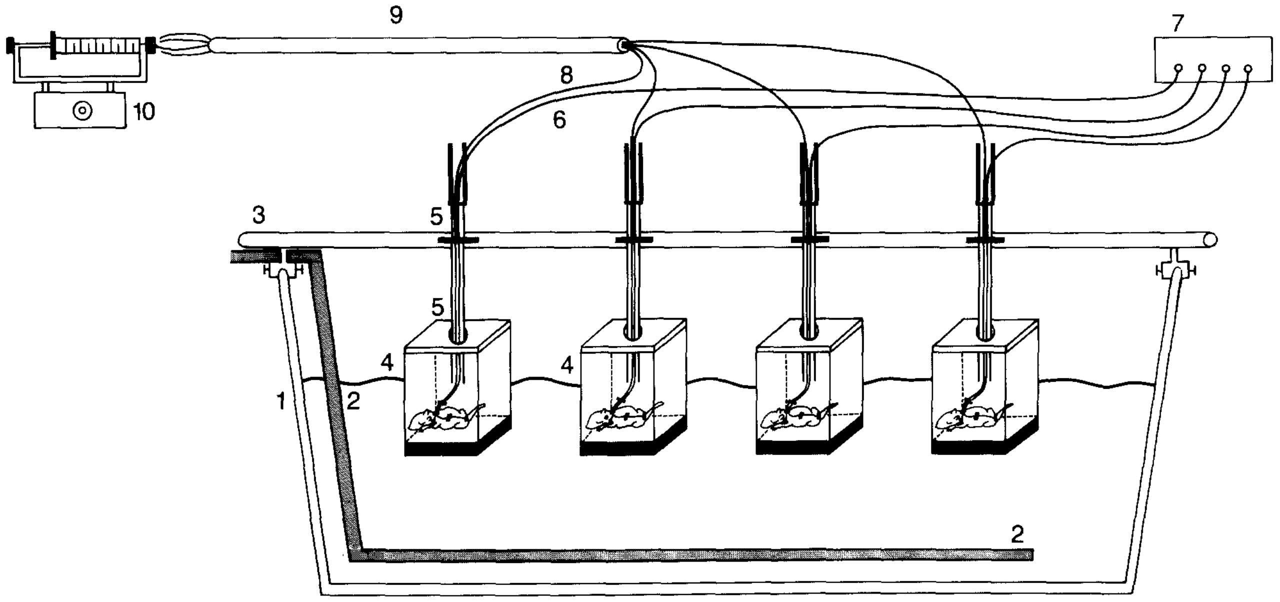

Fig. 1. Schematic diagram of the experimental set-up showing 4 of the 12 animal chambers. 1, water-filled plastic tub; 2, heating coil; 3, metal grid; 4, translucent animal chamber; 5, metal tubing for aspirating air samples; 6, thermocouple; 7, amplifier and recording units; 8, milk line; 9, cooling device; 10, refrigerated milk pump

humidity. In one room lights were on from 7:00 to 19:00 (LD-colony) and in the other, from 19:00 to 7:00 (DL-colony). Routine care was performed at the onset of the daily activity phase, shortly before the lights went out. Vaginal smears were used to control breeding so that pups in both colonies were born on the same day. Pups were mother-reared in the colonies until the experiments began.

Artificial rearing

Artificial rearing was started at 14:00 when the pups were 3 to 4 days old (birth = day 0). Intragastric catheters were implanted, as described by Hall (1975), during a few minutes of anesthesia. Briefly, a PP10 catheter was orally introduced and externalized at the left flank with the help of a wire stylet. One end of the catheter was secured in the stomach by a snugly fitting 1 mm wide silastic sleeve; the other end was looped and anchored (again with silastic sleeves) at the nape of the neck. Because of a 20% surgical mortality rate with the originally used halothane anesthesia, catheters were implanted after producing respiratory arrest with CO_2 anesthesia. All pups survived the CO_2 anesthesia and were fully awake within 10 min.

Synthetic milk – prepared from condensed milk, corn oil, and water, with vitamin and mineral supplements (Messer et al. 1969) – was continuously infused by multichannel infusion pumps. The infusion rate was increased stepwise from 1.5 ml $\cdot$ day^{-1} for 3-day-old rats to 3.5 ml $\cdot$ day^{-1} for 8-day-old rats.

Artificial rearing took place in a walk-in climatic chamber maintained at a relative humidity of 55%. To maintain an approximately constant thermal load for the rapidly growing animals, the temperature of the climatic chamber was set to 32 °C for 4-day-old pups and was gradually decreased to 28 °C for 8-day-old pups. Air temperature in the climatic chamber oscillated with an amplitude of ±0.6 °C and a frequency of 12 cycles per h around the set mean value. The mean value was stable to within 0.2 °C over 24 h. Up to 12 isolated pups could be singly reared in 8 × 8 × 10 cm clear plastic containers floating in a temperature-controlled water bath kept 1 °C warmer than the controlled air temperature to avoid condensation (Fig. 1). Ambient temperature in the animal containers was thus 1 °C

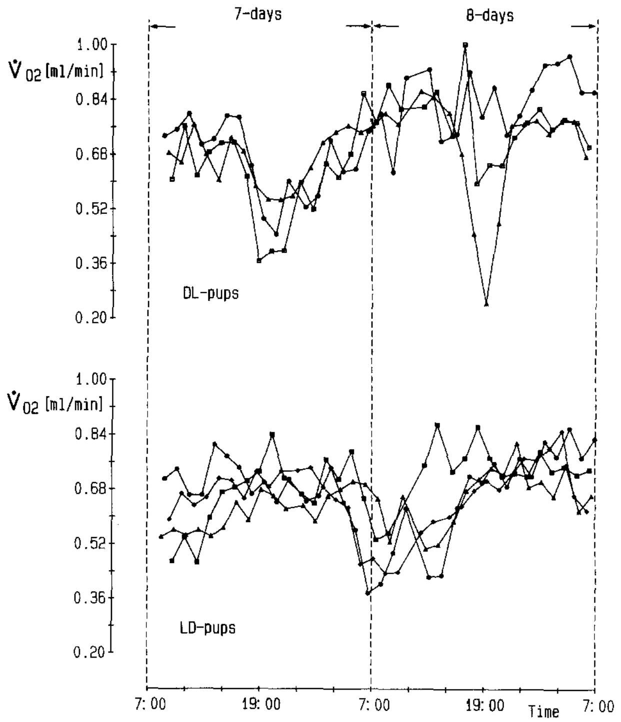

Fig. 2. Oxygen consumption of 7- and 8-day-old pups from two litters artificially reared together in constant dim light. Pups were born and mother-reared up to 4 days of age in colonies with lights on from 19:00 to 7:00 for DL-pups or from 7:00 to 19:00 for the LD-pups

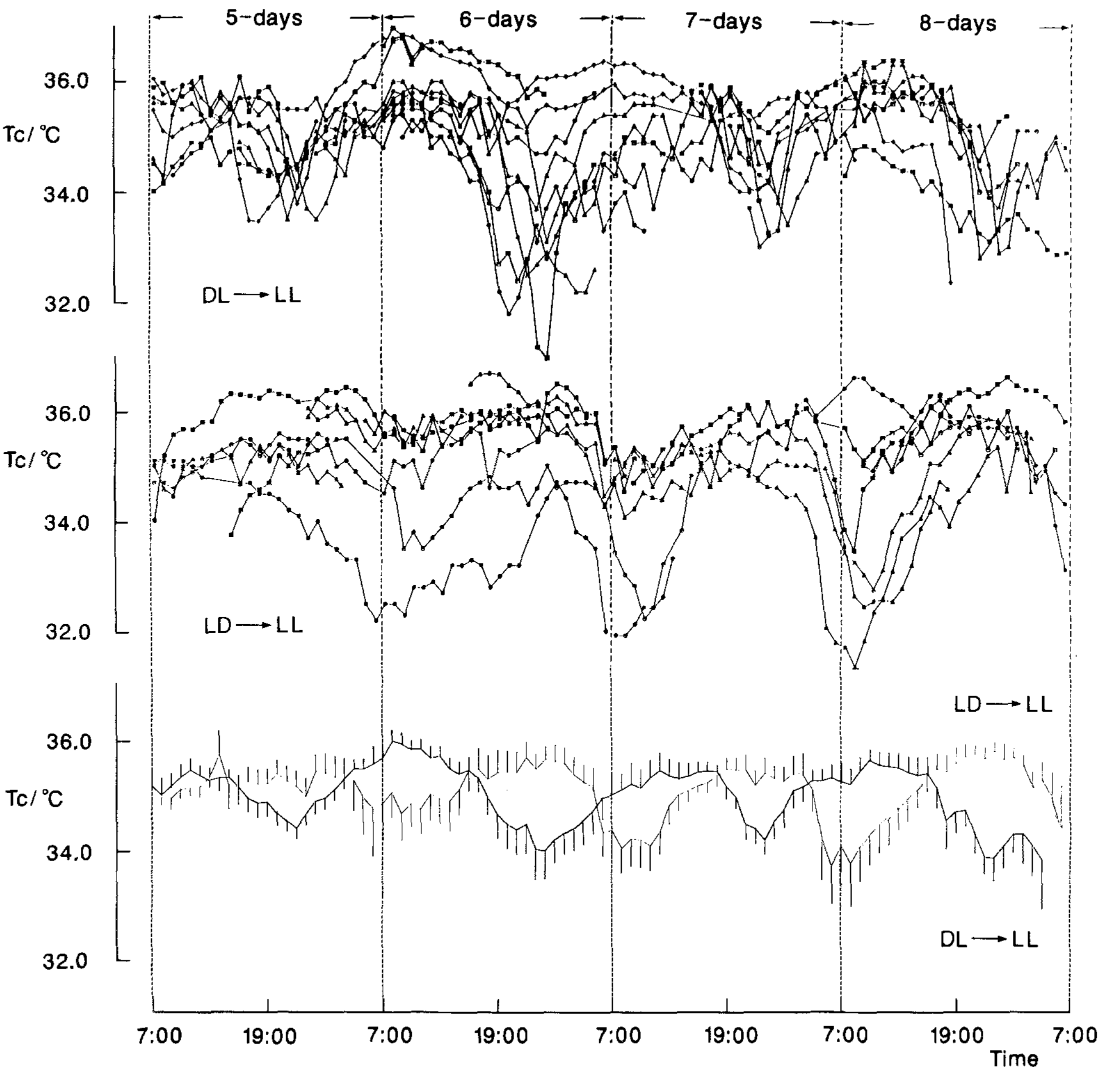

Fig. 3. Hourly mean core temperatures during artificial rearing in constant dim light of individual DL-pups (top), individual LD-pups (middle) and mean values ± standard error for both groups (bottom). Pups were born and mother-reared up to 4 days of age in colonies with lights on from 19:00 to 7:00 for DL-pups or from 7:00 to 19:00 for the LD-pups

above the set temperature of the climatic chamber. The animal containers could freely rotate around metal tubes to prevent tangling of connecting lines. Through these tubes air was continuously aspirated from the bottom of the container at a rate adjusted to keep oxygen extraction at about 0.5%.

Measurement

The airstream from each animal container passed through a column of anhydrous $CaSO_4$ (Drierite) to remove water vapor. Every 10 min a timer circuit connected to 3-way magnetic values sequentially diverted the dried air continuously aspirated from each of the seven animal containers (and one empty reference container) through a Tylan mass flow meter. Water manometers were used to set and monitor bypass airflows equal to the airflow during the 10 min measuring periods. Downstream from the mass flow meter and main flow air-pump, an aliquot of this airstream was aspirated through an electrochemical oxygen analyzer (Ametek S-3A). The position of the magnetic valves, as well as airflow and oxygen content of aspirated air, were simultaneously recorded on both chart paper by a multichannel linewriter and on floppy discs by a personal computer.

In each experiment the colonic temperature (Tc) of six rats was continuously recorded, starting on the day following surgery, with fine (0.06 mm) copper-constantan thermocouples (California Finewire) sheathed in soft silastic tubing (Dow-Corning 602-105). Thermocouples were first taped to the tail and then led to the neck. Milk lines and thermocouples were securely taped to a grid straddling the water bath to prevent tangling of the connecting lines without restricting movement of the pups (Fig. 1). Core temperatures were recorded on a multichannel point printer as well as on the computer. Climatic chamber and water bath temperatures were also continuously recorded.

Procedure

Pups born in the DL-colony (DL-pups) were reared simultaneously with pups born in the LD-colony (LD-pups) in constant dim light (80 lux). All animals were routinely tended at 16-h intervals – at 6:00 and 22:00 of even-numbered days and at 14:00 of odd-numbered days, or vice versa. At these tending times the absorbent padding on the floor of the containers was changed, and the condition of each animal was evaluated. A common sign of all different kinds of health problems during artificial rearing was an obvious failure of stomach emptying associated with hypothermia. Animals with overfilled stomachs were removed from the experiment. Attachment of the thermocouples was checked at each tending, and broken thermocouples were repaired only during the regularly scheduled tending periods. Thermocouples were routinely removed and repositioned at every second tending when the pups were weighed. Feeding rate, ambient temperature, and airflow were also adjusted, when required, only at the 16-h tending intervals.

Evaluation

For six experiments a total of 48 pups underwent surgery, and data from 25 could be evaluated. To avoid inclusion of artifactual minima from pups found with overfilled stomachs, we excluded all data from these animals which were gathered after the immediately preceding daily Tc maximum (defined below). Hourly mean values of core temperatures were calculated from the computer data stored every 15 s. Assuming an RQ of 0.75 (Planche et al. 1983), the equation for a flow-through mask was used to calculate each animal's oxygen consumption from the flow rate and oxygen content of the dried air aspirated from the animal containers (Withers 1977). To exclude mixing artifacts associated with switching the analyzed airstream between animals, oxygen consumption was calculated only from data collected during the second half of each 10-min measuring period. Body masses for calculating mass-specific metabolic rates were linearly extrapolated during the 32-h intervals between weighings.

Data from both LD- and DL-pups were grouped into 24-h days starting at 7:00 when the animals were 5 days old. For each animal the daily minimum and maximum for both core temperature and oxygen consumption were defined as the mean of the three lowest and highest consecutive values. Data are presented as mean values ± SE, and differences between LD- and DL-pups were tested for significance ($P<0.05$) with the Mann-Whitney U-test.

Results

Body masses of the healthy, artificially reared rats which yielded data for evaluation increased by more than 0.7 g per day. These pups grew from 9.4 ± 0.2 g ($n=25$) at 5 days of age to 12.3 ± 0.3 g at 8 days of age. Daily mean oxygen consumption increased during this time from 0.45 ± 0.05 ml · min^{-1} to 0.70 ± 0.07 ml · min^{-1}. The increase of metabolic rate was, however, not continuous. As Fig. 2 shows for a typical example, the oxygen consumption of simultaneously reared LD- and DL-pups decreased every day at about the time the light period started in the colony of origin – i.e., about 12 h out of phase between the two litters. This corresponds roughly to the time at which a minimum of core temperature defense occurs in mother-reared pups briefly separated from the dam (Schmidt et al. 1986).

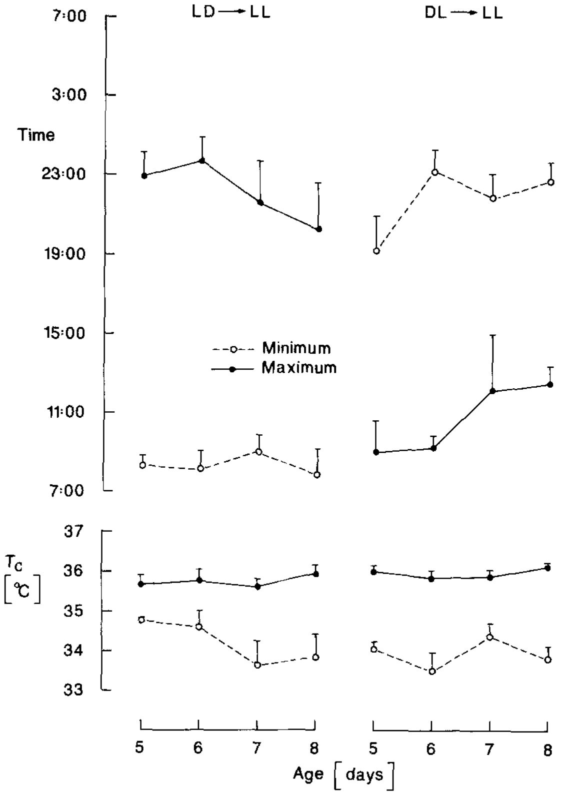

Fig. 4. Bottom: Daily minima (open circles) and maxima (closed circles) of body temperature. Top: Times of day at which these values occurred. Daily minimum and maximum were defined as the averages of the three lowest and highest consecutive hourly means on each day of artificial rearing. Mean values ± standard error. Left side: $n=5$–7 LD-pups; right side: $n=5$–8 DL-pups

Continuous measurement of core temperature in the artificially reared pups yielded the hourly mean values shown in Fig. 3. Despite considerable variability in the Tc of different pups or of the same pups on different days, a 24-h rhythm in core temperature is readily apparent in the population data. For pups commonly reared in dim light from 4 days of age, the minima and maxima of those derived from the LD-colony are clearly out of phase with those derived from the DL-colony. The average times at which the daily minimum and maximum Tc occurred – shown in the upper part of Fig. 4 – for the LD-pups were significantly different from those of the DL-pups, and the phase differences for the minima never varied significantly from 12 hours. The times at which the daily maxima occurred were more variable, but they were also roughly 12 h out of phase between the LD- and DL-pups. Neither the time of the Tc minimum nor that of the Tc maximum systematically shifted during the 4 experimental days. The daily minimum and maximum core temperatures were

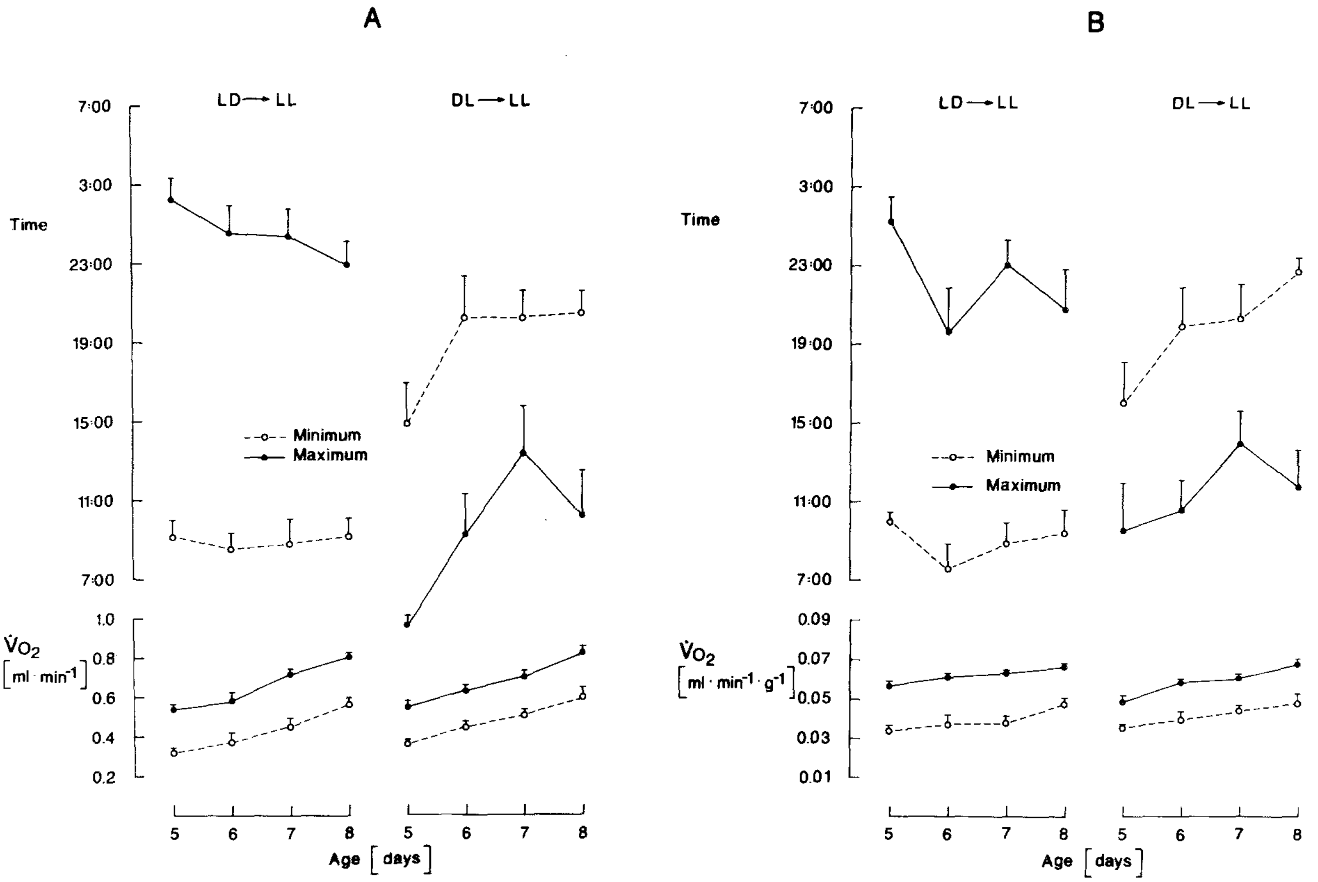

Fig. 5A, B. Bottom: Daily minima (open circles) and maxima (closed circles) of oxygen consumption per animal **A** and per gram body mass **B**. Top: The times of day at which these values occurred. Mean values ± standard error. Left side: $n = 8$–9 LD-pups; right side: $n = 6$–10 DL-pups

not significantly different for LD- and DL-pups (lower part of Fig. 4). Daily maxima of core temperature of 5- to 8-day-old pups cluster very tightly around 36 °C, while the minima range between 33° and 35 °C. In neither LD- nor DL-pups did the amplitude of the Tc rhythm decrease during artificial rearing.

Daily minima and maxima of oxygen consumption per animal (Fig. 5A) and per g body mass (Fig. 5B) were both more variable than the corresponding temperature parameters. Throughout artificial rearing the times at which the metabolic maxima occured in DL-pups were significantly different from the times at which they occurred in LD-pups. Time differences between metabolic minima of LD-pups and DL-pups were not significantly different from 12 h on days 6 through 8. The metabolic parameters were also like the temperature parameters in that the metabolism of LD-pups did not differ from that of DL-pups and the amplitude of the metabolic rhythm did not decrease as the pups were artificially reared from 5 to 8 days of age.

Discussion

Although rats have been thought not to display overt endogenous circadian rhythms during the first 2 weeks of life (Davis 1981), 5- to 8-day-old Zucker rat pups huddling in the nest in the absence of their mother show 24-h cycles of core temperature (Schmidt et al. 1986). Circadian variations in the metabolic rate of 2- to 7-day-old lean Zucker rats have also been observed during short-term separation from the dam (Planche and Joliff 1987). Careful measurements by Spiers (1988) have recently documented diurnal variations in the core temperature and oxygen consumption of 2- to 11-day-old albino rats during short separations from the dam, revealing that these diurnal variations are not restricted to the Zucker rat strain. Spiers' data moreover showed that the temperature rhythm in albino rats disappeared at about 15 days of age, supporting the report of Kittrell and Satinoff (1986) who did not find circadian core temperature fluctuations between 15 and 25 days of age. Because the thermal and nutritional status of suckling-age rats is so dependent on maternal behavior, though, it cannot be inferred whether the observed circadian fluctuations reflect endogenous or exogenous rhythmicity.

The first evidence for an endogenous character, which came from continuous measurements of a juvenile temperature rhythm in artificially reared rats (Schmidt et al. 1987), did not exclude artifactual generation of the rhythm by uncontrolled environmental factors. Simultaneous artificial rearing, however, of pups originating in colonies with light cycles 180° out of phase ensured that any external influences were coincident and equal for all pups. The persistence in these animals of 24-h cycles in core temperature and oxygen consumption – with undamped amplitude and in phase with the light/dark cycle of the colony of origin – is clear evidence that the observed oscillation is not caused by exogenous factors.

The demonstration of free-running rhythms is traditionally considered necessary for proving the endogenous origin of circadian oscillations. As this is not possible in short-term studies we instead used an experimental protocol that ensures that the observed oscillation cannot be the passive reflection of environmental changes by simultaneously investigating two groups of animals that had been entrained to diurnal conditions 12 h out of phase with each other. We conclude that the week-old rat pup possesses an endogenous timekeeping mechanism allowing it to maintain undamped daily Tc oscillations in phase with the colony of origin for at least 4 days in constant light. The question of whether the observed Tc oscillation is driven by the SCN, which is thought to start functioning as a biological clock even before birth (Reppert 1985; Hiroshige 1986) or by any other central nervous pacemaker is not addressed in this study. Preliminary results (Imai-Matsumura et al. 1988) indicate that the SCN is not involved in the control of the juvenile Tc rhythm.

Maintenance of a self-sustained oscillation, though suggested by amplitudes which do not decrease, cannot be definitely proven by this study because of the short period of observation. Further studies in lean Zucker rat pups artificially reared from either 4 or 10 days of age (Schmidt and Nuesslein 1989), however, have revealed an increase in the amplitude of this temperature rhythm during the 2nd week of life and have confirmed its disappearance during the 3rd week of life, as reported by Spiers (1988) for mother-reared albino rats. The time of disappearance of the rhythm was age-dependent and not influenced by the length of isolation under constant conditions, thus confirming the endogenous character of the observed oscillation (Schmidt and Nuesslein 1989). Studies in artificially reared, week-old Wistar rats have furthermore demonstrated that the ability to maintain overt daily Tc rhythms under constant conditions is not a peculiarity of the Zucker rat strain (Mumm et al. 1989).

The absence of a discernable Tc rhythm around the time of weaning suggests a transition between temperature rhythms of different origin. While the circadian pattern of core temperature in an adult rat has a rather sinusoidal form, the juvenile Tc rhythm resembles a sustained plateau with a sharp decline to a minimum Tc occuring about 6 h before the adult's daily minimum Tc. Furthermore, the amplitude of the endogenous juvenile rhythm in Zucker rats is strongly dependent on ambient temperature, disappearing at thermoneutrality (Nuesslein et al. 1989). Considering the obscurity of mechanisms for producing the well-known circadian core temperature cycle in mature rats, a discussion of possible causes for the 24-h rhythm observed in immature rats seems premature.

Acknowledgement. This study was supported by an Alexander von Humboldt award to RK.

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