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Circadian and Ultradian Rhythmicities in Very Premature Neonates Maintained in Incubators

M. Schimmel[1], J. Waterhouse[2], M.D. Marques[3] and D. Weinert[4]

[1]Dept of Geophysics, IAG-Universidade de Sao Paulo, Sao Paulo, SP, Brazil; [2]Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UK; [3]Museu de Zoologia, Universidade de Sao Paulo, Sao Paulo, SP, Brazil; [4]Dept of Zoology, University of Halle, Halle, Germany

Abstract

Six very premature babies (born at 26–28 weeks gestational age) have been studied in hospital for 11–17 weeks, while in intensive care and in an incubator. Apart from suffering occasionally from the neonatal disorders of haemolytic jaundice and 'respiratory distress of the newborn', the babies were healthy and developed normally.

Initially, the babies were continuously fed intravenously, and the lighting in the ward was on continuously. Routine care was given round the clock. When their medical condition permitted it, the babies were moved in their incubator to an adjacent ward, where they took frequent (2–4 hourly) small meals by mouth, the lighting was dimmed at night, and routine care tended to be given more in the daytime.

Hourly recordings of insulated skin temperature were taken throughout the study, and it is the detection of rhythmicity in these measurements that has been the subject of the present study. The methods used were Phase-weighted Stacks, Phasor Walkout and Power Spectral Analysis. These methods have previously been used mainly in geophysical studies, and their value is that they can detect weak signals in noisy data and do not assume a particular waveform of any signal.

Circadian rhythmicity was found in all babies for much of the time that were in the constant environment provided by the incubator. Ultradian rhythms were sometimes present also, but they were shorter-lived, and showed a wide range of changing periods, generally in excess of 8 h. When the babies were being treated for jaundice or respiratory distress, there was a tendency for the circadian rhythms to become weaker and for a broader spectrum of ultradian periods to appear.

Address general correspondence to: J. Waterhouse, Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Trueman Building, Henry Cotton Campus, 15–21 Webster Street, Liverpool L3 2ET, UK. Tel.: (UK) + 161-432-6352; Fax: (UK) + 151-231-4353; E-mail: waterhouseathome@hotmail.com
Address correspondence relating to the analytical methods to: M. Schimmel, Dept of Geophysics, IAG-USP, Rua do Matão 1226, 05508-900 São Paulo, SP, Brazil. E-mail:- schimmel@ija.csic.es

Placing babies in the 12 h : 12 h light : dark environment provided by the ward, and instituting feeding by mouth, had, in most cases, only modest effects upon either circadian or ultradian rhythms. Thus, circadian rhythms continued (but generally with a period not exactly equal to 24 h), and ultradian rhythms, when present, often did not show periods that could be related easily to feeding or care-giving.

These results are discussed in terms of evidence for endogenous and exogenous origins of the observed rhythms, and of theories that have postulated the relationship between circadian and ultradian rhythms. It is concluded that the results from the present analyses are difficult to reconcile with the view that circadian rhythms develop from interactions between ultradian oscillators. We suggest that they indicate a maturation of the circadian system as a consequence of increasing associations between the circadian elements that are present in the suprachiasmatic nuclei and in other oscillators of the circadian system. The new analytical methods used here also indicate that the results obtained from time-frequency analysis depend to some extent upon the method used.

Keywords: Time-frequency analysis, light-dark cycle, eating-feeding cycle, circadian rhythms, ultradian rhythms, pre-term infants.

Introduction

Reviews of the development of rhythmicity in humans describe how, immediately after birth, full-term babies show rhythmicity that tends to be ultradian (with periodicities of 4 h or less) rather than circadian. As the baby gets older, there is an increase in the amplitude of circadian rhythms and a decrease in that of ultradian rhythms. In premature neonates, there is a tendency for ultradian rhythms to be more marked than in full-term babies. (Hellbrugge, 1974, 1977; Minors & Waterhouse, 1981; Sitka et al., 1984, 1990; Mirmiran & Kok, 1991). These changes take place at the same time as the infant is developing neurologically, and is beginning to become more responsive to its environment.

More recent studies (Weinert et al., 1994, 1997; Glotzbach et al., 1995; Bollani et al., 1997; McGraw et al., 1999) have confirmed that circadian rhythms are weak immediately after birth. Such studies have generally, but not always (see Guilleminault et al., 1996), established that a low-amplitude circadian rhythm of core temperature is present at, or very soon after, birth.

Interpreting such results raises two issues. The first is the extent to which the measured rhythms are caused directly by ('masked' by) environmental rhythms and rhythms of feeding and care (in which case they could be described as being 'exogenous') rather than are caused by actions of one or more oscillating systems in the body (in which case they could be described as 'endogenous'). The second and related issue is: accepting that ultradian and circadian rhythms are not due entirely to exogenous influences, what is the relationship between the endogenous ultradian and circadian rhythms? This is particularly pertinent when, during infancy, circadian rhythms seem to increase in strength at the expense of ultradian ones.

With regard to the first issue, the ideal method would be to study an individual in 'constant routine' conditions (Mills et al., 1978; Czeisler et al., 1985). In this protocol, the subject is required, for a period of at least 24 h: to be in a constant environment with regard to lighting (LL), temperature and humidity; to remain awake but in the same posture; and to eat regular, identical meals. In such circumstances, masking influences have been minimised, and any rhythms that remain can reasonably be described as being 'endogenous'. Clearly, such a protocol is impossible to carry out in a neonate and unacceptable ethically (although conditions similar to this were used in a study by Martin-du-Pan (1970), and are present in infants studied in intensive care in incubators (Thomas, 1991; Glotzbach et al., 1995)).

Nevertheless, there is indirect evidence that the circadian rhythm of core temperature is present independently of environmental rhythms and the sleep-wake cycle. Thus, the circadian rhythm of core temperature arises before the sleep-wake cycle has been established (Guilleminault et al., 1996; McGraw et al., 1999). Also, the phase of the temperature rhythm is widely spread between different babies, indicating that it cannot be produced by a direct effect of the light-dark (LD) cycle (Bollani et al., 1997). There is also evidence that in some (Kleitman & Engelmann, 1953), but by no means all cases (for example, Shimada et al., 1999), the sleep-wake cycle of babies living in an environment with a natural LD cycle shows a circadian rhythm that is not adjusted to a 24–h rhythm; instead, it seems to free-run.

For ultradian rhythms with a period of 3–4 h, however, the picture seems to be different, these being present soon after birth with a timing that matches the rhythm of feeding and care-giving (Glotzbach et al., 1995). However, an exogenous source with an appropriate period is not readily apparent for ultradian rhythms in the range 6–20 h, for example.

Many of these points have been found in recent studies of ultradian and circadian rhythms in a group of full-term, healthy neonates. These were assessed on two occasions, 2 days and 4 weeks after birth (Weinert et al, 1994, 1997), during which, the sleep-wake cycle and rhythms of heart rate, systolic blood pressure and core temperature (rectal and insulated abdominal skin temperatures) were measured for 24 h. The babies were demand-fed (about every 4 h) and lived alone in a room exposed to the natural LD cycle (with approximately equal amounts of daylight and darkness). This study was different from others in that the records of rectal and skin temperatures, heart rate and systolic blood pressure were corrected for the direct effects of the sleep-wake cycle and, in the later study, for the LD cycle also. Two days after birth, a low-amplitude circadian rhythm in core temperature was present, but the acrophases of these circadian rhythms showed considerable inter-individual variation. By contrast, heart rate, systolic blood pressure and activity did not show significant circadian rhythms; instead they showed ultradian rhythms with a period of about 4 h. By the fourth week, the amplitudes of the circadian rhythms of core temperature had risen, and the acrophases showed less inter-individual variation, falling in the first half of the light phase of the LD cycle. Circadian rhythms in activity, heart rate and systolic blood pressure remained non-significant. Ultradian rhythms were increased in amplitude in all variables at 4 weeks compared with 2 days. Those for activity,

heart rate and systolic blood pressure were synchronous with the pattern of feeding and care-giving, and those for the two estimates of core temperature could also be related to these exogenous influences.

These two studies indicate that a weak circadian rhythm in core temperature was present immediately after birth and that its development was not a consequence of the development of circadian rhythms in the sleep-wake cycle or the cardiovascular system. Since the circadian rhythms in rectal and skin temperature were present after correction of the records for the masking effects of the sleep-wake and LD cycles, they could be described as endogenous.

However, in this as in most other studies, when rhythms are measured some days after birth, the babies have been exposed to the alternation of light and dark and other external circadian rhythmicities, and this might have induced the expression of circadian rhythms in some way. (This reservation does not apply to measurements made in the days immediately after birth, however). To remove this objection, it would be necessary to study babies whose exposure to since birth to such rhythmicities, as well as to ultradian rhythmicities, had been minimal. Such a 'constant routine' protocol is approximated in very premature neonates, in whom 'respiratory distress of the newborn' and jaundice are common, and who have to be treated in an incubator, sometimes for extended periods of time.

In a previous study (Tenreiro et al., 1991), heart rate and core temperature (insulated skin temperature) were observed in very premature neonates placed in incubators for these disorders. The environment of the babies in the incubators approximated to that required by a constant routine (ward lighting was on at all times, the LL condition), care was given whenever required, and feeding was by a continuous intravenous infusion pump. Maximal Entropy Spectral Analysis (MESA) and autocorrelation indicated: that circadian and ultradian (periods ranging from 3–20 h) rhythms were simultaneously present; that the circadian rhythms tended to increase in strength, and the ultradian rhythms to decrease, as the post-natal age of the baby increased; and that all rhythms were unstable, with regard to both whether or not present and, if present, their phasing.

Taken together, these studies indicate that circadian and ultradian rhythms are present in neonates, and that they cannot always be attributed to an exogenous cause. This evidence raises the second issue, namely the relationship between the two types of rhythms, and an explanation of the observation that the process of development in neonates is associated with a shift in emphasis from ultradian to circadian rhythmicity.

To pursue these two issues, the aim of the present report, two basic conditions need to be fulfilled. The first is that the neonate must be studied in an environment where masking influences — ultradian influences (from feeding and care-giving, for example) and circadian influences (from the LD cycle, for example) — are as small as possible. The second requirement is for a mathematical treatment of the results that can indicate if the rhythmicity present at any time necessitates the presence of ultradian oscillators. The study upon very premature neonates in incubators (Tenreiro et al., 1991) enabled exogenous influences to be minimised, but the mathematical

analyses, MESA and autocorrelation, while establishing the simultaneous presence of ultradian and circadian rhythms, did not address the origin of these components.

In the current study, we have re-analysed the data from some of the babies used in this earlier study, but used a new set of methods for analysing the periodicities, methods that have been used previously in geophysical studies. The methods consequently provide new and independent constraints, and have two advantages over the previous analysis. These advantages are, first, that, in addition to detecting sinusoidal rhythms, they enable non-sinusoidal rhythms to be detected, since the approach is not based on sine-wave decomposition. (Most spectral analysis methods, including MESA, are based on sine-wave decomposition and such methods, especially in the presence of other rhythms and noise, give rise to difficulties in the identification of non-sinusoidal signals after their decomposition into several frequency components. Non-sinusoidal signal detection enables the issue to be addressed of whether any observed rhythmicities necessitate the presence of an ultradian oscillator). The second advantage of these analytical methods is that our approach is not restricted to a special noise model. (In general, periodogram methods are based on least square or variance reduction approaches and, therefore, are restricted to Gaussian noise. Deviations from such noise models can lead to misleading results which generally are referred as non-robustness (Huber, 1981; Chave et al., 1987; Schimmel, 2001a, b). Our approach employs a coherence measure which is explicitly amplitude-unbiased and therefore suited for the detection of a coherent rhythm of weak amplitude which is concealed in noise of larger amplitude.)

In addition (see Methods), the hospital policy relating to the care of the neonates resulted in the babies being fed discontinuously and/or being transferred to a room where there was an alternation of light and dark. These changes enable the immediate and direct effects of these interventions to be assessed and the babies' response to an imposed ultradian rhythm (of feeding) or circadian rhythm (of the LD cycle) to be tracked. To do this, the above methods have been employed together with moving data windows.

Methods

Subjects and general protocol

In the Neonatal Medical Unit at St. Mary's hospital, Manchester, heart rate (not analysed here) and skin temperature were continuously and routinely monitored for periods of 11–17 weeks in 6 pre-term babies of 26–28 weeks gestational age and 792–1200 g birth weight. The start of recordings was always within 24 h of birth. The end of the recording was determined by there being no need to keep the baby any longer in intensive care. Some details of the babies included are given in Table 1. As would be expected, the babies had various neonatal conditions including idiopathic respiratory distress and jaundice but, as far as could be judged, all would be expected to develop normally and without obvious neurological disorder.

Table 1. Some details of the babies.

Name	Sex	Gestational Age at Birth (weeks)	Birth Weight (g)	Length of Study (weeks)	Discontinuous Lighting	Discontinuous Feeding	ill *
CB	F	26	1032	17	10+	10+	1–3,6,7
SB	F	26	957	17	14+	15+	1,2,4,5,8–10,13
AK	M	26	800	15	7+	11+	1,2,4–6
KEV	M	28	1200	14	14	14	1,2,4–6
GRIF	F	26	792	11	10+	9+	1–4
HARR	M	27	800	17	17	15+	1,2,5,6,9,11,12

*Weeks when phototherapy for jaundice, or respiratory support, was given.

On admission to the intensive care ward after birth, each baby was placed in an incubator, the air temperature and humidity of which were kept constant. Medical care was given whenever required, most commonly ventilatory support to combat respiratory distress, and the continuous use of phototherapy if jaundice were present. Routine nursing care for the babies was given whenever required. Nutrition was provided by solutions containing glucose, electrolytes, amino acids and lipids, which were administered intravenously by continuous infusion pumps. Antibiotics and aminophylline were administered when indicated by the medical condition. Ward lighting was continuous (LL, about 1300 lx), but the ward had windows and so the natural LD cycle would have been evident to some extent, at least in the ward itself. Parents were encouraged to visit and tend their child when possible; in practice this was in the daytime, but not normally at a regular time of day. Hence, the environment of the baby was comparatively constant and any interventions were performed as required rather than as part of any regular regimen.

As the baby's condition improved, two major changes were instituted though these need not have occurred simultaneously (Table 1). First, discontinuous feeding was instituted, this being 2–, 3–, or 4–hourly as considered appropriate ('discontinuous feeding', EF). Second, the incubator was moved to another ward in which artificial lighting was 150–200 lx in the daytime and the lights were dimmed (less than 10 lux) during the night (20:00–08:00 h), though auxiliary lighting was available if required and, as before, the natural LD cycle would have entered the ward ('discontinuous lighting', LD).

Measurements

As part of their intensive care, the babies' heart rate and chestwall or abdominal skin temperature were monitored continuously throughout their stay in intensive care. Skin temperature was monitored by a thermally-insulated surface electrode, further insulation being provided by a layer of lint and adhesive tape. Hourly temperature readings of skin temperature were taken by trained staff, who also checked that the baby was not lying on the electrode. There was some latitude in the exact time the readings were taken to ensure that it was not just after the baby had been disturbed for medical attention, for example. Data sets were complete except for baby AK, where the sixth week (days 36–42) was missing.

In addition, a weekly assessment of the babies' medical condition was made on the basis of their medical records. During weeks when, on average, a baby was particularly in need of respiratory support or phototherapy, it was described as 'ill', and in weeks after this as having 'recovered' (see also Table 1).

A short description of methods applied in the analysis

In this section we briefly present the techniques and tools employed to search for the hidden periodicities. These are the Fourier theory, the phasor-walkouts (e.g., Zürn & Rydelek, 1994) and the phase weighted stacking (PWS) technique (Schimmel & Paulssen, 1997; Hoenen et al., 2001). These techniques are based on different design

philosophies and can therefore provide independent constraints and help to focus on different characteristics of the data.

Fourier theory

The Fourier theory (e.g., Bracewell, 1965) is used to determine the power spectral density function or just the power spectrum of the data. This method decomposes the data into a set of independent sine functions at distinct frequencies. The power at each frequency represents the contribution of that frequency component to the data variance. This method assumes Gaussian noise and is suited to detect sinusoidal rhythms. Non-sinusoidal rhythms, however, might be missed since they are decomposed into a suite of sine functions of different frequencies rather than into one single frequency component. Note that it is this method that has often been used in studies of neonate rhythmicity in the past.

Phasor-walkouts

The phasor-walkout technique — see Zürn and Rydelek (1994) for a revision and further references — illustrates graphically how a single component of the Fourier amplitude spectrum sums up successively with time during the Fourier transform. It pictures the transform at one frequency as a vector summation which occurs in the complex domain. Every sample contributes as one vector with lengths equal to its sample amplitude, and angle (phase) determined by the time of that sample and the considered frequency. The sum of all vectors corresponds to the Fourier amplitude at that frequency and its square to the power. If the vectors point into the same direction then they sum constructively, producing a large amplitude. Random directions yield, depending on the individual sample amplitudes, a destructive interference pattern, and the corresponding amplitude in the spectrum depends on this interference. Hence, this graphical Fourier transform can reveal whether a spectral peak almost hidden in the background noise is caused by fortuitous constructive interference of noise or a small harmonic signal. The discrimination is based on a qualitative inspection of the walkout pattern.

Phase weighted stacks

PWS does not perform a spectral decomposition of the data and is fundamentally different from a Fourier analysis. Signals are detected by their repeated occurrence and waveform similarity. PWS, such as presented in Hoenen et al. (2001), are based on an actogram representation of the time series and the systematic time-shifting and summation of the traces. Here we apply a modified algorithm based on these principles. One single time series is used rather than building up an actogram and as a consequence *differential slowness* (Hoenen et al., 2001) can be replaced by *period*. In order to enable the detection of small amplitude signals concealed in larger amplitude noise, a weighting procedure based on waveform similarity is employed to suppress noise (Schimmel & Paulssen, 1997). This further enhances the signals. In

addition, the application of the PWS method handles non-sinusoidal signals and permits the detection of multiple signals as a function of time. The coherence measure (PS) used to suppress the incoherent noise during the summation of the traces is explicitly independent of the signal amplitudes. Hence the coherence is not decreased by varying signal amplitude; as a result, coherent signals of small amplitude are detected as well as coherent ones of a large amplitude. The PS values range between 0 and 1, with 1 indicating a complete signal waveform similarity. The larger is PS, the better is the rhythm period constrained. If the signal waveforms are unstable, then the PS values are small and the signal detection becomes more ambiguous. However, the systematic appearance of signatures for consecutive stretches of data can indicate the presence of rhythm.

PWS and PS reveal the signals as a function of the time of the day and the rhythm period (or differential slowness). Inherent to the method, a long duration rhythm manifests itself at periods which correspond to the rhythm period (P) and its multiples ($2 \times P$, $3 \times P$, etc.). This is best understood when picturing the sum-and-shift procedure (Hoenen et al., 2001) at the rhythm period and its multiples.

The time-frequency analyses

In order to access the rhythms as function of frequency (or period) and the post-natal day when temperature acquisition took place, we apply a moving data window of certain width. The detected frequencies for the data selected by the window are ascribed to the centre time of that window. The successive shifts of the data window, and application of the Fourier theory or PWS to each in turn, form the backbone of the time-frequency analysis.

In the case of the Fourier analysis, we use Gaussian shaped windows to determine the power spectra as a function of the centre time. The Gaussian window decreases the spectral leakage and gives best simultaneous time frequency resolution (e.g., Harris, 1978). The window width is a function of frequency. We define the standard deviation of the Gaussian window as a fixed number of cycles of the search period. This frequency-dependent data window changes the variance of the data, and this is corrected to enable a comparison of the amplitudes obtained at the different frequencies. The amplitudes obtained, as a function of the centre time and frequency, form a time-frequency matrix which is represented as a contour plot.

The phasor-walkout technique does not need to be adapted since it already shows the evolution of the Fourier amplitude with the progress of time. However, only one frequency at any time can be tested.

For the PWS technique, the moving window is a boxcar (rectangle window) centred every day. (A window which tapers the selected stretch of data is not required since there is no transform to the frequency domain.) The length of the boxcar is defined by a fixed number of cycles of the search period. That is, if 10 cycles are used then the window is 10 days long for a 24–h test period, and 20 h long for a 2–h test period. With PWS, signals are detected as a function of time of day but this information is not included in our time-frequency analysis. For each frequency, we take

the largest detected value, regardless to its time of day, to construct the time-frequency matrix. This matrix is then represented as a contour plot.

The choice of the boxcar employed depends mainly on the data characteristics. A large data window favours the suppression of noise and increases the period resolution, but it also decreases the resolution at the start and end of a data set of finite duration (as in the present study). Further, a large window means that short-duration rhythms or alterations of strength or frequency are less well resolved due to the averaging process. Conversely, if the time window is too small then the signals cannot be detected unambiguously owing to the diminished suppression of noise. Processing the data with the different parameters gives a good feeling for the robust features in the data and selection of optimum window size.

The period discretization — that is, the time difference between neighboring test periods — in the PWS analysis was chosen to be 6 min. In the Fourier analysis we are restricted to equally-spaced frequencies which are harmonics of a fundamental frequency determined by the number of samples and their time separation. We show the Fourier power spectra as a function of the period which is consequently based on an increasing period discretization for increasing periods.

Table 2. Main results for constant conditions. Only distinct long-duration signatures in the contour plots are indicated.

	Ultradian Rhythms		Circadian Rhythms	
	Post-Natal Age	Periods	Post-Natal Age	Periods
Name	(days)	(hours:minutes)	(days)	(hours:minutes)
CB	5–12	17:30–17:50	28–36	23:00–25:00
	15–20	13:50–14:30	49–54	22:00–22:30
	16–21	11:50–12:10	53–62	25:10–25:40
	55–59	14:40–14:50	53–65	23:50–24:10
SB	5–10	16:30–17:00	7–13	22:50–23:50
	30–38	7:00–8:00	25–28	23:00
	50–56	11:50–12:10	40–58	23:50–25:15
	54–61	14:50–15:40		
AK	27–31	15:10–15:30	7–26	24:00–24:45
KEV	77–83	16:00–16:15	34–47	23:10–24:50
			51–57	24:40
			61–76, 83–87	23:50–24:20
GRIF	24–27	10:30	15–28	25:30–26:00
			29–56	23:45–24:30
HARR	37–41	17:30–18:00	7–13	20:30–21:00
	54–57	18:30	12–18	25:50–26:50
	60–62	15:20–15:30	7–38	23:50–25:30
	70–77	11:00–12:00	39–44	23:00–24:00
	76–80	16:00	64–74	23:30–24:15
			77–89	21:30–21:50

Observations and results

The hourly measured temperatures of each neonate have been de-meaned and then analysed using the approaches described above. Our observations and results are arranged according to the different environmental conditions of the babies. Figures 2–7 show the PS and PWS results obtained for each baby, Figure 8 is an example of Fourier power spectra, and Figure 9 shows examples of Phasor-Walkout.

In the contour plots, Figures 2–7, we mark in grey tones the different environmental conditions and health of the babies, and indicate by crosses the two largest amplitudes per day. The boxcar windows are uniformly 13 cycles long, which enables direct comparisons between Figures 2–7. The doubled standard deviation of the Gaussian window is 13 cycles long. Owing to the data window employed, there are no results available for the first and last days of data acquisition.

The main results during constant conditions, and LD and/or EF conditions are summarised in Tables 2 and 3, respectively. The quality of the listed signals varies and can be assessed through Figures 2–7.

Rhythms during constant environmental conditions (Table 2)

The PWS technique and the Fourier power spectra show the presence of a mostly dominant, sometimes intermittent, circadian rhythm for all babies. The quality

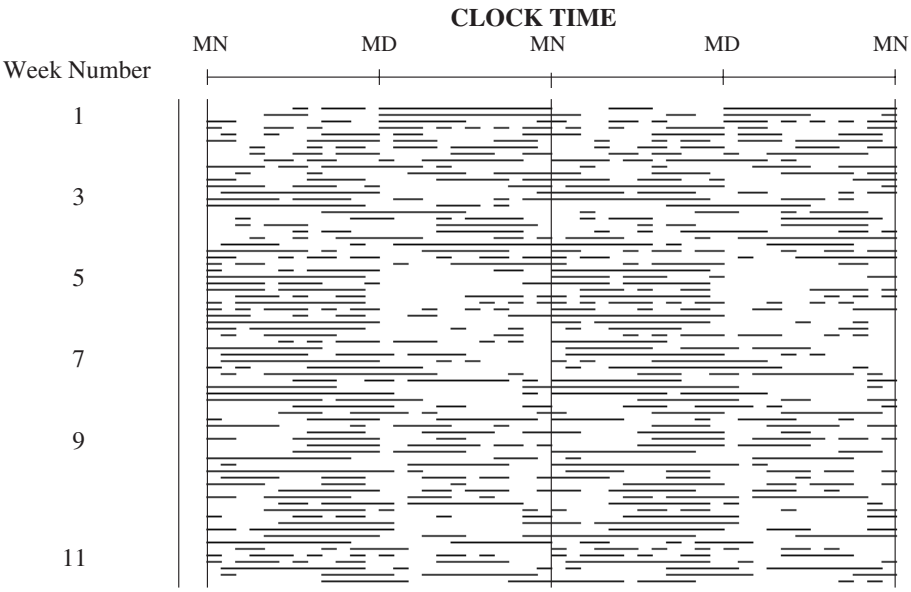

Figure 1. Temperature data of GRIF. A horizontal line indicates the times when the temperature was above the daily mean. Results double-plotted to facilitate observation of circadian rhythmicity.

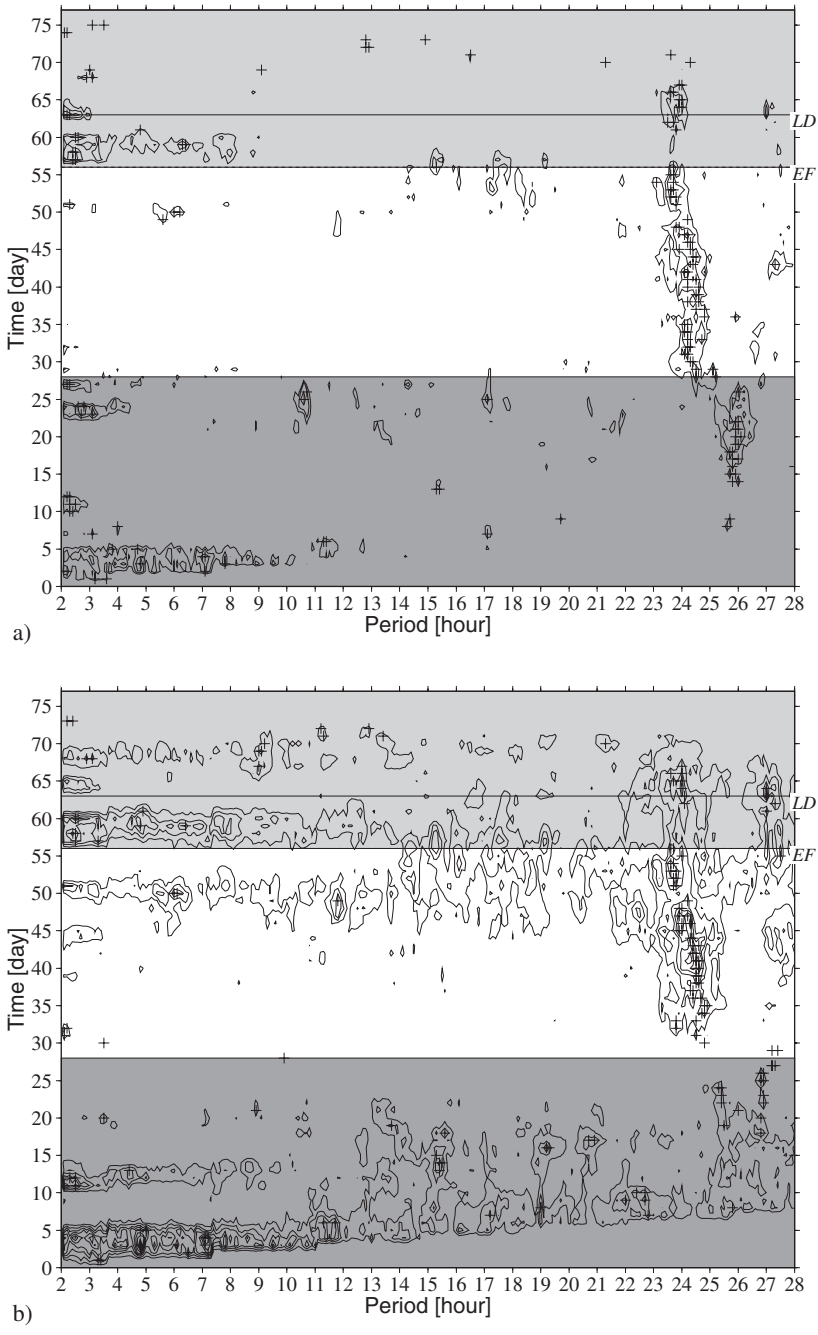

Figure 2. PS (a) and PWS (b) amplitudes for baby GRIF. The moving data window is 13 periods long. The grey tones mark the days the baby was ill, exposed to a light dark cycle (LD) or discontinuously fed (EF). The contouring starts at 0.4 (a) and 0.1 (b), with intervals of 0.1 between each line (a and b). Crosses are used to mark the two largest amplitudes per day.

(signal-to-noise ratio) of the detections varies from baby to baby. Baby GRIF produced some of the clearest results, and will first be described in some detail.

Baby GRIF. The best example for an unambiguous circadian rhythm is obtained for the baby GRIF. Figure 1 shows a double-plot of all the temperature data. In this figure, a horizontal line indicates the hours when the temperature was above the daily mean. Inspection of this plot indicates the presence of circadian rhythmicity with a period of greater than 24 h during weeks 3–8. At other times, in the first two and the last three weeks, the record is less clear, and visual inspection becomes inadequate.

The contour plots in Figure 2a, b show the maximum PS and PWS amplitudes as a function of the rhythm period and the day of temperature acquisition. This Figure indicates that GRIF's temperature profile reflects a circadian rhythm which starts immediately after the baby's recovery from illness on about day 29. This signal last until day 56 which is when the EF condition started. The period of this rhythm has a decreasing trend from about 24 h 30 min on day 29 to 23 h 45 min on day 56. Earlier in the baby's life, during the last 10 days of its illness, a rhythm of about 25 h 30 min to almost 27 h can be identified in Figure 2b. The amplitude-independent coherence of this (Fig. 2a) is largest at about 26 h. It is smaller in amplitude than the circadian one (present after recovery, see above) but its coherence values are of the same order.

At least after recovery the circadian rhythm is not accompanied by any unambiguous ultradian signal; that is, no ultradian rhythm is observed as a long duration and/or a large amplitude signal. This observation does not change when decreasing the length of the moving data window. Increasing the window length further stabilises the signatures of the circadian components. Some short-duration ultradian signals are present during the weeks of illness. A 10 h 30 min signal is detected by its coherence (PS amplitude in Fig. 2a) at about day 25; however, its amplitude is very low and, therefore, it did not appear in the PWS. During the first week of illness (the first post-natal week), rhythms with periods of 4 h 50 min and 7 h are detected, and periods of 11 h 20 min and 15 h 10 min might also be present. They appear with small amplitudes in the PS, and so they are not coherent features. All the other rhythms are smaller in amplitude and/or have a shorter duration; they are likely to be noise.

The Fourier power spectra (Fig. 8) also indicate the presence of the circadian and some of the short duration rhythms at the higher frequencies. Note a confirmation of the change of the circadian rhythm after recovery from illness at about day 29. Decreasing the double-standard deviation to 7 cycles slightly changes the time-frequency resolution, but does not change the overall pattern of detected signals.

The phasor-walkout for a 24–h test period is shown in Figure 9a. The walk starts with the first sample of the time series at 0,0 and the black line corresponds to the stretch of data acquired under constant conditions. The linear parts of the walkout indicate the presence of a circadian rhythm and, since straight walks in different directions signal phase changes, it seems that this rhythm switches between two distinct phases. Slight period changes (23.75–24.25 h, in increments of 0.05 h) lead to small deviations of the walkout but without any clear improvement overall. (This is also reflected by the width of the circadian signals in Fig. 8). Phasor-walkouts were

determined at the distinct ultradian frequencies constrained by amplitude maxima in the Fourier spectra; none of these frequencies gave results that could be attributed to a coherent rhythm over a considerable time.

The other babies will now be considered in slightly less detail.

Baby AK. This baby was ill during much of the time spent in constant conditions; nevertheless a significant circadian rhythm can be detected in all the analyses. Figure 3 shows that the circadian rhythm is observed from the last days of the first bout of illness to the first days of the second bout; thereafter, the signal weakens. Intermittently, the period is exactly 24 h, which could mean that this baby picked up an external diurnal signal. Decreasing the data window to 7 cycles confirms that this 24–h rhythm is confined to the last days of the first illness and the first days of the second. Between these two bouts of illness, the coherence of the circadian rhythm decreases and the period lengthens from 24 h 30 min to 24 h 45 min. The decrease of coherence could have been caused by a decrease in the signal-to-noise ratio or by a less stable rhythm. In addition, there are signals at about 8 h and 16 h, but these are visible only transiently, for not more than 5 days. The evidence for ultradian periods is strongest during the times of illness, but no distinct and isolated signal is observed. (From days 35 to 42 no data were available, see Methods; the gap was filled with zeros and was best resolved at the lowest periods since the data window was smaller than the gap. For PS, the coherence values are 1 when zeros only are included in the data window, since they are perfectly coherent as long as no other data are included. For these data windows, the PWS amplitude is zero, since the amplitude data sum to zero).

Baby HARR. Circadian rhythms (23 h 30 min–25 h 10 min) are observed in this baby also (Figs. 4a, b). On days 77–89, a coherent signal of almost 22–h was identified, even though this does not appear in the PWS analysis due to its small amplitude. The strength, coherence and period of the circadian rhythms show an alternating pattern, possibly responses to the varying state of health of the baby and amount of medical support it received during the four periods of illness. Three distinct time intervals with increased amplitudes at all frequencies (representing noise) can be observed in Figure 4b. They appear to be related to the periods of illness and could explain the intermittent decreases in coherence of the circadian signal (Fig. 4a). A fourth interval with increased noise could have been averaged out owing to its short duration compared to the data window used. Figure 4 indicates that the circadian rhythm was not accompanied by other marked rhythms during days 10–30, 50–70 and 85–98; however, on days 9–11 and 64–68, the neonate has a rhythm of exactly 24 h and so seems to have picked up an external rhythm. Additional rhythms, with periods of about 11–12 h, 15 h 30 min, 16 h, 17 h 30 min, and 18 h 30 min, can be identified. Each occurs only once, has a duration shorter than 10 days, and seems to be related to one of the periods of illness.

Babies CB, KEV and SB. Circadian rhythms alternating in strength and period were also detected for CB (Fig. 5), KEV (Fig. 6), and SB (Fig. 7). For CB and KEV, circadian rhythms are observed mostly during the times without illness. The data of SB

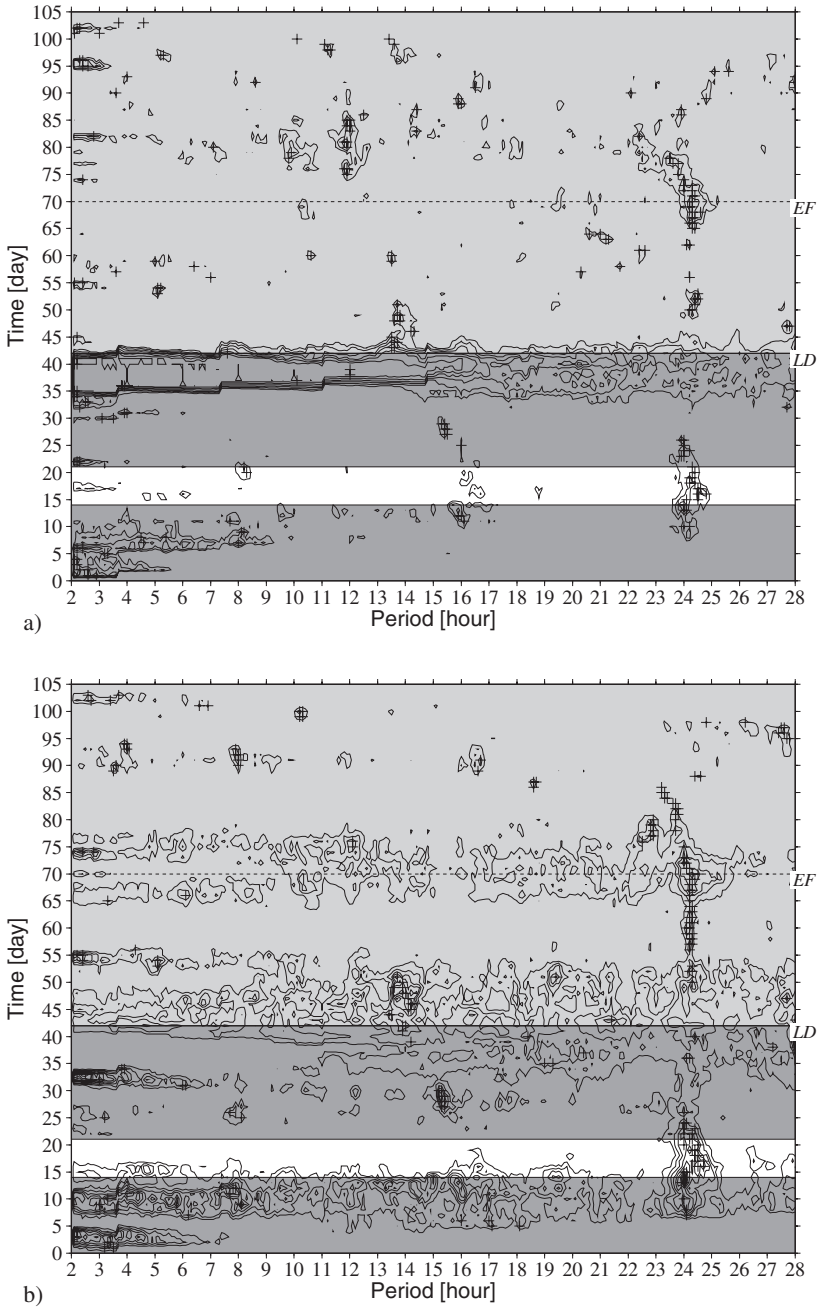

Figure 3. Same as Figure 2 but for baby AK.

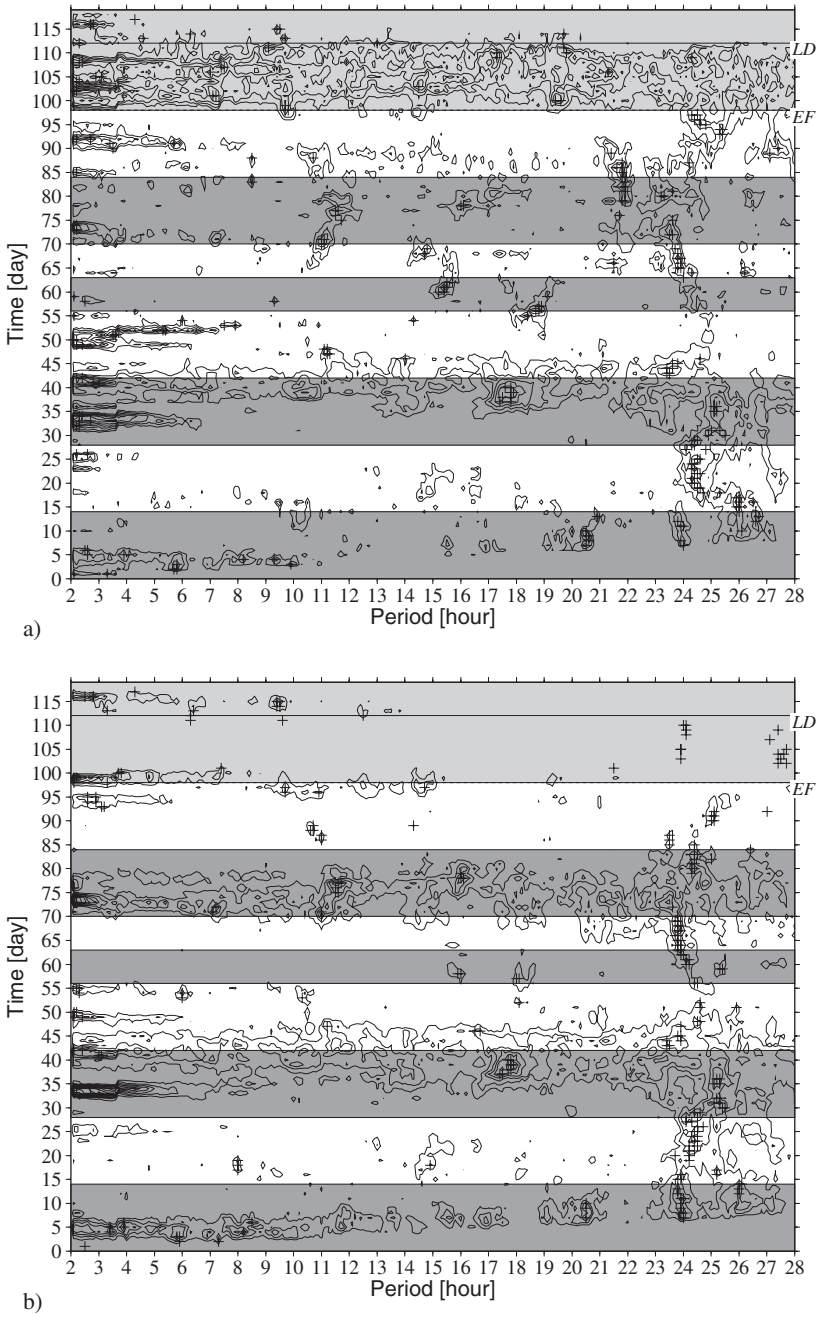

Figure 4. Same as Figure 2 but for baby HARR. The contouring of the PS values (a) starts at 0.3.

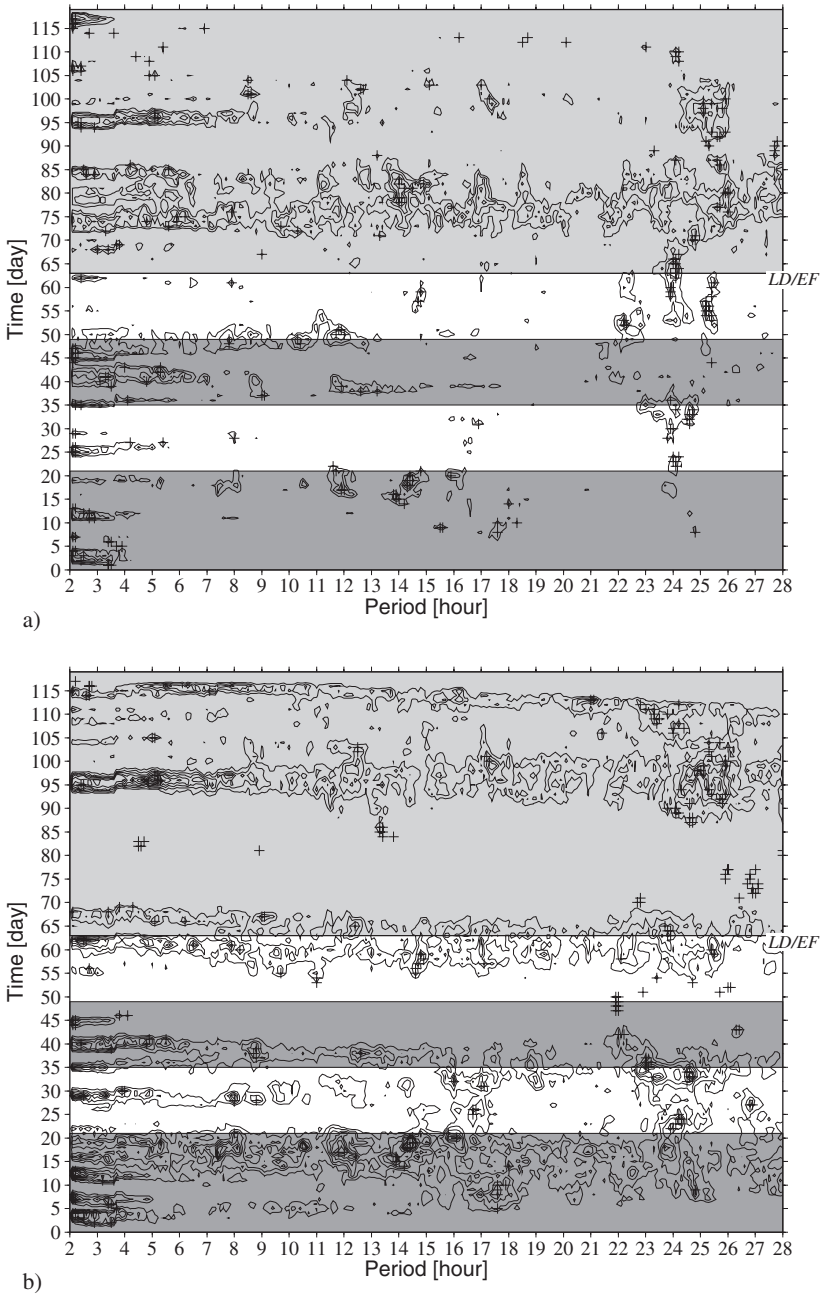

Figure 5. Same as Figure 2 but for baby CB.

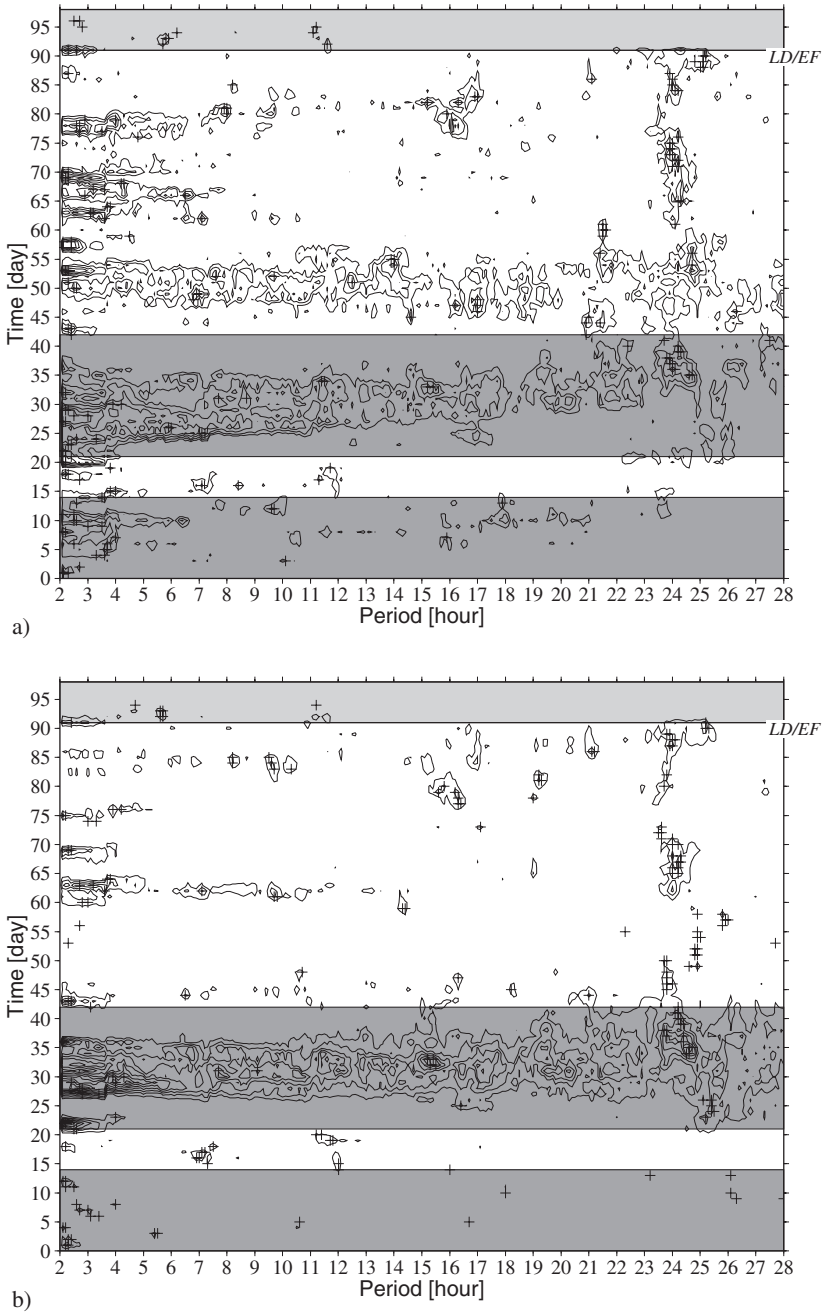

Figure 6. Same as Figure 2 but for baby KEV. The contouring of the PS values (a) starts at 0.3.

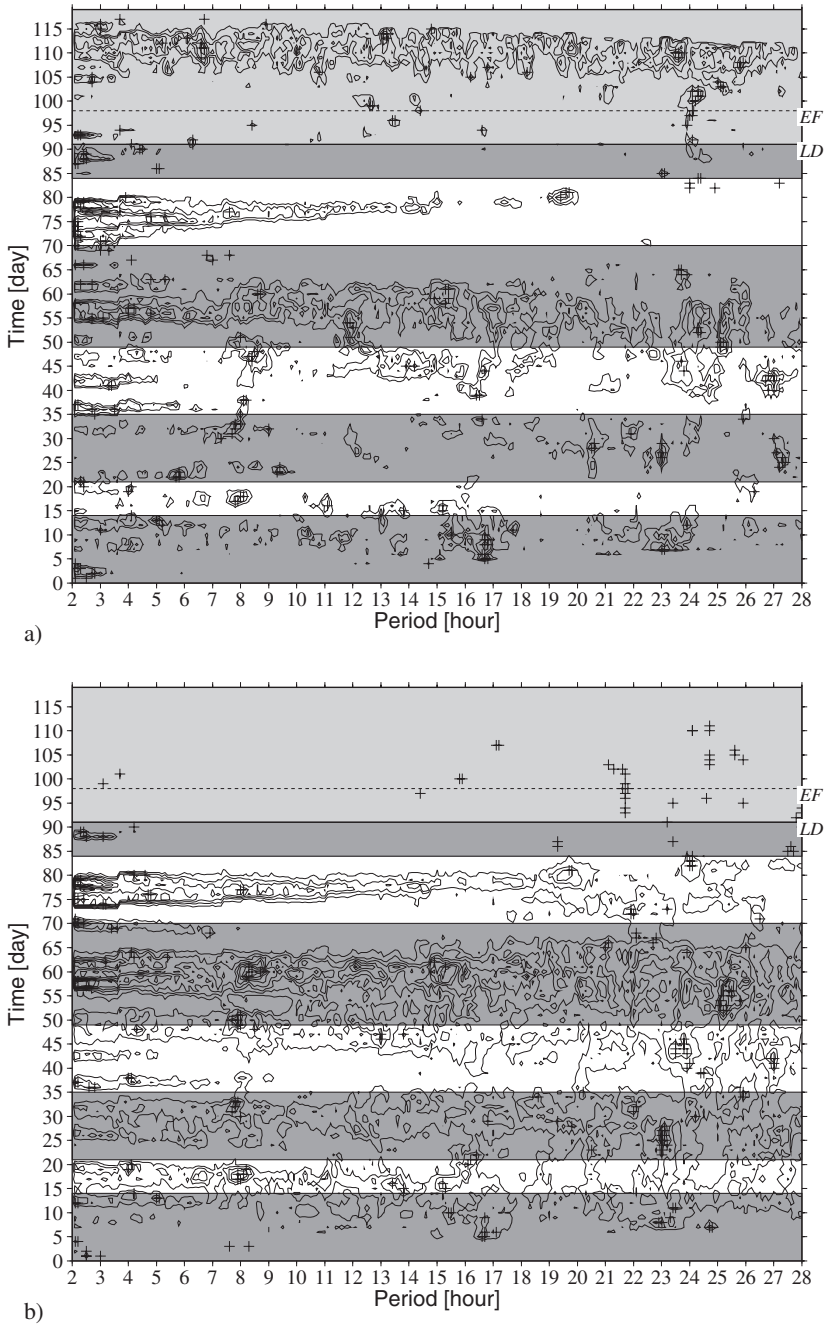

Figure 7. Same as Figure 2 but for baby SB.

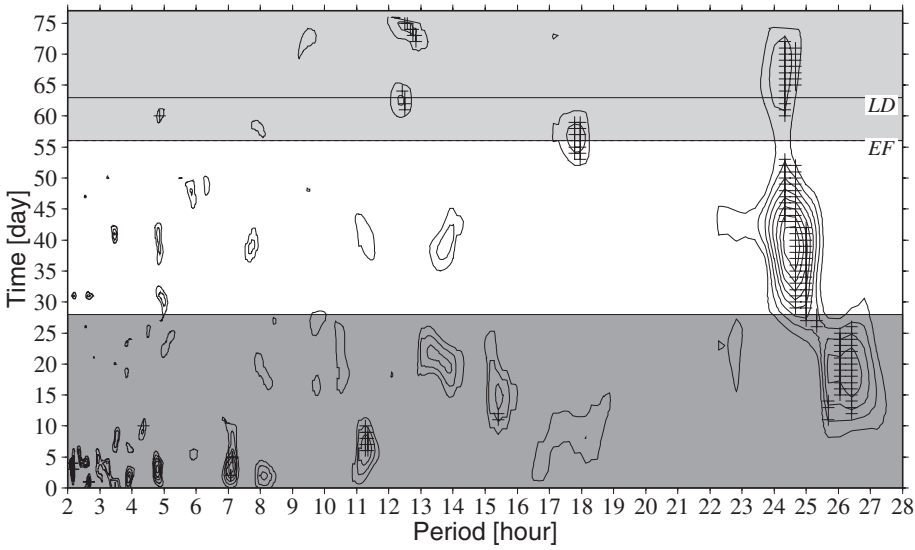

Figure 8. Fourier Power Spectra for baby GRIF. The moving data window is a Gaussian curve with doubled standard deviation equal to 13 periods. An amplitude normalisation accounts for the frequency-dependent window. Crosses are used to mark the two largest amplitudes per day.

have a decreased signal-to-noise level which hampers signal detection. Nevertheless, the data of these three babies contain time intervals where the circadian rhythm stands out from noise or from a signal at other frequencies. For SB this becomes visible when using more cycles in the moving window. Increasing the data window stabilises the circadian rhythm and decreases the presence of short duration rhythms and noise. For these neonates the circadian rhythm intermittently becomes exactly 24 h; this happens at about days 28, 34 and 54 for CB, days 34–36, 46, 62, 65–69, 72 and 85–86 for KEV, and 47, 57 and 58 for SB, respectively.

In addition to the circadian signals, there is evidence for ultradian frequencies which mostly occurred during times when there was an increase in amplitude at all frequencies. They could have been responses to medical support. Most are of very short duration and none of the signals has a time interval larger than 9 days. The signals of longer duration are listed in Table 2.

Rhythms during discontinuous feeding and/or lighting (Table 3)

The time intervals during which there was discontinuous feeding (EF) and/or lighting (LD) are marked with light grey in the contour plots (Figs. 2–7).

Baby GRIF. From Figure 2a, it can be observed that the circadian rhythm becomes less coherent during EF. During this time, an increase in signal amplitudes is observed

Table 3. Main results for LD/EF conditions. Only distinct signatures in the contour plots are indicated.

Name	Ultradian Rhythms Post-Natal Age (days)	Periods (hours:minutes)	Circadian Rhythms Post-Natal Age (days)	Periods (hours:minutes)
CB	73–84	13:50–14:30	64–72	24:00–24:50
	98–103	17:00–17:20	75–86	25:30–26:00
	98–102	12:00–12:50	92–100	25:00–25:50
SB	108–115	6:30–6:45	92–102	24:00–24:20
			92–101	21:45
AK	45–51	13:30–14:00	52–72	24:10–24:30
	76–85	12:00	76–82	22:30–23:30
	89–94	4:00		
KEV	92–94	5:50–6:10	91–97	~24:00
	92–95	11:05–11:35		
GRIF	57–60	2:30	63–67	23:45–24:00
HARR			103–111	23:50–24:10

at the ultradian periods (2–8 h) with amplitude maxima at about 2 h 30 min, 5 h, and 6 h 30 min; however, a lack of distinct ultradian rhythms is indicated by the absence of multiples of these periods. This could mean that the duration of the ultradian rhythms is too short, compared to the moving window, to appear also at the lower frequencies. Using a data window of 7 rather than 13 cycles does not change the result.

It is striking that the ultradian periods weaken after introduction of the LD cycle, and that, at the same time, there emerges a circadian signal with a maximum period at exactly 24 h. Figure 9a shows the corresponding phasor-walkout. The transition from the light grey to the mid-grey tone marks the beginning of LD conditions. It can be seen that the walkout becomes linear after the more random path during the EF conditions. The Fourier power spectrum also (Fig. 8) confirms the increased power of the circadian rhythm after the LD condition was applied.

During the transitions to conditions EF and LD, the power is increased at periods of 18 h and 12 h 30 min, respectively. At 18 h, a weak signature is also observed in the PS (Fig. 2a); however, no signal is observed at 12 h 30 min in this Figure.

Baby AK. In this baby, the LD cycle was applied before EF. Circadian rhythms are evident during LD and at the beginning of EF. The PS (Fig. 3a) shows that the coherence of the circadian alternates. This could be due to fluctuations in the signal waveforms, caused, for instance, by an increase in noise, or to fluctuations in the phase or period of the rhythm. However, examination of the PWS (Fig. 3b) shows that a circadian is present almost all the time in LD; its period is slightly larger than 24 h. The small coherence of this signal might indicate that its circadian period was not well constrained. Figure 3 shows also the presence of a 14–h rhythm. It is observed in the first week of the LD cycle at 14 h and 28 h. (The 28–h period is the first multiple

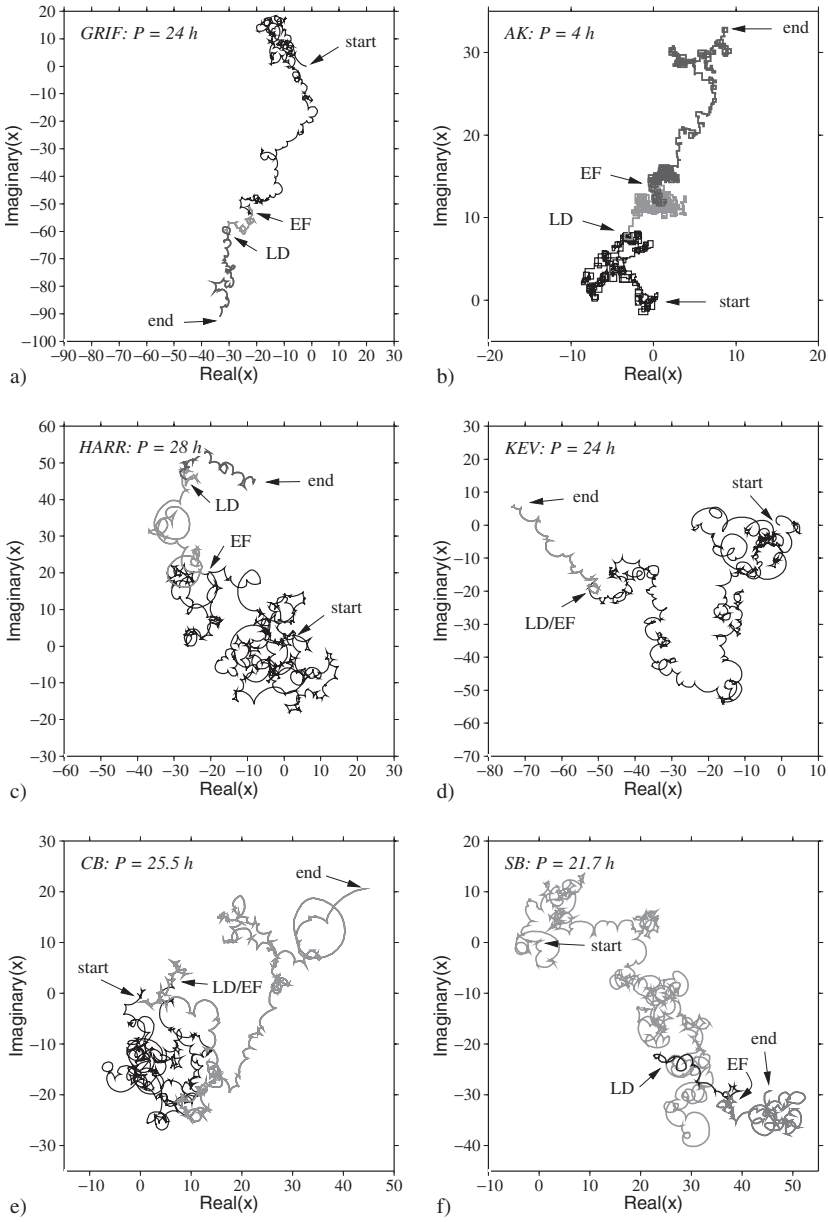

Figure 9. Phasor-walkouts for GRIF (a), AK (b), HARR (c), KEV (d), CB (e), and SB (f) at selected frequencies. The frequency value is given in the title of each figure. The walks start at 0,0 (start) and finishes at 'end'. The different environmental conditions of the baby are shown in black, light grey and dark grey; the baby starts in LL with continuous feeding, and the points at which the conditions are changed are also marked by LD of EF (see text for more details). The axes denote the real and imaginary part of the complex numbers, X, used in the Fourier sum to obtain the complex spectrum at test period P. The amplitude of the Fourier spectrum at this period is the distance between the first and last sample of the walkout.

expected for a 14–h rhythm.) All the other features during LD are less prominent and can probably be attributed to noise.

The circadian rhythm weakens after the introduction of EF. From PS (Fig. 3a) it can be seen that the coherence at the higher frequencies increases but the signal amplitudes is not large (see Fig. 3b). The coherence is increased at 4 h, 10 h, 12 h and 14 h. At days 89 to 94 a 4 h rhythm is evident from the PWS (Fig. 3b). Its signatures are also visible at 8 h, 12 h, and 16 h. The phasor-walkout is shown in Figure 9b. The light and dark grey tones mark the LD and EF conditions, respectively. Only for a short time during the EF conditions does the walk show a linear trend, which can be attributed to a weak rhythm in the presence of noise. At its beginning and end the 4–h component clearly has a random character. A phasor-walkout analysis at other test frequencies did not reveal any long duration ultradian signal.

Baby CB. The EF and LD cycles started simultaneously. An intermittent circadian rhythm (with periods between 24–27 h) is detected (Fig. 5a); on days 66, 67 the period is exactly 24 h and the signal has a considerable coherence of 0.69. The signal-to-noise ratio is low and an unambiguous signal is not observed. Figure 9e shows, as an example, the phasor-walkout at 25.5 h; it shows that a rhythm with this period occurs on two distinct occasions.

An increase of coherence can be observed transiently at isolated ultradian periods, such as a period of 14 h at about day 80; however, stable, long-duration signatures are not observed.

Baby KEV. The EF and LD cycles started simultaneously. A clear 24–h rhythm is detected, though it cannot be seen in the contour plots of Figure 6 since the 13–cycle data window cannot be centred on the last 6 days of recording. Figure 9d, however, shows the phasor-walkout at 24 h, and gives clear evidence for a 24–h signal starting at the beginning of the LD/EF cycle.

Weak features at about 2 h 50 min, 6 h and 11 h 20 min can be observed in Figure 6, but no unambiguous ultradian signal was detected.

Baby HARR. The temperatures show increases in coherence (Fig. 4a) and decreases in PWS amplitude (Fig. 4b) at all frequencies during EF. The PWS amplitudes become maximal at about 24 h and 27 h 30 min. During LD conditions (the last week of data collection), the data are ambiguous; there might have been a rhythm with a period of 27–29 h, but this cannot be seen in Figure 4 since the data window could not be centred during the last week. The walkout for 28–h period is shown in Figure 9c; the dark grey line corresponds to the LD conditions. The phasor-walkout at a period of 24 h is not linear during either EF or LD.

There is some evidence for an incoherent signal at periods slightly larger than 3 h; the signatures, at 3, 6, 9, and 12 h, are visible only in the PWS and on about day 113.

Baby SB. The situation is similar insofar as (Fig. 7) signals can be detected at the circadian periods, but their coherence and amplitude are small. Again, the detection

of signals is not unambiguous. The period during LD conditions might be about 21 h 45 min, observed when using a smaller data window. Figure 9f shows the corresponding phasor-walkout; the section corresponding to LD is marked in black. After institution of EF, coherence increases at almost all frequencies, but the PWS maxima are still clustered between 23 h and 25 h. At days 108–115 a 6 h 30 min–6 h 45 min signal is indicated by PS maxima in Figure 7a. Other PS maxima with durations of 2–3 days are observed at 3 h and 13 h.

Discussion

A brief rationale for the analytical methods used in the present study has been given in the Methods section of this report; for more details of the application of these methods, see Hoenen et al. (2001) and Schimmel (2001b). Here, the chronobiological implications of the findings will be discussed.

The main finding from this analysis of core temperature data collected from very premature neonates living in the fairly constant conditions of an incubator (summarised in Table 2) is that circadian rhythms of core temperature were reliably present, but ultradian rhythms were less reliably so.

When in LL and fed continuously by intravenous pumps, all babies showed circadian rhythms with periods between about 22–27 h, though the period and phase varied (relative to external time) during the course of the study. Such observations, of the presence of labile circadian rhythms, accord with those of Martin-du-Pan (1970), made in babies living in constant conditions, and with those of Kleitman and Engelmann (1953), who found a circadian rhythm in babies living in a LD environment. It also accords with the inferences that could be drawn from the studies of Weinert et al. (1994, 1997), where the presence of circadian rhythms — rhythms that showed considerable inter-individual variation in their phase adjustment relative to the LD cycle — was inferred from temperature data that had been ‘purified’ so that effects of the sleep-activity and LD cycles had been minimised.

Although ultradian rhythms were frequently present, a wide diversity of periods was detected both in LL (Table 2) and LD (Table 3). At this point, it should be emphasized that ‘ultradian’ implies many rhythms, not necessarily in the same variable or from the same origin, and the diversity of periods observed by us, together with their lack of consistency, seems to bear this out. Thus, when an ultradian frequency was singled out from any of the series, it can be seen that, even if it were expressed with a high amplitude, this expression vanished after a few days. The opposite situation existed with circadian frequencies. They were present on occasions when ultradian rhythms were not detected at all and, more importantly, showed a consistency that was absent from the ultradian rhythms. Therefore, these findings do not support the view that circadian rhythms arise from interactions between ultradian oscillators (Hellbrugge, 1977; Dowse & Ringo, 1987; Barrio et al., 1997), particularly since it has been argued that such a process is delayed in premature babies (Hellbrugge, 1974), and all our babies were very premature.

Others have considered that the presence of circadian rhythms depends, instead, upon interactions between cells that have similar (circadian) periods (Enright, 1980;

Diez-Noguera, 1994; Bouskila & Edward-Dudek, 1995). A strong candidate for such a source of circadian rhythmicity is the suprachiasmatic nuclei (SCN), even though they are believed not to be fully developed at birth (Rivkees & Reppert, 1992; Rivkees, 1997). Recent studies of mammalian SCN cells in culture (Honma et al., 1998) and *in vivo* (Yamazaki et al., 1998) indicate that circadian rather than ultradian rhythms are the main ones generated, and it is electrical coupling between these elements that is believed to generate a robust circadian output (Mirmiran et al., 1995; Zhang et al., 1995; Liu et al., 1997; Miller, 1998; Honma et al., 2000; Shirakawa et al., 2000). Registering the firing activity of single cells of the SCN (Liu et al., 1997) showed a wide range of periods, and the circadian output is the result of a mutual synchronization of these units. Further, descriptions of the genetic and molecular basis for rhythmicity concentrate on the production of circadian rather than ultradian periods (Hastings, 1998; Dunlap, 1999; Roenneberg & Merrow, 1999; Hastings & Maywood, 2000).

Until circadian rhythmicity has been firmly established, couplings between cells are likely to be weaker and more transient than in the adult. Weak couplings mean that phase differences between the circadian rhythms change with time, and this can cause energy at ultradian frequencies in the Fourier spectral analysis. With a single spectral computation upon a particular stretch of data (as was performed in Tenreiro et al., 1991), this might be misinterpreted as indicating an ultradian signature. However, in a moving window analysis (such as used in the present analysis), the energy would be unlikely to appear at the same frequency for consecutive data stretches, as is illustrated in the Fourier transform (Fig. 8). Indeed, the moving window Fourier analysis of all babies showed — with only one exception, a 4–h period during days 87–93 for baby AK — no rhythm with a period of less than 8 h that lasted longer than 3 days. With PWS also, large signatures at ultradian frequencies were observed, but the use of partly overlapping data windows indicated that these signatures were of short duration.

During time spent in LL, all babies were ‘ill’ on at least one occasion, during which they needed ventilatory support and/or phototherapy (see Figs. 2–7). For baby AK (Fig. 3), this was associated with the development of a rhythm with a period of exactly 24 h, which might have been a direct response to the treatment it received. For all babies, however, there was an increased presence of ultradian rhythms (with a wide range of frequencies), nearly always with a simultaneous fall in the strength of circadian rhythms. This partial switch from circadian to ultradian rhythmicity has been observed also in adults in intensive care (Waterhouse et al., 1996), a switch that reversed when patients were recovering. Whether, in adults, this temporary switch originates in the SCN itself, or whether, instead, it originates ‘downstream’ from the circadian oscillator (due to drug therapy, for example), is unknown; it is also unknown if the phenomenon in the present group of babies has the same explanation as in adults.

When babies were transferred into the room where discontinuous (EF) feeding was instituted and a LD cycle was present, there were some changes in the observed rhythmicities (Table 3).

Instituting the EF condition produced a slight increase in the strength of ultradian rhythms, particularly in babies GRIF and AK (and to a lesser extent in KEV and

HARR), and this might have been a direct response to this rhythmic input. The response was weaker than that found in full-term, healthy babies studied 2 days and 4 weeks after birth (Weinert et al., 1994, 1997). One possible reason for this is that our babies had suffered from disorders due to being very premature and, even at the end of the study, they still had gestational ages of only 37–44 weeks (see Table 1); by contrast, in the study of Weinert et al., the responses to feeding in the healthy babies increased between day 2 and week 4 (about 40 and 44 weeks gestational age, respectively).

A moderate increase in strength of circadian rhythms in response to the imposed LD cycle was present in most babies and, in those babies in which it was a period of exactly 24 h that increased (GRIF, CB, KEV), this can be attributed to a direct effect of the LD cycle. This result is comparable to those of other studies where a masking effect of light has been shown in very premature human (D'Souza et al., 1995) and primate (Rivkees et al., 1997; Hao & Rivkees, 1999) infants.

Notwithstanding the strong influence of the LD cycle, feeding must be considered also. A recent study showed that a feeding cycle entrained the circadian rhythm of liver activity, independently of the SCN, that remained entrained by the environmental LD with a different phase (Stokkan et al., 2001). Similarly, a restricted-feeding schedule phase-shifted the expression of clock genes in cerebral, but SCN-independent, areas (Wakamatsu et al., 2001). This action of the feeding cycle could explain the changes observed in the present study in the general pattern of frequencies when EF was introduced. Even so, there was very little evidence that EF acted to promote circadian rhythmicity (by freqency demultiplication, for example).

As for the ultradian frequencies, although it is known that the SCN and other brain areas in mammals (Yamazaki et al., 1998) possess cells which show ultradian rhythms, they may be expressing rather different processes. Short cycles that are not controlled by a clock, such as those involved with digestion, for example, are commonly found in all organisms and might account for the transient ultradian rhythms shown in the current study.

We conclude that the presence ultradian frequencies, even with high amplitudes, is fortuitous and could lead to a misleading interpretation of the role played by these rhythms in neonates. Suitable methods of analysis that allow an assessment of the consistency of a frequency in a time-series may help in the detection of persistent rhythms, even ones of low amplitude. The early development of a circadian rhythm of core temperature in very premature neonates, shown here, did not coincide with the disappearance of ultradian rhythms; on the contrary, transient, ultradian cycles were detected when circadian rhythms were already well established.

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