The Development of Circadian Rhythms in Human Infants

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Abstract

Introduction
The first four postnatal months, for a newborn infant, is a period of rapid adaptation and change. Infants undergo a series of integrated physiological changes that culminate in mature physiological diurnal rhythms by which they establish equilibrium with the new environment, all of which are under the genetic control of the biological clock.

This is a longitudinal study of 35 infants in which the age related changes in physiology, are assessed during night time sleep and related to circadian genes, melatonin and cortisol.

Aim
The aim of the study is to monitor the physiological development of normal full-term human infants concomitantly assessing the expression of circadian genes.

Method
Full term healthy infants were selected. Infants were recruited into the study from 6 weeks until 18 weeks of age. Fortnightly home visits were conducted in which the overnight deep body temperature of infants was monitored. On each night of study, actigraphy was used to study infant and maternal sleep. Longitudinal measurements of melatonin and cortisol secretion by paired day-night urine collection and peripheral gene expression using buccal swabs were taken by mothers.

Results
There is evidence of a sequential and ordered development of circadian rhythms in human infant physiology. There was a temporal relationship demonstrated in the maturation of the infant circadian rhythms. Core body temperature demonstrated a robust rhythm, characterised by an abrupt change, the timing of which varied from infant to infant. Night time melatonin secretion increased with age. Cortisol played a key role. Infant sleep improved with physiological maturation. A number of complex relationships between aspects of the physiology and circadian gene expression were elucidated.

Conclusion
There is a demonstrable integration of genetic and physiological changes, during the immediate postnatal period when infants are most vulnerable to illnesses and particularly to sudden and unexpected death.

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Chapter 1

Introduction

1 Introduction

Happiness is not a matter of intensity but of balance, order, rhythm and harmony. ***Thomas Merton 1915 - 1968***

1.1 Introduction

The first four postnatal months of life are one of the most critical periods in human growth and development. During this time, complex developmental and physiological changes are occurring at an incredible rate over a brief period of time; one which accounts for a significant portion of the infant's total lifespan to date. The consequences of a delay or malfunction during the assembly and stabilisation of the physiological apparatus at this stage could be devastating; contributing and leading to long term morbidity, increased vulnerability to disease and even mortality as characterised by Sudden Infant Death Syndrome.

Neonatal physiology in the normally developing infant is an area that has not traditionally been studied in great detail, when compared to other periods. In order to understand malfunction; one must understand function and normality. This thesis will seek to explore the complexities of the maturing physiological systems in newborn infants, within the first four postnatal months; in the home environment within which they are cared. To probe further into this critical and key stage of development, the order, timing, interaction and relationships between the separate components of physiology will be examined. Components parts of the physiological systems will be considered in terms of 24 hour biological rhythms – as dictated by the body's preset 'master clock' - the suprachiasmic nucleus (SCN) and in terms of outputs of the clock – genes, hormones and their influence on behaviour as manifest by infant sleep.

At delivery, an infant's body undergoes a series of changes which allows it to survive outside the womb disconnected from its mother and the placenta for the first time. Spontaneous independent respiration is established, and the newborn's cardiovascular system undergoes a series of adaptations to change from foetal to neonatal circulation. The adaptations which occur at delivery enable the newborn to cope with their new environment (Sinha & Donn, 2006).

The physiological changes which occur over the first 2 to 4 months of postnatal life, culminating in the establishment of 24 hour circadian rhythms, may perhaps also be 'life saving' and allow the infant to adapt to the environment outside the womb. They denote a change of state. It is during this time that an infant moves from the 'immature' to the 'mature' physiological state; from 'unstable' to 'stable' physiological systems; from 'susceptible and exposed' to 'insusceptible and protected.' Remaining in an immature, unstable physiological state confers vulnerability (Guntheroth & Spiers, 2002, Bajanowski *et al.*, 2007); the vulnerability, pre-programmed from foetal life, may continue through into adult life (Barker, 1988, Barker *et al.*, 1989).

Many of the individual components of physiological maturation during infancy have been studied in isolation; the development of a circadian pattern of cortisol secretion; changes in night time melatonin secretion contributing to sleep; day- night patterns of heart rate, respiratory rate, blood pressure and temperature.

Each of these independently is important in its own right, however what is the relationship between them? What determines their order? Is the development of each system sequential and predetermined or does it occur at random? What switches on the body's internal clock mechanism? What delays it? Can it be advanced? What is the influence of genes and the environment? How is infant health and well being impacted?

The period of passive 'feeding and growing' in the first months of life is in fact one of complex activity and physiological change. It hosts a concert of complex interactions of genes, hormones, neuronal messages which culminate in the final maturation which set a baby on its life journey into childhood and beyond…….

It is hoped that this study will provide an important platform for further and fuller understanding of infant developmental physiology. All measurements were conducted within the home setting. Particular attention was paid to the period of night time sleep in the infants.

The thesis will be presented in the following way:

The introduction will examine themes; physiological development in humans and circadian rhythmicity. Individual components of physiology investigated in this study are then dealt with in turn - temperature development; melatonin and cortisol secretion; the development of infant sleep; and the topic of human clock genes. The methods and results from the data collected pertaining to each of these areas are presented followed by a discussion of the findings and their implications on infant health.

1.2 Physiological Development in Humans

Human development occurs in a set sequence. The timing of the sequence may be altered but in most cases the integrity of the order is always maintained and is the same, irrespective of external factors (Falkner & Tanner 1986). Physiological state, although preset from before birth, matures with increase in age. There are certain periods in development when the pace of change is at its greatest, and it is then that the developing infant is at its most vulnerable (Chamley *et al*, 2005, Slater & Lewis, 2002). This describes well the journey an infant undergoes in the first few postnatal months, moving from newborn to full 'adult' physiological maturation.

A comprehensive description of the different aspects of development was given by Boyd and Bee (Bee & Boyd, 2007). Puberty is a period of development which illustrates this clearly. It is a common human experience. It cannot be reversed. The sexual development occurs in a set order, however the timing of the change can be altered by external factors, the environment and illness.

Aspects of development change as an individual gets older (Chamley *et al* 2005). Previous work by this research group has demonstrated that the majority of important developmental physiological changes occur within the early period during months one to four, as a continuum of those which occur in the foetal period (Lodemore *et al.*, 1991, Petersen *et al.*, 1991, Jackson, 2000, Jackson *et al.*, 2004). The main aspect of physiology investigated in this work led by Wailoo and Petersen was the development of changes in core body temperature during night time sleep in infancy. For the purpose of this study we chose to concentrate on the same period of development (first four months of life) but to investigate several aspects of development in parallel.

There is general agreement, that although the exact timing of maturation of physiological systems in infants cannot be pinpointed, most system maturation has occurred by 6 months of age, and definitely by 12 months. Previous work by our research group has pointed toward months two to four as being crucial, in terms of physiological maturation and so it was decided to concentrate on this period.

The notion of 'passiveness' associated with the first few postnatal months, with a baby just 'feeding and growing' with a doubling in birth weight (Chamley *et al* 2005) is being dispelled. It is in fact a time when the speed of change in developmental parameters (Tanner, 1989) may create a period of increased vulnerability and there is a move towards strengthened diurnal rhythms and systems (Bee & Boyd, 2007). This study will focus on the rapid and unique changes which occur during infancy only.

The fact that physiological changes occur at within a narrow and critical time period confers increased vulnerability and susceptibility to the infant. This may be borne out by the fact that the peak of mortality in the post natal period still occurs at 3 to 4 months of age with Sudden Infant Death Syndrome being the main and the leading cause of death in the western world, despite successful public health campaigns and improved healthcare (Mitchell *et al.*, 2007). The question has been raised - are there any other health interventions that could be introduced that would allow for SIDS rates to fall even further? And if so what is the science behind them that may explain why it in fact occurs?

How does an infant navigate this potentially 'stormy' period of life and successfully make it to the other side? What determines how and when physiological changes

occur? What influences them? How do we minimise the impact of an external ‘insult’ and ensure an appropriate achievement of a mature stable infant physiology?

The next section will focus on the normal patterns of physiological development previously studied in human infants and will seek to examine the factors which are known to affect them.

Summary

1. Development encompasses a range of physiological, biological and social changes in humans that move the individual from a simple to a more complex organism.

2. Development is ordered, occurs in all organisms and is sequential and irreversible.

3. Although the order remains the same, the rate of maturation is different and is under the influence of internal and external factors.

4. Infancy is a critical period of development due to the complexity and rapidity of the changes which occur.

1.3 Circadian Rhythms

There are several methods which may be employed to investigate infant development. One method involves examining longitudinal physiological changes over time. This may be quantified by measuring the development of circadian rhythms in infants.

Circadian comes from the Latin '*Circa diem'* which means "about a day." Circadian rhythms exist in both animal and plant species and have been conserved across all the species. They are fundamental to survival as they provided a bridge between the physiological systems which operate with an organism and the external environment as well as time. The 24 hour rhythms allow an organism to adapt quickly internally to changes on the outside (Brady, 1979, Squire *et al.*, 2009).

Chronobiology involves the study of biological rhythms which are characterised by cycles that oscillate around a 24 hour period, a *circadian* rhythm. Shorter rhythms are known as *ultradian* while longer rhythms are referred to as *infradian*. A classic example of a circadian rhythm is the sleep wake cycle (Brady, 1979, Minors, 1981).

Familiarity with some of the terminology used in chronobiology is helpful for the purpose of understanding of this work. Figure 1.1 provides a visual aid.

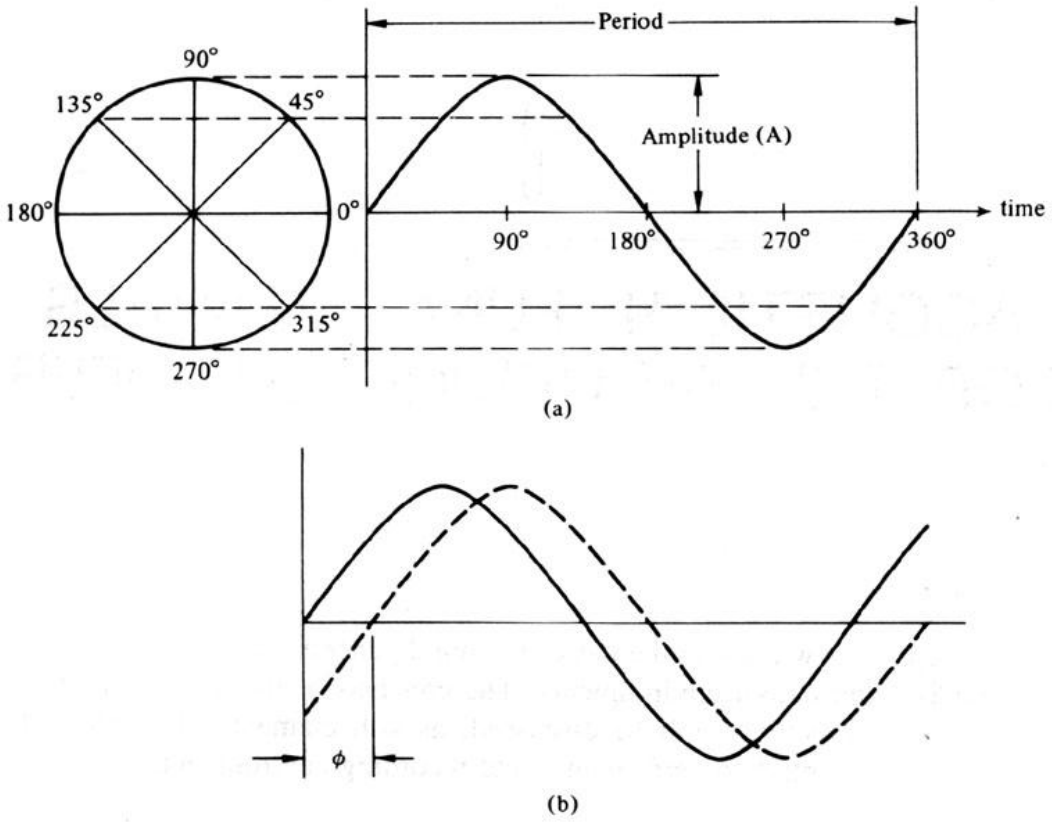

Figure 1.1 - Diagram illustrating circadian oscillation and associated terms.

Amplitude is denoted as (A); ø denotes a phase shift.
(Reference http://scienceblogs.com/clock/upload/2007/07/oscillation.jpg)

The 24 hour biological rhythms can be drawn as sinusoidal, symmetrical or asymmetrical curves on a graph. If the rhythm can be drawn so that it most closely resembles a cosine curve, the degree best fit is referred to as *mesor*.

The **period** is the time taken for the measured rhythm to complete one cycle or oscillation. It is the time interval between two peaks or troughs. Another method of calculating it is to take the reciprocal of frequency.

Amplitude is defined in two different ways. It is the maximum between the highest and lowest points on the graph. In some cases it is reported as half that value (see Fig 1.1 – similar to the radius rather than the diameter of the wheel). For the purpose of this study, the second definition is utilised.

Frequency is the number of oscillations per unit time. It can be determined by taking the reciprocal of the duration of an entire cycle or period. If a rhythm speeds up, then the frequency of the oscillation increases while the period decreases.

Any single time points selected on the cycle is known as **phase.** Commonly the minimum or maximum or the minimum points on the curve may be selected. An alternative definition is that phase is the time at which the cycle reaches a pre-selected or identifiable point.

Acrophase is the time on the x-axis of the maximum of the cosine function if a cosine function wave has been fitted to the data.

A **phase shift** is used to describe a movement of the rhythm (whilst maintaining its shape or pattern) along the time axis to the left or to the right. A **phase advance** occurs when the rhythm shifts to the left indicating an earlier achievement of the rhythm. A **phase delay** is the opposite with the rhythm occurring later.

Nychthemeral refers to when there are no alterations to the environment while the subject is being studied for the purposes of chronobiological research. All measurements in this study were nychthemeral. Infants were cared for in normal lighting conditions irrespective of the season. Temperature varied according to the heating within the home and maternal preference and the normal routine of the infant was maintained. No factors were controlled for as might be done more easily in a sleep lab setting.

1.3.1 Detection of Rhythm

In order to detect a rhythm a variable must be measured over more than one cycle and the cycle must be shown to be repeated. Adequate and frequent sampling is necessary.

1.3.2 Exogenous and Endogenous Rhythm

(Adapted from Minors 1981)

Exogenous rhythms are characterised by the environment and outside factors which are thought to control them. If the external factor is taken away or a change made to the environment which is critical, then the rhythm will fail to exist.

Endogenous rhythms have an internal driving factor which 'compels' it to run and directs it. It does not depend on the environment and will continue to oscillate if all external factors are obliterated or extinguished. They have an internal 'clock' or 'pacemaker.'

Entrainment is the process by which factors which are not governed by an internal pacemaker; but which are related to the environment (known as *zeitgebers* or time givers) which synchronise or change the timing of a rhythm. An example is darkness which is a cue giver for the secretion of melatonin within in the body (Minors, 1981).

1.4 Ontogeny in Human Infants

Well defined day night rhythms are thought to be absent in newborn babies. Within the womb, there is evidence of short (ultradian) rhythms or initial suggestions of rhythmicity. These are thought to be a consequence of the foetal entrainment by the mother. A clear emergence of day night differences in physiological systems and patterns of behaviour does not occur till a few months of age.

One study conducted involving 12 premature infants (29 to 35 weeks) did not find a clear diurnal 24 hour rhythm in core body temperature over the first weeks of life. Instead they demonstrated the presence of multiple ultradian (short) rhythms in body temperature with no clear diurnal day night rhythm. Patterns for heart rate and sleep-wake cycles were more variable and showed a less distinct pattern (Mirmiran & Kok, 1991).

Below is a description of how circadian rhythms emerge with regards temperature control, hormone secretion and the development of sleeping patterns in infants. The ontogeny (origin and biological development) of clock genes has not previously been described in human infants and consideration is given to the published literature related to this work.

1.5 Temperature

Temperature was selected as the primary outcome measure of physiological maturation in the infant. It exhibits a robust, repeatable rhythm over a 24 hour period, with sleep and is easily measurable (see introduction section 1.5.3). Core body

temperature is one of the most tightly regulated systems in the body. Deviation from 'normal' physiological development, as characterised by the development of adult mature temperature pattern during sleep, may be a marker for future morbidity, mortality or be the basis for increased vulnerability such as to the disease entity known as SIDS.

1.5.1 The Mechanism of Thermal Regulation

The hypothalamus is responsible for the neuronal control of core body temperature. Peripheral receptors send signals to the posterior hypothalamus at the level of the mammillary bodies. These signals integrate with those from the anterior hypothalamus to regulate body temperature.

Body temperature is lowered by several methods: vasodilation of peripheral blood vessels, sweating and a decrease in metabolism. Conversely, temperature is raised in response to low temperatures by vasoconstriction, piloerection and the generation of heat by processes controlled by the sympathetic nervous system.

Core body temperature is maintained within a strict, narrow and precise range of +/- 0.6º Celsius, which is critical for enzyme function. There is a set point of 37.1º Celsius above which heat is conserved and below which heat is produced. This occurs in order to maintain a normothermic environment. Temperature only changes in response to a significant insult such as illness, vaccination or profound hypothermia.

1.5.2 Circadian Rhythms in Temperature

The pattern of change in core body temperature within a 24 hour period has been well described in adult chronobiology research. The tight control of core body temperature is thought to be part of the mechanism which determines the sleep/wake cycle (Aschoff, 1981).

During the day it had been well documented that body temperature is high with a gradual fall over the first few hours after the onset of sleep based largely on work in the adult population (Minors, 1981). Early summary reports investigating the development of circadian rhythms with regard temperature control in infancy and childhood, showed a drop occurring through the night before a gradual increase towards day time values based on the analysis of data from 100 children (Hellbrügge, 1960). The maximum drop was reported to occur later in the night for older children and earlier in infants. However the time of onset of sleep was not taken into account. Therefore the finding of a later drop occurring in older children, per say, when compared to an infant, is likely to be related to a later bed time, rather than denoting a change in maturity state. Another method of determining the timing of temperature circadian rhythmicity relates that the measurement of the depth of fall in the first four hours of sleep at any age, as being more important. Normalising the data to timing of sleep onset reveals the maximum drop occurring in the first few months of life, not the first few years. This technique was used by Lodemore et al in their description of the development core body temperature rhythms. To be more precise the timing of the maturity in infancy of core body temperature changes occurred, at 2-3 months of age. This is described in more detail below as it is the method selected in this study as a marker of physiological maturity (Lodemore *et al.*, 1991).

1.5.3 Ontogeny of Temperature Control

When a baby is first born, there is thought to be little difference in day and night time core body temperature. After a few weeks of age, a day and night difference begins to emerge, with night time temperature falling within the first four hours of sleep. The maximum fall is to 36.7ºC. The infant does not have a fully mature circadian rhythm at this point. As the baby gets older, just prior to sleep onset, there is an appreciable increase in the depth of drop in core body temperature just prior to sleep onset within the first four hours. Once the temperature is able to fall to below 36.5ºC, the infant is described to be exhibiting a 'mature' or 'adult-like' temperature rhythm (Lodemore *et al.*, 1991).

Once this mature rhythm has been established there is no further drop in core body temperature during night time sleep. Therefore if subsequent measurements are done on an infant once 'maturity' has taken place the temperature plots look the same. This pattern in the core body temperature is stable throughout the rest of the childhood and adulthood. The core body temperature will only rise if an individual is exposed to an infection or has immunisation (Lodemore *et al.*, 1992, Jackson *et al.*, 1994, Jackson *et al.*, 2001). Similar descriptions of core body temperature patterns in night time sleep have been given in other studies. In addition Glotzbach and Weinert have also demonstrated changes in sleep-wake levels as well as heart rate, occurring over the same period (Glotzbach *et al.*, 1994, Weinert *et al.*, 1994).

1.5.4 Factors affecting Temperature Control

Intrauterine uterine growth retardation (IUGR) (Jackson *et al.*, 2004), being from particular ethnic minority groups (Petersen & Wailoo, 1994), coming from a poor economic background (Wailoo *et al.*, 2003), as well as exposure to environmental tobacco smoke, male gender and high birth order; have all been shown to cause a delay in the achievement of the maturation of adult temperature pattern (Lodemore *et al.*, 1992, Tuffnell *et al.*, 1995). These factors overlap with those demonstrated in epidemiological studies to be associated with Sudden Infant Death Syndrome (SIDS). The peak age in incidence coincides with a possible period of vulnerability caused by a delay in the maturation of the physiological systems in humans (Bajanowski *et al.*, 2007, Rognum & Saugstad, 1993).

In the next section another physiological parameter which displays a circadian rhythm, melatonin, will be described.

Summary

1. Normal thermoregulation is controlled by the hypothalamus.

2. Infants develop adult-like circadian rhythm core body temperature around 3 months of age. This is related to night time sleep.

3. Factors directly related to the infant, socio-economic factors as well as those related to infant care practices and the environment, affect the age of the maturation of temperature control.

4. Mature temperature circadian rhythms are stable from 3 months of age with a minimum drop to just below 36.5ºC unless the infant is ill or has been immunised.

1.6 Melatonin

(Reviewed in Cardinal 1998)

Melatonin is a primary chemical messenger of the internal 'clock' which controls all physiological systems. It plays a key and central role in transmitting and translating messages regarding the external environment to the central nervous system which in turn allows circadian rhythms and peripheral oscillators to be synchronised. The infants' night time sleep was selected as the period for study in this project as it is during night time sleep that the evolution of circadian rhythms in infancy can be mapped. It is also during night time sleep that the predominance of cot deaths occurs (Blair *et al.*, 2006), which is why it was originally chosen by this research group as its primary focus.

1.6.1 Production and Regulation of Melatonin Secretion

Melatonin, a methoxyindole, is mainly made and secreted by the pineal gland during darkness. It is thought to be the primary messenger of the body which conveys messages regarding light and dark to cells in the body, specifically retinal cells in the eye. It helps stabilise circadian day and night rhythms and may determine patterns of physiological development. It is an important signal of the central biological clock.

Melatonin is made from tryptophan and is found in circulating blood. Tryptophan is converted to 5-hydroxy tryptophan by hydroxylase enzyme. The product made as a result of this reaction is converted to serotonin. As a result of the action of two enzymes, namely sNAT and HIOMT, serotonin is converted to melatonin (Reiter, 1991, Vijayalaxmi Jr *et al.*, 2002). Melatonin is not stored in the body. Therefore if

melatonin is measured in the blood circulating volume, it is a direct measure of the quantity of melatonin produced.

The secretion of melatonin is under direct control of the suprachiasmatic nucleus (SCN). Information about darkness and light is relayed to the SCN from the retina, via specialist nerve pathway called the retinohypothalamic fibres. During the day, bright lights inhibit melatonin production. It reaches maximum concentrations during periods of darkness (Reiter, 1991).

1.6.2 Actions of Melatonin

A major role of melatonin involves determination of circadian rhythmicity. Body temperature and night time sleep, are directly influenced by levels of melatonin within the body.

In animal studies, melatonin has also been identified as a potent free radical scavenger with anti-oxidative properties. Its mode of action is thought to be related to enhancing the amount of detectable specific active enzymes. There is little data on how this translates in the human body as reviewed by Claustrat (Claustrat *et al.*, 2005).

Melatonin is also thought to have a role in immunity by being involved in some way in the interaction between the immune system and the central nervous system (Cardinali & Pévet, 1998, Guerrero & Reiter, 2002, Claustrat *et al.*, 2005). It is involved with T helper cell recruitment. It also is thought to increase natural killer cell activity. Some work suggests that it works in direct opposition to steroid induced immunosuppression and prevents T cell death (Shneerson, 2005). The exact

mechanism by which this occurs is not clear. There is clearly a great deal to be found out about the messenger of the clock.

1.6.3 Circadian Pattern of Melatonin Production

The majority of studies which have demonstrated the circadian pattern of melatonin secretion (day night differences) involve adult participants. Melatonin peak onset time is commonly quoted as between 3am and 4am during periods of darkness. Values fall during the daytime with exposure to light at dawn.

1.6.4 Ontogeny of Melatonin Secretion

Melatonin receptors have been identified in the foetal SCN. Several studies have investigated the presence of melatonin in the blood immediately after birth. Melatonin has been detected in newborns but there has been no recognisable day night rhythm in the first few days and weeks; as reported by Kivela, Munoz-Hoyes and Thomas (Kivela *et al.*, 1990, Munoz-Hoyos *et al.*, 1993, Thomas *et al.*, 1998). Interpretation of the results from these studies is limited by the manner by which they were conducted. Participant numbers in one of these studies was small. Kivela et al included only 19 mother-infant pairs and used a twice a day sampling schedule. The spread of sampling was very wide (8:00 to 20:00 for day time urine sample and 20:00 to 8:00 for night time sample). This may account for the finding that a diurnal rhythm was not detected in the first few weeks. Using a minimum number of samples per day in attempts to minimise disruption and maximise recruitment and retention study design may compromise study design and make results less interpretable. The study by Munoz-Hoyos el al demonstrates this. Although the number of participants was larger (n=119) single samples was taken per infant (for the reasons stated above), which would make comment on diurnal rhythm within any infant impossible and meant that different infants were being compared at different times.

The actual age of the emergence of the first age-related day night difference in melatonin production has been described as occurring at around 3 months of age (Kennaway, 2000).

It is at this time that infant sleep is thought to become consolidated with parents reporting that the infant has 'learnt to sleep though the night' and night time sleep exceeds day time sleep (Rivkees, 2003).

1.6.5 Factors affecting Melatonin Secretion

Prematurity is thought to delay the development of melatonin rhythm by up to 9 weeks (Kennaway *et al.*, 1992).

Exercise and prolonged fasting affect the pattern of melatonin secretion but these effects are minimal; especially when compared to by far the strongest zeitgeber light (Vijayalaxmi Jr *et al.*, 2002, Reiter & Richardson, 1992).

Melatonin has an antiphase partner hormone cortisol. This is examined in the next section.

Summary

1. Melatonin is produced by the pineal gland.

2. It has an important role in determining day and night rhythms in human physiology.

3. Its synthesis is mainly determined by light/dark cycles.

4. Peak melatonin production is during the night time period with a fall during the day.

5. The age when the melatonin rhythm is thought to stabilise is approximately 3 months of age.

1.7 Cortisol

Cortisol is a hormone, secreted by the adrenal glands, which has been shown to exhibit one of the most robust circadian rhythms. It is produced in antiphase to melatonin. The role it plays in the 'clock' and circadian rhythmicity is an emerging and exciting area of research. It has traditionally been a 'cross over' measure quoted frequently in psychosocial and biological work as a physical measure of 'stress.' In molecular biology work is has been found to synchronise the clock (see introduction section 1.9.6). It was included as a measure of interest in this study as it may be the 'key' that unlocks the door to physiological maturation. We do not know the precise role it plays in chronobiology and the consequence of early or late maturation of the corticosteroid axis/rhythm; therefore it is worth exploring.

1.7.1 Production and Regulation of Cortisol Secretion

Cortisol is a hormone which is essential for life. It accounts for 95% of glucocorticoid activity. It has a major role in the body's ability to process and metabolise glucose, protein and fats. It also facilitates the body's normal response to stress. In its absence, a minor illness can cause death.

Cortisol is made from cholesterol. It is produced in the adrenal cortex. Cholesterol is acted upon by the enzyme cholesterol desmolase and as a result pregnenolone is made. This is then converted to 17-hydroxypregnenolone; then to 17-hydroxyprogesterone; onto 17-deoxycortisol and finally to cortisol.

1.7.2 Actions of Cortisol

Cortisol has several functions. Firstly it is involved in gluconeogenesis; the production of glucose from protein and other substrates. This occurs in the liver. Cortisol also helps to regulate blood sugar levels by decreasing the utilisation of sugars in cells other than in the liver. It promotes the synthesis of protein within the liver. Simultaneously, protein is broken down to its constituent amino acids outside of the liver in order to increase availability for gluconeogenesis within that organ. Fatty acids are made available by the breakdown of adipose tissue (Guyton & Hall, 1997).

Cortisol has a direct effect on vessels which make up part of the cardio-vascular system. In times of stress, such as severe hypovolaemia (or shock) or in times of prolonged fasting, peripheral blood vessels constrict and allow diversion of blood to vital organs.

Cortisol also has a role in infection, healing and inflammation (Nelson, 1980).

Adrenocorticotrophic hormone (ACTH) is a substance produced by the pituitary gland which overall is responsible for regulating the production of cortisol from the adrenals. It is made in response to another hormone corticotrophin releasing hormone (CRH) which is made by the hypothalamus. CRH secretion is under direct control of the central nervous system. High levels of cortisol suppress ACTH secretion forming a negative feedback control 'switch' which can be used to reduce cortisol production. The negative feedback system that controls cortisol can be switched off or blocked by several factors including stress (Bravo, 1989).

1.7.3 Circadian pattern of Cortisol Secretion

In adults, cortisol has a robust day/night rhythm of secretion; with a peak at dawn, in order to get ready for the 'stresses' of a new day and a trough occurring normally in the middle of the night. Melatonin and cortisol are 'phase locked' with the cortisol trough occurring at the time of melatonin peak.

The pattern of cortisol secretion is also linked to the sleep wake cycle. A change in night time sleep onset will shift the timing of the peaks and troughs.

1.7.4 Ontogeny of Cortisol Secretion

A small study confirmed work by previous researchers that a robust day-night pattern of secretion of cortisol is not present at birth (de Weerth *et al.*, 2003). In other work conducted in term, premature and small for dates infants, cortisol levels were found to be high initially straight after delivery. This may confer protection to the infant during the delivery itself or initial high levels may enable the newborn to cope with the rapid adaptation required ex-utero in the brand new external environment. The foetal adrenal gland then involutes quickly within the first week with an accompanying drop in levels (Rokicki *et al.*, 1990, Atkinson, 1995).

The amount of cortisol then produced starts to rise again over the next few weeks in response to increased ACTH production. This results in a tripling or quadrupling in blood cortisol levels (Winter, 1985). The infant then begins to develop a circadian pattern over the first 4 postnatal months of life.

There then fails to be consensus on the exact timing of the appearance of an adult circadian pattern as reported by de Weerth et al (de Weerth *et al.,* 2003).

Differences in the timing of maturity of hypothalamic- pituitary- adrenal axis, as found by the individual research groups, may be due to the fact that each group used different definitions as to what constituted a circadian rhythm. Also the frequency samples were taken and methodologies in the studies were not comparable (see discussion section 11.3). In some cases sample sizes were relatively small. There have been a range of ages, quoted in the literature, at which infants have been thought to obtain a mature circadian rhythm of cortisol production: from as early as 8 weeks of age (Santiago *et al.*, 1996); to as late as 9 months of age (de Weerth & van Geert, 2002). Clearly the occurrence of the event has not been pinpointed to an exact time and the variability is wide. Use of a uniform and consistent definition of timing of circadian rhythmicity will allow meaningful comparisons and measuring different aspect of physiological maturation in the same babies at the same time has an obvious advantage.

In a separate study, Weerth et al used a multi-level random and fixed effects model in an attempt to show the emergency of the circadian pattern of cortisol production in a group of term infants. They showed the emergence of a diurnal rhythm occurred on average after 8 weeks of age. There were individual differences. The paper suggested that the differences in age of circadian maturity of cortisol for selected infants may be a marker of normal development, when other factors are corrected for, as was in this case using hierarchal modelling (de Weerth *et al.*, 2003). It should be noted that although samples were taken at five separate time points for each infant on each day of monitoring; no samples were taken between midnight and seven in the morning; which may again limit interpretation of the results.

There are clear differences in infant circadian patterns when compared to those achieved in adult hood. There may be an increasing conferring of stability and irreversibility, as one progresses from infancy to childhood with decreasing likelihood to revert to an immature state. This echoed the sentiment of early work by Vermes et al in 1980 which showed short, ultradian variations in secretion of cortisol in the early postnatal weeks, which emerged as a more distinct 24 hour rhythms from 3 months of age. Once the cortisol rhythm was fully synchronised and stable, there was little regression back to the previous pattern of secretion (Vermes *et al.*, 1980).

1.7.5 Factors Affecting Cortisol Secretion

Stress has a direct effect on cortisol production via the hypothalamic pituitary adrenal axis. It switches off the negative feedback loop and results in an increase of cortisol production.

Infants with mature cortisol axes are more likely to have longer undisturbed periods of night time sleep.

Having small frequent feeds as opposed to larger volumes less frequently, when introduced into the model used by de Weerth did not significantly promote or delay the maturation of the circadian cortisol rhythm but the timing of solids increases overall cortisol levels (de Weerth *et al* 2003). The analysis used to formulate these conclusions was a complex form of modelling using data from 14 infants; within which there was limited heterogeneity; which may affect the validity of these conclusions, with regards there application to the general population.

Identifying factors, which may influence circadian rhythmicity of cortisol secretion, and rhythmicity for the other parameters measured in this study is important, as there may have a role in entrainment of the infant clock by different infant care practices (see discussion 11.5). This may have long term impact on infant physiological development, illness and disease.

The above described aspects of physiology were measured during night time sleep. A fuller description of sleep and its ontogeny are described in the next section.

Summary

1. Cortisol is crucial to an organism's survival.

2. It controls the body's response to stress from minor illness to major trauma.

3. It acts on inflammatory processes and is involved in inhibiting healing at high levels.

4. Cortisol is regulated by ACTH production from the anterior pituitary.

5. In adults it has a diurnal pattern of secretion with the highest levels early in the morning and the lowest levels at night.

6. With most studies, there is a suggestion that there was an early development of circadian pattern by 2 or 3 months of age.

7. There are several factors which affect cortisol production including stress.

1.8 Sleep

Sleep in the postnatal period is important to the infant as a biological function, but it is equally as important, in the social context, to the parents and family. It is an overriding and sometimes a consuming goal of the parents of a newborn to have their offspring "sleep through the night". The physiological processes that lead to this are not well described in the literature.

In this study infant night time sleep was measured, as during this period many physiological changes can be 'tracked' longitudinally. These physiological changes with age can be used as a marker of developmental maturity.

During sleep, an organism has a reduced level of alertness, a lower response to the external environment, and reduced movement (Stedman, 2004). Physiologically there is a range of brain wave activity during sleep which has a characteristic pattern and this is used clinically in the determination of distinct sleep stages using electroencephalography (EEG) neurophysiology (Erwin et al 1984).

Normal adult sleep can be divided into two types - non Rapid Eye Movement (NREM) or Rapid Eye Movement (REM).

NREM sleep has 4 stages. It occupies the majority of adult sleep time. As an individual progresses from stage 1 to 4 of sleep, the depth and level of unconsciousness increases. There are characteristic physiological changes which occur. Heart rate and core body temperature fall as do systolic blood pressure and respiratory rate. Parasympathetic nervous tone increases whilst there is a corresponding fall in sympathetic tone. NREM sleep has characteristic external physical changes which can be observed. Although there is a slower rate of breathing, the respiratory pattern in NREM sleep is regular. Eye movements are slow (hence the name non rapid eye movements). Rather than be flaccid and atonic, body muscle tone is preserved.

During REM sleep, overall sleep physiology is similar to NREM (in comparison to wakefulness), but there are some distinct differences. Breathing is more irregular and eye movements rather than being slow, are rapid. Total body tone is reduced which acts as a protective mechanism and prevents one from enacting one's dreams.

In infants, sleep states have alternative nomenclature. Sleep is divided into Quiet Sleep (QS or Non REM sleep equivalent) and Active Sleep (AS or REM sleep equivalent).

All of the above sleep stages, whether being observed in children or adults, have set criteria which allow the appropriate sleep stage to be assigned. Full detail of the electrophysiological changes in corresponding sleep stages are found in published

technical manuals, for example in Rechtschaffen & Kales (Rechtschaffen & Kales, 1968).

The full investigation of sleep is possible using a method called polysomnography (see below introduction section 1.8.4). It includes measuring eye movements (EOG), muscle activity (EMG), heart variability (ECG) and measuring pattern of breathing and brain wave activity by EEG (Curzi-Dascalova & Mirmiran, 1996, Kahn *et al.*, 1996). At present all other sleep investigations are compared to this gold standard test.

Complex sleep staging was not possible in this study due to the type of equipment available. The equipment purchased was limited by cost and the practicality of using certain equipment in the home. Full polysomnography was not available. Sleep diary supplementation with actigraphy was used as an alternative. The study protocol had to be simplified as much as possible, to take into account the other measurements that were been made alongside that of sleep (see introduction section 1.8.4).

1.8.1 Functions of Sleep

The functions of sleep remain unclear. One theory proposes it helps prepare for the next time period of when one is awake; by allowing the sufficient build-up of energy stores for metabolic processes and activity via specific anabolic pathways. In addition sleep allows time for general repair and recovery. The function of sleep, however, is likely to be more sophisticated than this. A body of evidence is building that in the same way sleep is not an homogenous entity, its function is multifaceted. New neuronal networks are established during sleep and there is synchronisation of existing nerve pathways. This is known as neuronal remodelling and is thought to lead to improved synaptic function, whilst contributing to memory consolidation and integration of complex motor sequences. The entire process is thought to be sleep-state specific with nREM cortical network synchronisation and REM associated new neuronal network formation, which may be used as a marker of neurodevelopmental maturation (Shneerson, 2005).

In infants specifically, sleep is thought to be necessary to maintain brain plasticity through child and adulthood (Krueger & Obál, 1999, Douglas, 2002, Graven & Browne, 2008).

1.8.2 Regulation of Sleep

The activation of specific parts of the central nervous system is important in determining sleep. The Reticular Activating System (RAS), including the pons and the mid brain is key in this regard. Activation of the RAS results in wakefulness. When the RAS is inhibited, sleep occurs (Douglas, 2002, Shneerson 2005). There are multiple brain nuclei, most notably the suprachiasmatic nuclei in the hypothalamus, nerve pathways and endocrine glands which are involved in the neuro-physiological control of sleep. Sleep regulation is driven by two distinct processes: process S which is an internal driver of sleep (known as Sleep homeostasis) and process C which is extrinsic and commonly referred to as Circadian drive. Sleep and wakefulness is a balance of the two (Salzarulo & Fagioli, 1992, Beersma, 1998, Borbély & Achermann, 1999, Shneerson, 2005).

1.8.3 Ontogeny of Sleep in Infants

Sleep patterns change with age. In neonates, there is reversal in the sleep stages as demonstrated in adult studies. In adults, sleep begins with non REM sleep (equivalent to quiet sleep in babies) or deep sleep. Sleep episodes, in infants, begin with active sleep, which is equivalent to REM sleep. In infants there is an alternating pattern of sleep between REM and non-REM which lasts for 60 minutes compared to the non-REM / REM cycle in adults which lasts for 90 minutes (Erwin *et al*., 1984, Coons & Guilleminault, 1984).

Inutero, below 28 weeks gestation, most brain wave activity is indeterminate and cannot be classified into active and quiet sleep. At 28 weeks there is some differentiation into rest activity cycles. REM (active) sleep predominates. This alternates with brief periods of quiet (non REM equivalent) sleep. At term, there is a decreases in the percentage of REM sleep which makes up around 50% of total sleep duration, according to one study group. At this time there is a corresponding increase in non-REM or quiet sleep. By 5 to 8 months of age postnatally, recordings show that non-REM occupies 80% and REM 20%. The EEG now approximates that found in sleep studies performed in older children/teens and adults as reported by Graven and Browne (Graven & Browne, 2008). This was similar to findings in a study which used 24 hour motility signals in term infants on pressure sensitive bedding (Thoman & Whitney 1989). The predominance of REM sleep in the early postnatal period is a finding common with newborn mammals other than the human infant and is thought to be related to the role of REM sleep in brain maturation and development (Frank &

Heller, 2003). Other studies have shown an increase in quiet sleep with age, with no change in the percentage of active sleep rather than a decrease, in both term and premature infants (Navelet *et al*., 1982, Mirmiran *et al*,. 2003).

Mirmiran suggested that there is a possibility that sleep is controlled by a mechanism which is inherent to the infant and that the timing of sleep consolidation and maturation is not as significantly affected by factors such as prematurity as might be expected (Mirmiran *et al.*, 2003).

Several studies pin point the timing of the change in sleep from immature to mature patterns to occur at around 3 to 4 months of age. There are several ways sleep maturation can be mapped. One way is to document the number of REM sleep onset periods. In a maturing infant these decrease with age (Coons & Guilleminault, 1984).

Another method used to track changes in sleep is to determine the age when sleep consolidation occurs, i.e. when night time sleep exceeds day time sleep (Glotzbach *et al.*, 1995, Ariagno *et al.*, 1997, Mirmiran & Ariagno, 2000).

Mirmiran demonstrated that at 36 weeks (corrected gestational age); infants spent a large proportion of time during both day and night asleep (65% of their day sleeping and 71% of the night period asleep). By 3 months of age, daytime sleep had fallen to significantly lower levels (as low as 18%) while night time sleep remained relatively unchanged at 65% (Mirmiran & Ariagno, 2000).

Sleep can clearly be used as marker of developmental maturity as an adult diurnal sleep pattern becomes firmly established. It is the 'most obvious' diurnal rhythm that develops and may have special significance if it represents a culmination of the other physiological measures 'maturing' and stabilising.

1.8.4 Measurement of Sleep

Most research in sleep medicine concentrates on the pathologies and the physiology of adult sleep. Work in children, infants especially, is thought to take much longer to set up, conduct and complete, yield much more complex and difficult data to interpret and which must be adjusted according to the age and maturation/developmental stage and generally be exceptionally complicated to perform (Thoman & Acebo, 1995). In this study all these issues had to be taken into account and a method of measuring sleep was chosen that gave limited detail but had maximum ease of use and acceptability of the equipment to the participants and carers.

Polysomnography (PSG) is the gold standard for sleep studies. It may be employed in the paediatric population (Sterman *et al.*, 1977, Hoppenbrouwers *et al.*, 1982).

However in children and infants it is fraught with technical difficulties – both in its set up and its interpretation. A good manner with parents and child, as well as perseverance, is needed to obtain a full study with interpretable data from this complex investigation. The operator must be specifically highly skilled in the setup of the EEG montage and the attachment of the multiple channels and probes (respiratory/muscle/eye movements etc.) in, most probably, a non cooperative child or

infant. Through the night there is a high risk of data loss if probes become detached (Kahne & Fisher, 1973).

Financial and time constraints in this project did not allow for full PSG to be performed in the home setting. Portable equipment is expensive, interpreting PSG is time consuming and it requires a high level of expertise and training.

Alternatives to full polysomnography are direct behavioural recording, time lapse video recording and motion sensor devices e.g. in a mattress (Thoman & Acebo, 1995, Anders & Keener, 1985, Thoman & Glazier, 1987). With the exception of the latter mentioned method, the others require hours of observer time. The data must be checked for inter observer agreement to ensure validity and again is time consuming. Families may not consent to having video recording or direct observation and this data must be treated sensitively as other parent/carer behaviour may be captured on the same footage in addition to infant/child sleeping patterns (Anders & Keener, 1985). Despite these drawbacks, all of the above methods have been reported to document changes in sleep patterns which it is claimed has contributed to research linking sleep with neurodevelopmental outcomes (Thoman *et al.*, 1981, Lombroso & Matsumiya, 1985, Thoman & Whitney, 1989, Freudigman & Thoman, 1993, Whitney & Thoman, 1993).

This type of claim would need to be tested and validated in studies with large sample sizes in order to prove or disprove this.

For the purpose of this study, the protocol had to be 'user friendly' and promote maximum participation so a simple method of measuring sleep (actigraphy) was employed.

Actigraphy allows home monitoring of infant sleep. The actiwatch is a lightweight, small, simple to use device which measures movement by use of an accelerometer. Using algorithms (mainly written for adult work) it translates movement counts into estimates of wake and rest (see methods section 2.7). The algorithms are written specifically for adult use. They do not allow sleep to be separated into different sleep states as it gives only a crude measure of movement rather than a precise measure of sleep (Acebo *et al*., 1999, Sadeh & Acebo, 2002, Morgenthaler et al., 2007). An actiwatch model, however, has been developed for use in infants which divides sleep into active and quiet sleep (Sadeh *et al.*, 1989).

Another drawback of actigraphy it is less able to differentiate between movement artefact during sleep from movement during 'genuine' wakefulness. Movement in infants induced by the carer (such as rocking) may also be scored incorrectly as a period of wakefulness when actually the infant is asleep. This is less of an issue in adult research as movement generated is by study participant only.

Actigraphy has variable correlation with specific sleep parameters. In adults it may have up to 90% agreement with polysomnography for detecting periods of sleep (Paquet *et al*, 2007, Morgenthaler *et al*, 2007). This is achieved in children at around 9 months of age. The use of actigraphy in younger infants have reportedly much

lower rates of agreement with polysomnography, as low as 70% at 3 months and 55 to 80% in newborns (Sadeh & Acebo, 2002, Acebo *et al.*, 1999). For detection of wakefulness the correlation is much weaker (quoted as 50% in adults) (Paquet *et al* 2007).

After considering the several different aspects of physiology measured in this study we will now consider the genetic control mechanisms, which determine the existence of 24 hour rhythms.

Summary

1. Sleep is defined by lack of response to awareness of your environment.

2. Sleep can broadly be divided in 2 cycles NREM (active) and REM (quiet) sleep.

3. The order and proportion of sleep changes with the increase in post natal age with an initial predominance of REM sleep.

4. Sleep function includes having a role in the development of neurological pathways, memory, consolidation and for later preservation of brain plasticity.

1.9 Central clock genes

Several biological rhythms, such as melatonin, cortisol and temperature control exist within a single individual. It is unknown in which manner those rhythms are linked. What ensures their synchronicity? Contemplating this question led to the realisation of the existence of a master clock. This section looks at what we know about the clock, how different aspects of physiology interact with it and how circadian rhythmicity is determined by it.

1.9.1 The Master Clock – The Suprachiasmatic nucleus

The master internal clock in mammals is located in the hypothalamic suprachiasmatic nucleus (SCN). It is made up of two nuclei, which lie adjacent to each other. Each nucleus is made up 10,000 neurons. The suprachiasmatic nucleus lies below the thalamus. It promotes wakefulness and has direct influence on other centres of the brain which induce sleep.

The SCN, located in the anterior hypothalamus, is divided in to a core and periphery in terms of its pacemaker functions (Takahashi *et al.*, 2008). The differentiation into the two zones is based on the difference in function of the two component parts. The neurones in the core of the SCN, which secrete vaso-intestinal peptide/gastrin releasing peptide, accept light messages via neuronal pathways, most notably the retinohypothalamic tract as they contain melanopsin pigment. This is turn relays messages which translate into effects on sleep. The periphery responds to stimuli other than light, such as exercise which, according to the time of day it is undertaken can advance or delay sleep (Shneerson, 2005).

Overall the SCN generates signals (nerve signals and the production of hormones) which allow the integration and synchronisation of daily body rhythms such as those being investigated in this study (melatonin and cortisol levels, development of night time sleep and temperature patterns).

The suprachiasmatic nuclei (SCN) neurons project to different parts of the brain. This includes the paraventricular nucleus and arcuate nucleus, which are responsible for the circadian rhythmicity of endocrine function. Temperature control is mediated via the preoptic nuclei and anterior hypothalamic nuclei. The main heat loss centres are located here. In contrast the posterior hypothalamus is thought to be involved in heat conservation and a lesion here causes hypothermia (especially common in neurodisability paediatric patients) or poikilothermy, which is the inability to adjust body temperature to changes in the external environment. The body's sleep wake functions involve multiple SCN nerve output pathways. Cortical arousal occurs via the posterior hypothalamus. Sleep centres are found in the preoptic nuclei (Shneerson, 2005, Young *et al.*, 2007, Takahashi *et al.*, 2008).

1.9.2 Peripheral Clock

There are peripheral circadian oscillators in tissue spread throughout the body (Reppert & Weaver, 2002). Tissue taken from the liver and from cardiac muscle, are examples of those commonly studied that have been shown to exhibit a robust circadian rhythm in peripheral gene expression. If the tissue is dissected and the cells grown in medium, independent from the central pacemaker, there is still a diurnal pattern of peripheral gene expression despite the communication with the central clock having been transected. Such a preparation can be maintained for several days

(Balsalobre *et al.*, 1998, Zylka *et al.*, 1998, Yamazaki *et al.*, 2000). The central pacemaker cells show their superiority as they can oscillate in medium for several weeks at time, compared with days for peripheral oscillators as demonstrated in Figure 1.2 (Reppert & Weaver, 2002).

In vivo, the peripheral clocks use the central clock as a main reference and time synchroniser. The SCN is therefore sometimes referred to as a 'conductor' to maintain order, rhythm and keep the diurnal patterns 'on cue' and coordinated rather than have a haphazard and chaotic pattern, with a lack of precision (Damiola *et al.*, 2000, Stokkan *et al.*, 2001, Albrecht & Eichele, 2003b).

In vitro, if temperature is adjusted or other small adaptations to the medium are made, there are corresponding changes in peripheral gene expression, showing that the peripheral clocks are dynamic and do not rely fully on the central pacemaker to determine their rhythm (Balsalobre *et al.*, 2000, Brown *et al.*, 2002).

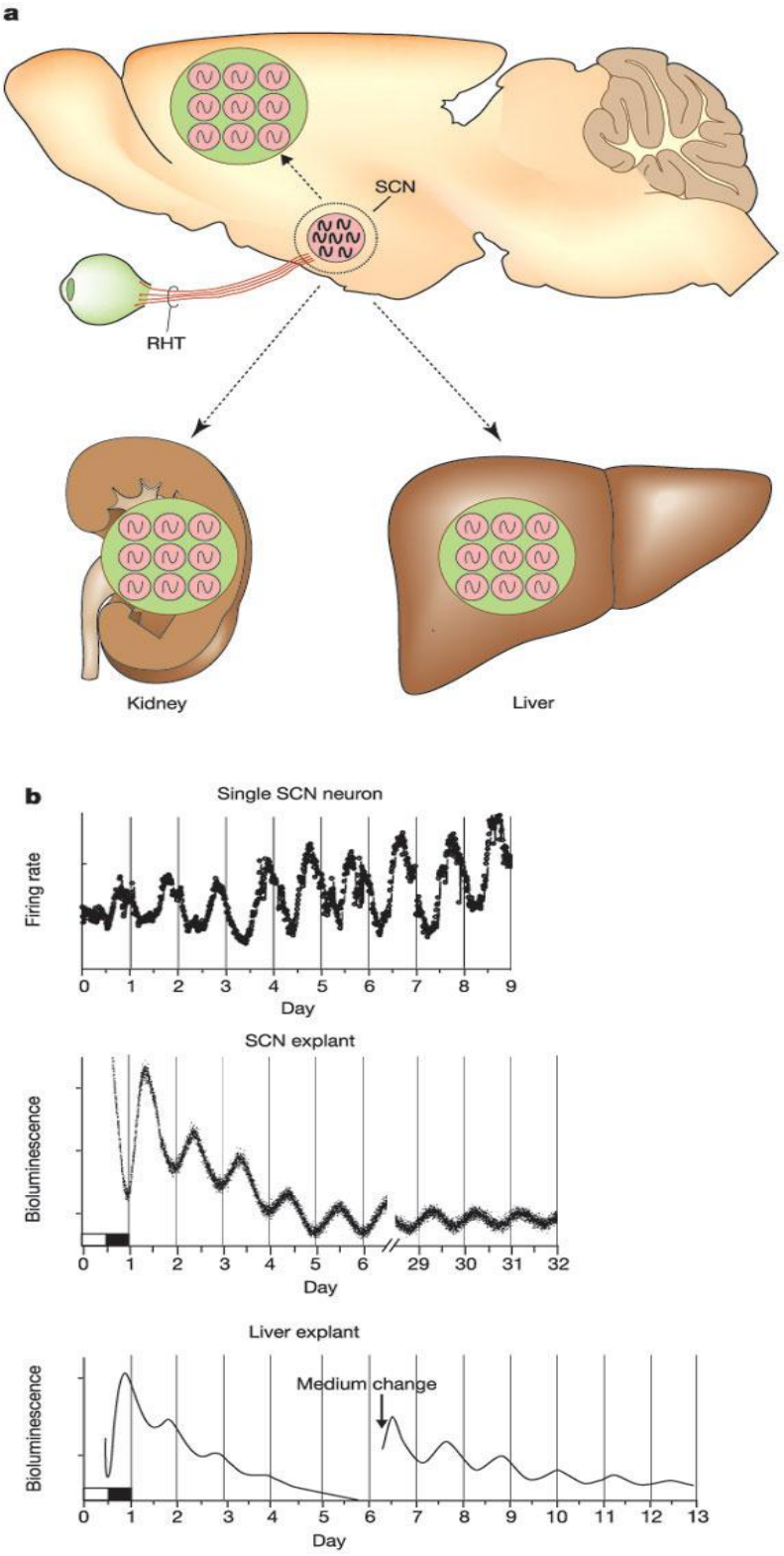

Figure 1.2 - The mammalian circadian timing system is a hierarchy of dispersed oscillators.
Reprinted with permission Nature 2002 from the following article: Coordination of circadian timing in mammals
Steven M. Reppert and David R. Weaver *Nature* **418**, 935-941(29 August 2002) doi:10.1038/nature00965

(a) The master clock SCN is entrained by light via the retinohypothalamic tract, this in turn entrains the peripheral clocks (b) A single SCN neuron can sustain an independent robust circadian rhythm for as long as 9 days in culture, which can continue for weeks in the same medium, although the response is dampened. Tissue from a peripheral oscillator can only self sustain for few days with a rapid dampening of signal, unless medium culture is changed which regenerates the rhythm.

However the overwhelming evidence suggests that the master SCN synchronises the timing of peripheral oscillators. These help determine circadian rhythmicity, the main research focus of this study (Reppert & Weaver, 2002, Pando *et al.*, 2002).

The mechanisms of both levels of the 'clock' are thought to be similar, so findings from peripheral clock work has been extrapolated to attempt to increase the understanding of the central clock (Reppert & Weaver, 2002, Yagita *et al.*, 2001). In this study although the intention was to study central clock genes, peripheral circadian gene expression was investigated as a proxy for central clock gene expression.

1.9.3 Molecular Components of the Clock

(Reviewed in Takahashi Nature 2008 & Hirayama & Sassone-Corsi Encyclopaedia of Neuroscience 2009)

There are several genes which are responsible for the maintenance of the synchrony and precision of the central clock. They are involved in the translation of numerous proteins which interlink to form a complex negative feedback system which constitutes the central clock (Takahashi *et al.*, 2008, Lowrey & Takahashi, 2004, Bell-Pedersen *et al.*, 2005). The genes include *Period Homologue 1* (Per1), *Per2, cryptochrome 1 (Cry1) and Cry2, Clock, Bmal* and casein kinase. The genes encoding the corresponding proteins are found mainly on chromosome 5 (Shneerson, 2005).

The circadian cycle begins in the early hours of the morning; the basic helix loop Clock forms a heterodimer with BMAL1. This complex binds to E-box elements (CACGTG) which are found on *Per* and *Cry* genes. This drives the translation of PER and CRY proteins which go on again to form further heterodimers. This forms the positive limb of the feedback loop. The *Per* and *Cry* genes reach their maximum level at lunchtime and the highest levels of the PER:CRY protein expression occurs approximately at 2 pm (Reppert & Weaver, 2002, Albrecht & Eichele, 2003b, Albrecht, 2004).

The PER:CRY protein pair works within the nucleus to switch off the CLOCK:BMAL1 function by protein to protein interaction. This forms the negative loop of the feedback system (Squire *et al.*, 2009).

The activation of the negative arm of the feedback loop results in the termination of the *Per* and *Cry* gene transcription (Lee *et al.*, 2001). During the night, PER protein becomes broken down in the cytoplasm. When its concentration falls in the nucleus, the negative feedback loop is terminated and the circadian cycle starts again. The molecular components of the clock take 24 hours to run.

The clock is guaranteed further precision by a second back up 'switch' or regulatory cycle (Albrecht & Eichele, 2003b). CLOCK:BMAL1 protein pair allows the expression of a nuclear hormone receptor gene called *Rev-erb-alpha.* The Rev-ERB alpha protein shuts off BMAL1 expression by binding to a promoter (Preitner *et al.*, 2002).

In an alternative part of the cycle CRY:PER heterodimers repress the *Rev-erb-alpha* gene. When the Rev-ERB-alpha protein is low, BMAL1 protein levels are depressed and new CLOCK:BMAL factors are made, starting a new circadian cycle.

The CLOCK:BMAL1 heterodimer has additional functions outside of the process described above. It activates other clock control genes known as CCGs. The production of proteins from these CCGs assist in maintaining 24 hour rhythms apart from the SCN (Albrecht & Eichele, 2003a).

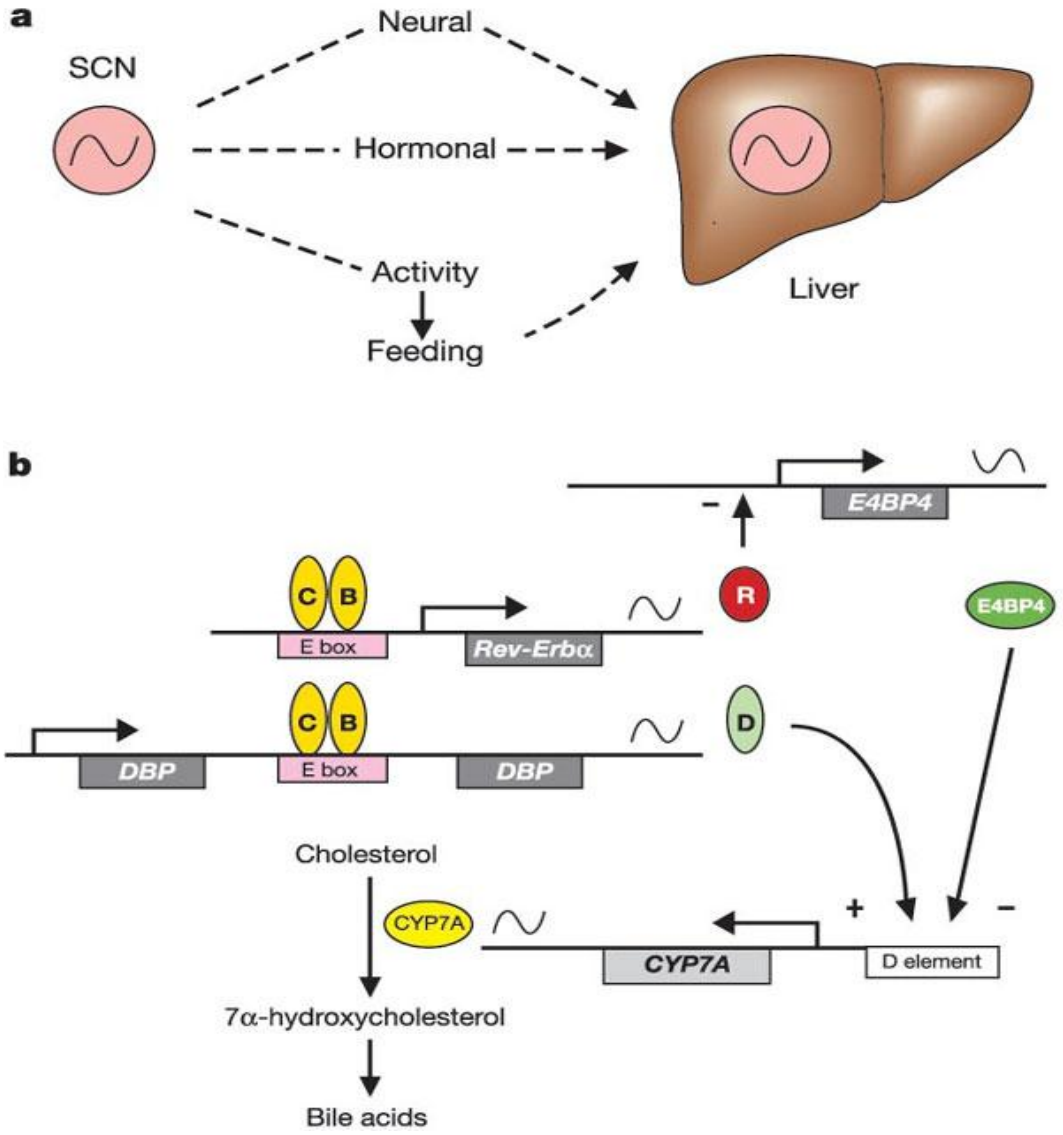

Figure 1.3 - Mechanisms from SCN to clock-controlled gene expression in liver.
Reprinted with permission Nature 2002 from the following article: Coordination of circadian timing in mammals Steven M. Reppert and David R. Weaver Nature *418, 935-941(29 August 2002) doi:10.1038/nature00965*

(a) There are several outputs of the central clock which mediate its effect on peripheral tissue. (b) Example of how peripheral rhythmicity is driven by central clock genes CLOCK –BMAL1 heterodimers through an E Box enhancer. The rhythmicity of the gene of interest E4BP4 is in antiphase and driven by the repressor REVERBα. Further genes DBP and E4BP4 act to determine the rhythmic transcription of an enzyme (in this case cholesterol 7α hydroxylase) whose function is to produce bile.

1.9.4 Clock Input

The SCN's main input is light, which allows entrainment of the central clock and synchronisation of peripheral clocks. Clues as to how this entrainment occurs are derived from animal studies. Melanopsin pigment in found in photoreceptors that are present in addition to rods and cones, and it is via these that light is translated into a signal to the SCN via the retinohypothalamic tract (Freedman *et al.*, 1999).

1.9.5 Clock Output

As the peripheral oscillators in mammals are not light sensitive, it has been postulated by Silver et al that alternative factors must be involved in synchronisation of peripheral clocks to the SCN and in getting messages regarding timing to the peripheral tissue where the diurnal rhythms occur (Silver *et al.*, 1996). Protein factors that are being investigated include TGF-alpha and prokineticin2 (PK2) which are both found in SCN (Kramer *et al.*, 2001, Cheng *et al.*, 2002). More research is required to identify other synchronisers (Albrecht & Eichele, 2003a).

1.9.6 Cortisol and the Clock

The rhythmic secretion of cortisol is indirectly under the control of the SCN via the paraventricular nucleus, the nerve cells containing corticotrophin releasing hormone (CRH). They control the release of Adrenocorticotrophic Hormone (ACTH) from the pituitary (Kalsbeek *et al.*, 1996, Buijs & Kalsbeek, 2001).

Conversely glucocorticoid produced by the adrenal glands can reset the circadian time by changing *Per* gene expression as measured in peripheral tissue (Balsalobre *et al.*, 2000).

1.9.7 Melatonin and Clock

Melatonin, a circadian hormone produced by the pineal gland acts as a messenger of the central clock encoding for darkness (Stehle *et al.*, 2003, Dubocovich, 2007).

Melatonin receptors are mainly found in the SCN, in the hypophyseal pars tuberalis (PT). It is thought that the activation of these receptors (MT_1 MT_2) in cells in the retina allows the message regarding light/dark conditions of the external environment to be relayed to the SCN via specialist nerves known collectively as the retinohypothalamic tract (Dubocovich & Markowska, 2005).

Melatonin is well documented to have a direct effect on sleep in diurnal animals (Cardinali & Pévet, 1998). Melatonin also has a role in the maturation of 24 hour rhythms by regulating phase relationships (Cardinali & Pévet, 1998, Claustrat *et al.*, 2005).

Melatonin phase shift (i.e. melatonin onset and offset occurring later or earlier in the day) can happen at two critical periods of the 24 hour day: dusk and dawn. If melatonin is administered at times other than dusk and dawn there is only minimal affect on the body's ability to secrete its own supply of endogenous melatonin (Dubocovich *et al.*, 1998).

Melatonin may have a role in affecting change in other physiological measures. It may be involved in the mechanism that results in a fall in core body temperature during night time sleep (Strassman *et al.*, 1991, van der Helm-van Mil, A. *et al.*, 2003).

1.9.8 Temperature and the Clock

Temperature control is an important output of the central clock. Both animal and human studies have demonstrated clear changes in body temperature that vary with the time of day of measurement and sleep stage (Heller, 2005).

The links between the central clock, clock inputs, clock outputs, hormones, neuronal activity, external cues, and physiological systems form a complex web of interactions feedback loops, and resultant effects on the body. The manner in which these interplay within the human infant at a time of maximum rate of growth and rapid development is an important area of investigation. Taking temperature maturity as a marker of physiological development into account, it may illuminate the transition from normal intrauterine development and normal foetal programming to healthy adult physiology versus abnormal intrauterine development and foetal programming of adult disease. It may also provide a window into the period when 'things go wrong' in term of physiology culminating in the ultimate failure of physiology – death, such as in cot death.

This study has involved investigating 'normal' physiology in order to enable a better understanding of the 'abnormal.'

The following questions are to be addressed in this thesis.

- What is the pattern of physiological development in human infants in overnight core body temperature during sleep during the first four postnatal months and what is the nature of the change (gradual versus abrupt)?
- Is there a pattern of change of cortisol secretion in human infants in the early postnatal period and how does it relate to maturation of body temperature?
- Is there a pattern in the secretion of melatonin with increase in postnatal age and how does it relate to physiological maturation state in terms of core body temperature?
- What are the characteristics changes in infant night time sleep as babies get older and is there any relationship with physiological development using overnight core body temperature as a marker of maturity?
- What if any are the factors which have an effect on the development of core body temperature maturation, secretion of cortisol and melatonin and the development of infant sleep?
- Is there any suggestion of ordering/timing of physiological events in human infants in the first four postnatal months?
- Can the genetic output of the clock be detected and measured in human infants from buccal swabs and are there any longitudinal changes with age?

Chapter 2

Methods

2 Methods

2.1 Overview of recruitment for study

The aim of the study was to investigate the normal development of the circadian rhythms in human infants. Deep body temperature during night time sleep was used as a measure of physiological development during the first postnatal months of life. Changes in the secretion of melatonin with age were measured using urinary estimation of the metabolite 6-sulphatoxymelatonin (aMT6s). Cortisol secretion was measured in urine. Paired infant maternal activity-rest actigraphy elucidated night-time sleep patterns. Finally the pattern of expression of peripheral genes was examined by extracting RNA from buccal smears. The project was conducted within the geographical location of Leicester, Leicestershire and Rutland.

The recruitment for this study occupied a concentrated period of time due to the delay in Research and Development and Ethics approval. The intended 24 month period of recruitment was limited to 16 months, from March 2007 to July 2008. Physiological monitoring took place in parallel to it. This put a significant time pressure on the completion of the study as a maximum of eight infants at a time could have physiological measurements made. Each recruitment and monitoring period lasted up to 5 months. The delay in the commencement of this project due to the processes associated with ethics (COREC) and Research and Development (R & D) approval was a total of two years.

There were strict inclusion and exclusion criteria used for the selection of participants. Thirty five normal healthy babies took part in the study. Full term healthy infants with no known congenital anomalies were selected at the time of birth from records of the local maternity facility (Leicester Royal Infirmary). Term was taken as a mature gestational age of 36 to 43 weeks at delivery. Extreme preterm and post term babies were not included. A healthy infant was defined as one who made an immediate transition to extra uterine life without significant requirement for active emergency resuscitation secondary to hypoxia. All babies selected were singletons. Method of delivery was not included as a selection criterion – therefore the babies in the study included those born by normal vaginal delivery, elective and emergency caesarean section, as well as instrumental delivery.

If infants were diagnosed with a congenital anomaly on antenatal scan or were subsequently diagnosed in the immediate postnatal period there were not included in the study. No infants less than 36 weeks gestation were recruited. This was in an attempted to reduce confounding factors, such as extreme prematurity which would be expected to have an effect on physiological development. Families who were known to social services and had infants who already had safeguarding child protection plans were not approached. The study aimed to study physiological profiles of Caucasian infants. Ethnicity is known to influence the timing of temperature maturity (the main physiological outcome of interest in this study).

The researcher attended the postnatal ward of the main university hospital teaching hospital. Midwives consulted ward lists (which included maternal basic

demographics; infant birth weight and gestation, medical history and delivery type) and advised as to suitable possible participant families.

Subjects were recruited during four monitoring periods; the first from April 2007 to June 2007; the second August 2007 to October 2007; the third period from January 2008 to March 2008 and the final period from April to June 2008.

The group of subjects were chosen randomly from the ward lists to try to represent the social class distribution of the geographical area where the study took place. The Leicester Royal Infirmary has over 6000 deliveries annually and is the largest maternity unit in Leicester. The spread of social groups among deliveries on the unit is thought to be representative of that in the local population.

2.1.1 Expression of interest contact on postnatal ward

Mothers were approached for an expression of interest to take part in the study from the postnatal wards within the first week after delivery. The ward based delivery record was consulted together with midwifery staff to ensure that the infant fulfilled the inclusion criteria.

Mothers were approached at bedside and a brief overview of the study given. They were given a flier inviting them to take part in the study and a patient information leaflet and a letter with further information about the study (appendix 1). Mothers who were expressed an interest completed a reply slip containing contact details: name, address and telephone number.

This personal contact on the postnatal ward allowed the mother to meet the researcher face to face before the home recruitment visit was made (should the family wish to participate). This was thought to contribute to the high recruitment and retention rate. The methods was chosen in preference to sending 'anonymous' letters in mail shots which may have had a much lower success rate as a recruitment strategy.

A follow up phone call was made to the family between 2 and 4 weeks later to elicit a continued expression of interest. Families were then offered a home recruitment visit to discuss in detail the project, the practicalities of participation and obtain written and verbal consent.

Recordings were expected to take place on the infants between 6 and 16 weeks of age, although in a few instances the monitoring took place just beyond these limits.

2.1.2 Recruitment visits in the home

If a mother consented, a recruitment visit was made following the confirmation of expression of interest. The study protocol was discussed in more detail. The family had the opportunity to see the equipment and kit that would be used for core body temperature monitoring (data logger including the temperature probes), collection of urine (for estimation of cortisol and melatonin), measurement of sleep (actiwatch) and the buccal swabs (for collection and extraction of RNA). Examples of the documentation used are in appendix 1. Participation was voluntary and a family could withdraw at any time. GPs were informed of participation.

Further information regarding maternal health, the pregnancy, the delivery, infant past medical history and demographics were taken. This included basic information regarding the infant such as gender, birth weight, and details related to obstetric history such as known concerns on antenatal scans (as related by mother), mode of delivery, gestation at delivery, Apgar scores, history of risk factors for sepsis, known antepartum complications, initial chosen method of feeding and any relevant past or family history if illness.

Also further information about the parents and family was obtained: maternal/paternal age, employment status and occupation of parents, marital status, whether the household was smoking or non-smoking. The type of housing (type of property as well as whether owner occupied, private rental or council) was recorded as well as household income (before and after delivery of infant).

The first monitoring visit generally took place 2 weeks after the recruitment visit at around or as close to 6 weeks of age as possible.

2.1.3 Home monitoring visits

The first monitoring visit generally occurred 2 weeks after the recruitment visit at around or as close to 6 weeks of age as possible. Appointments were made to fit in with the family schedule and normal infant bedtime which may have been as late as 11pm in some instances. The researcher set up the temperature monitoring equipment and attached the actiwatch to mum and baby. Mothers (and fathers) were trained in urine and buccal swab collection methods. They were given a sample collection schedule for the following fortnight. Questionnaires were completed enquiring about infant health over the preceding days. Details were also taken of the proposed sleep environment for that night (wrapping, place of sleep). Mothers completed a sleep diary to supplement the actigraphy.

The equipment was collected the morning after for downloading and analysis. The samples were transported to the University of Leicester for storage, until processing.

Information was obtained by questionnaire including details of recent illness (using CONI symptom diary) and recent vaccinations. A sleep diary was completed (appendix 2). Other details collected are listed below. Social class used Office Population Census classification (OPCS, 1991).

2.1.4 Further monitoring information required

Infant details

Age of infant
Method of feeding (breast/bottle/solids)
Room location and type of bed baby sleeps on
Heating in room
Night time clothing and wrapping of baby
Sleep position
Date and type of immunisations

Infant wrapping/insulation calculation

The Shirley institute provided scores for the level of insulation around the infant provided by clothing, blankets and wrapping using tog scores. A higher score indicates a greater level of wrapping. Allowances were made for single or double wrappings.

Tog values used included:

nappy	2.0	cotton sheet	0.2
vest	0.2	flannelette sheet	0.5
babygro	1.0	shawl	2.0
velour babygro	1.5	old blanket	1.5
night gown	0.6	new (thermal) blanket	2.0
pyjamas	2.0	thin quilt	2.5
cardigan	2.0	medium quilt	4.0
trousers	2.0	thick (cot) quilt	9.0
jumper	2.0	baby nest	4.0
tights	2.0	sleep suit	4.0
socks	2.0		
mittens	0.2		

2.2 Infant temperature monitoring

Night time core body temperature changes were taken on several occasions for each infant till temperature maturity was achieved. Once this had occurred a further recording was conducted to confirm maturity attainment.

Parents were instructed to continue their routine infant care practices with respect to heating, wrapping and clothing the baby, as well as feeding, in order to study the baby in his/her normal home environment. The length of recordings was dependent on the routine of the household, and was from the last nappy change of the evening until waking in the morning.

The temperature data were recorded via temperature probes (room, infant peripheral temperature and infant core temperature) attached to a small portable Grand Squirrel data logger (model 1201/1202). These were set to record temperature every 60 seconds. The data were 'cleaned' to disregard any readings were the probe had become dislodged and for most infants included a period one hour pre bed time to eight hours after. On direct questioning mothers found the loggers easy to use and non-intrusive. This experience in practice was in direct contradiction to the theoretical concerns voiced during the ethical permission process, that mothers would find participating in the study too difficult, time consuming and impractical while looking after a newborn and that the rectal thermometry in particular would be problematic.

Core body temperature was taken simultaneously with peripheral (shin) temperature and room temperature in degrees Celsius. All probes were made of stainless steel apart from the rectal probe which was made from soft grade medical PVC.

The room temperature probe was 3 mm wide and 50 mm long. It was placed in a non-static bag along with the data logger for portability. The skin probe was attached to the surface of the infant's calf/shin (5.5 mm wide, 14 mm long). The rectal probe had the smallest diameter of 2 mm, with a similar length to that of the room temperature probe. It was inserted 5 cm from the anal margin and the cabling taped with Micropore tape to the infant buttock. No discomfort was caused during insertion or throughout the monitoring. The probes had an accuracy of +/-0.2 ^{o}C at 0 to70 ^{o}C. The data loggers had an accuracy of 0.05^{o}C.

Temperature probes (Viamed YSI 400 compatible)

Alternatives to the Grant temperature probes were also available. The YSI 400 series reusable probes were selected for their highly accurate temperature technology. These were calibrated by the medical physics department to ensure accurate use and compatibility with the Grant data loggers. The probe specifications were as follows:

Range of operation: 0 to 60 ^{o}C

Accuracy: +/-0.2 ^{o}C

Compliant with EEC CE medical device directives and EN1270

Termination: standard .25" phone plug

Skin probe – 4.8mm sensor disk diameter

Rectal probe – 9Fr., 3.0mm diameter

Calibration of the temperature probes

Temperature probes (Grant and YSI) were calibrated for use with the Grant data loggers. Their accuracy was tested against total immersion using Emil Goldline mercury thermometers. The accuracy was found to be correct to 0.15^{o}C for all measurements and to 0.05^{o}C for the range 35.5^{o}C to 40^{o}C.

2.3 Collection of urine samples

Paired urine samples were collected (night and day) on consecutive days for each week of monitoring. In some cases 48 hours of samples were collected for each week. Three methods of collection were available to parents: use of Hollister U bag, clean catch or urine pad.

U bag

The U bag required a clean surface free from creams and ointments to try to encourage close adhesion of the sticky backing to the perineum. This method was a victim of its own success, as some families reported a reluctance to detach the bags once they had collected a sample as they felt removing the adhesive may cause discomfort to the baby.

Clean catch urine

A second option of urine collection from the babies was given to mothers. This involved clean catch urine samples – this is used in clinical practice on children's wards around the country. This does require patience on behalf of the mother as it can be particularly time consuming. This did not prove as popular an option (as was the case for the U bag) with the mothers, so permission was sought from the ethics committee to add an additional method of urine collection to the study protocol.

Alternative method of urine collection - pads

The Newcastle urine collector Uricol pads (Redlands, Newcastle UK) were used as a third alternative method by mothers to collect the night time/day time urine samples. Firstly the infant's pubic and perianal area was cleaned and clear of powder, oils and creams. The Uricol pad was accurately positioned over the expected void area cotton side down. The adhesive tape on the back of the pad was removed and the sticky backing attached to the baby nappy. The nappy was then secured. At 10 minute intervals the pad was checked for wetness. As soon as this was confirmed the pad was removed and laid on a flat surface, wet side up. Using the 5ml syringe provided, the tip was placed on the pad at an angle of 45 degrees and 5 ml of urine extracted. The urine was then decanted into a sterile universal container and stored.

Parents placed collected urine in universal containers which were placed in household refrigeration facilities. These were then transported the following day to university where they were kept at -20 degrees till processing.

2.4 Estimation of creatinine

Creatinine was estimated using the RANDOX Creatinine Colorimetric method, using commercially available reagents (piric acid and sodium hydroxide). The creatinine reacts with the piric acid to form a coloured complex. The amount of complex formed is proportional to the concentration of creatinine (Stockgrand Ltd, Surrey UK). Equal volumes of piric acid and sodium hydroxide are added to make the standard solution. The urine samples are diluted to 1:29 concentration with water. The standard piric acid/sodium hydroxide solution was added to aliquots of the diluted urine samples and quality control high/low creatinine solutions. The samples

were read against air at 429 nm after 10 minutes. Concentrations of creatinine were measured in mg ml^{1}.

2.5 Estimation of urinary melatonin

Values for melatonin estimation were derived by using an assay for a metabolite of melatonin; 6-sulphatoxymelatonin (aMT6s) (Arendt *et al.*, 1985).

There is good correlation between urinary 6-sulphatoxymelatonin and urinary/serum melatonin (Bojkowski *et al.*, 1987). The data were log transformed. All values were corrected for renal metabolism using creatinine.

Urinary aMT6s assay was conducted using a radioimmunoassay using as ^{125}I labelled tracer (Stockgrand Ltd, Surrey UK). The diluted urine sample was incubated with a specific sheep antiserum to aMT6s and trace amounts of ^{125}I-aMT6s was added. The samples are incubated for 15-18 hours at 4 ^{o}C. The free and antibody bound fractions of 6-sulphatoxymelatonin are then separated and centrifuged. The non-antibody bound fraction in the pellet is counted in a gamma radiation counter and the 6-sulphatoxymelatonin concentration in the samples determined from the standard dose-response curve that was simultaneously constructed. All samples are processed in duplicate.

2.6 Estimation of urinary cortisol

Cortisol was measured by radioimmunoassay using Cortisol-3-0-carboxymethyloximino-^{125} iodohistamine as the radiolabel. The assay utilised an antiserum (rabbit anti cortisol bovine serum albumin conjugate) and used a cellulose linked donkey antirabbit IgG (Dassep, Stockgrand Ltd, UK) as the solid phase separation system. The cortisol standard (1 mg/ml) was suitable for use with urinary samples (sigma Ltd). All samples were processed by Stockgrand laboratory (Surrey, UK).

Assays measuring cortisol from urine have been used previously in work involving infants (Jackson *et al.*, 2001, Wailoo *et al.*, 2003, Petersen *et al.*, 1994, Westaway *et al.*, 1995).

2.7 Use of Actiwatch for activity-rest measurement

The serial longitudinal measurements of night time activity -rest in the infant maternal pairs was conducted by using Actiwatch mini (Cambridge Neurotechnology Ltd). It is an ultra light device which detects and logs the duration and intensity of movement. Actiwatches record a digitally integrated measure of gross motor activity that is assessed by an internal accelerometer. The watches are waterproof and shockproof. The use of Actiwatches to monitor rest-activity patterns and sleep efficiency in children and adults has been validated (Finn *et al.*, 2001, Leger *et al.*, 2002) and has been validated against polysomnography, the gold standard of sleep investigations.

Downloading and configuration of data were done via the interface between the Actiwatch and a reader to a personal computer. The Actiwatch software package

allowed the display and comprehensive analysis of Actiwatch-derived data. Using this approach, double-plot actigrams of rest-activity patterns and counts of activity distributed during the period of monitoring, were plotted for infant and mother. The data were analysed to determine the time of sleep onset and waking. Sleep efficiency was also calculated as the ratio of total sleep time to sleep period (Zhdanova *et al.*, 2001) as well as sleep latency, fragmentation index as well as other sleep parameters.

The device specifications are as follows:

- weight 7.5g
- battery life 500 days memory 128K
- diameter 24mm depth 7mm
- Epoch range 2 s -15 min.

Concurrently measurements of ambient light were taken using the Actiwatch mini L. The device which has the same size and weight has the Actiwatch mini. It detects and logs light intensity with a resolution of 0.1 Lux and a range of 1 to 50 000 Lux. There is an inbuilt infra red filter.

All processing and analysis of infant and mother sleep data were performed by the researcher with statistical support and guidance.

2.8 Gene expression measurement

Sample collection was conducted in the home setting by the parents who were trained in Buccal cell collection using a foam tip Catch All sample collection swab (Epicentre, Madison Wisconsin) (see appendix 2). The cells were preserved in RNAlater to prevent RNA degradation. Samples were labelled with date and time of collection. They were then stored in the household freezer at -20 degrees Celsius until transportation in dry ice and long term storage at -80 degrees at University of Leicester Genetics department until analysis.

The samples were processed in a three step process. Firstly the RNA was extracted. Secondly, the mRNA was converted to cDNA by reverse transcriptase. The last step involved qPCR which was used to examine clock gene expression.

A hemocytometer was used to estimate the cell yield from a series of adult samples for comparison with the samples taken from the infant population. An average was taken for adult samples. The sample size from an adult buccal swab was estimated to be 50 000 to 100 000 cells. The yield from an infant swab was 5000 to 15 000 cells.

2.8.1 RNA preparation and cDNA synthesis

RNA was extracted from each sample using the Qiagen RNAeasy mini kit (Qiagen) according to the manufacturer's instructions with the exception being the elution of the RNA into 28µl of elution buffer. This method had optimal extraction from <100 000 cells. It uses a silica based filter.

cDNA synthesis was carried out using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science) as per instructions. The oligo-dT and random primers allowed high yields from whole transcript including 5' ends. The reverse transcription reaction was performed using the anchored oligo-dT primers provided. 1µl of RT-PCR product was amplified for 50 cycles with initial GAP primers. 1ng amplified while 100pg did not amplify (the suggested extraction from buccal samples lay between 500pg and 1ng).

2.8.2 Quantitative PCR (qPCR)

Quantitative PCRs were carried out on a MJR Opticon 1 quantitative PCR machine. qPCR is a modified polymerase chain reaction that can be used to rapidly measure the quantity of DNA, cDNA or ribonucleic acid present in a sample by recording the amount of DNA present after every cycle by the use of fluorescent markers. The qPCR amplifies the DNA exponentially; therefore the number of cycles and the point at which the fluorescence passes a decided point is used as the ct value.

SYBER Green (SG) was selected as the fluorescent marker of choice. It is an asymmetrical Cyanide dye. It binds to double-stranded DNA. The resulting DNA-dye-complex absorbs blue light (λmax = 498 nm) and emits green light (λmax = 522 nm). SYBR green was a less expensive option, but as it is not sequence specific it will detect any double stranded primer-dimers and any non-specific product.

Two primer sets were used. The first pair 5′-GGTATCGTGGAAGGACTCATG-3′ and 5′- GGATGATGTTCTGGAGAGCC-3′ to amplify the constitutively expressed gene *GAPDH*, and the second pair 5′ - TGCAGGAGGCTAGCGAAGC-3′ and 5′- GGCGAGCCAACTGGATGTC-3′ for amplification of the gene of interest, *H3F3B*.

The reactions were performed with final concentrations of 0.3μM for *H3F3B* and 0.1μM for *GAPDH* using 2x SYBER green (Eurogentec Ltd) as per instructions. 1:5 diluted cDNA was added to the reaction mix and amplified using the following PCR conditions: 2 minutes at 50^0C, 10 minutes at 95^0C, and then 95^0C for 15 seconds and 60^0C for 1 minute for 49 cycles. Each sample was amplified in triplicate and the *H3F3B* was then normalised to *GAPDH* expression.

The processing of the genetic samples was conducted in the Department of Genetics at University of Leicester by a post doctorate genetics researcher, under the direction of genetics and molecular biology senior academics. Gene data analysis was a combined effort of the postdoctoral student, researcher and statistics team.

In total three methods were trialled for RNA extraction segment of the experiment. The phenol based method (Trizol) is optimum for low quantities of cells but it proved difficult to recover RNA in a third of the samples therefore it was not used. Two other methods were tried. Qiagen RNA Easy was selected rather than the RNAqeous micro extraction method. The qPCR had specific problems too. A large number of cycles had to be run due to difficult amplification of DNA. This contributed to the need to rigorously reduce the levels of contamination.

2.9 Supplementary questionnaires

There were several supplementary questionnaires that were employed for the purposes of this study to gain further background information about the families and carers (mainly mothers) of the infants enrolled in the project. The social circumstances of the family and the wellbeing and health of the mother may have direct impact on the care of the infant and indeed their physiological maturation. Questions about the impact of having a newborn baby were included in the lifestyle questionnaire. Mothers also listed adverse life events which may have coincided with the birth of their infants (Corney, 1988). It was felt important to have a screening questionnaire to assess maternal mental state. The Edinburgh Postnatal Depression Score (EPDS) was selected as a well known and validated tool (Cox *et al.*, 1987). If a mother scored between 9 and 11 she was deemed to be at risk. The family health visitor and GP were informed, with permission. A score of above 12 was of more concern as it pointed to a possible diagnosis of postnatal depression. Thirdly a questionnaire with a scoring scheme for the amount of support (physical/emotional/professional) each mother had available was included (Murray: personal communication). Each questionnaire is included in appendix 2.

2.10 Analysis

The software packages used for the analysis were Microsoft Excel, SPSS versions 14 & 16, STATA and R version 2.8 statistical packages.

2.11 Statistical methods

For comparison between normally distributed sets of data Student's t tests have been used, paired where appropriate. Student's t test was used, for example, in the comparison of the mean age of mothers of participant infants with that of mothers who declined, in this case unpaired (see results section 3.2.1). This method determines whether two normally distributed sets of numbers could reasonably come from the same population.

Data that were found to be positively skewed (such as reported melatonin and cortisol lab values), were logged for analysis where required (see results chapters 5 & 6).
The non parametric tests Mann-Whitney U (for unpaired data) and the Wilcoxon signed rank statistic were also used (for paired data). Mann Whitney U test shows differences between overall distributions rather than between distribution means.
The Pearson's product moment r statistic was used to test for correlation between variables, such as investigating the relationship between night and day melatonin.

Linear regression was used to investigate whether the change in one variable (x) was associated with a change in a second variable (y) in order to predict y for a given value of x. The equation $y = \alpha + \beta x$ was defined as the linear regression equation, where α is the intercept and β is the regression coefficient. α is the value of the equation when $x = 0$ and is the slope of the line. When x increases by one unit, y will change by β units. This modelling was applied to temperature, sleep, melatonin, and cortisol data in order to investigate the factors influencing these within the infants group (see results chapter 9).

A random effect modelling is an extension of linear regression, which has the advantage that within the model itself, it is able to distinguish between babies. It takes into account the structure of the data, i.e. for the group of infants who had data, each infant was observed on a number of occasions on different weeks. It was used for the more complex aspects of the analysis and was done by a specialist statistician, following expert advice which was sought as to a suitable method to analyse the data set. It is a hierarchical, multilevel, repeated measures modelling (Field, 2009). It was used to investigate the change with age for several measurements for all infants. The measurements included melatonin, cortisol, infant sleep and circadian gene expression data (see sections 5.1.1, 6.1.1, 7.3.2 & 8.3). The data were fitted using STATA, a comprehensive statistical package.

In the first part of the random effects modelling an average of each individual baby slope was taken and it was assumed each infant had the same slope ANCOVA (analysis of covariance). The height of the line (intercept) was calculated separately for each infant's data; and an estimate of the slope given. In the next step of the modelling, the slope of each infant remained the same for each infant, however the estimates of the height of the line or the (height) of the y-intercept were adjusted to give an inherent estimate of the amount of gene expression per baby at a given age and a presumed normal distribution was drawn.

The final step was an extended random effects model which allowed the slope to change for each infant. The height given (y intercept) was normally distributed and the slope given was also normally distributed for each infant. It was then possible to

test that each infant's height and slope differed from each other; more than what would be expected at random.

The height and slope were correlated and this was allowed for in the model. The random effects modelling were therefore used to examine the relationship between which measure was being analysed (e.g. night time melatonin) and postnatal age (in the given example looking for an overall increase or decrease in night melatonin with increasing age).

Further detail on the use of linear regression and random effects modelling are given in the relevant results chapters.

The first stage of the gene expression data analysis was to plot the sinusoidal curve that best fitted the observed data for each baby week. The goodness of fit for each graph for each baby week was ascertained in two ways. Firstly the amplitude was calculated; this was a separate value for each baby week. We chose amplitude as the best indicator of goodness of fit as it showed the strength of the relationship and is read in the same units as gene expression. It gave a value for how well the H3f3b/GAP ratio fitted a diurnal pattern. Secondly as a secondary measure and as a robust check for goodness of fit, a commonly cited statistical measure R-squared was also used. This was also calculated for each baby week, so any given baby has several values for each week of measurement. R-squared showed the proportion of the total variation (variance) in the observed values that is explained by the fitted curve.

In addition we wanted to ascertain, how well the sinusoidal curves fitted all the combined data to give an overall indication of how well the model of diurnal variation fitted the data.

There may have been different rhythms which fit the gene data better and a diurnal (24 hour) rhythm may not have been the optimum or best. Alternatives may have been ultradian rhythms which cycle over shorter periods: six hours, eight hours, twelve hours rather than rhythms based on full 24 hours. These shorter time periods were not included in the modelling used in this thesis.

Error bars are omitted from figures, where appropriate, for ease of illustration.

Power calculation

Sample size:

Fifty babies will constitute a study of age related physiological development and clock gene expression. The distribution of clock gene expression is unknown.

In order to detect a minimum clinically significant difference between the average time to maturity in core body temperature circadian pattern and the timing of maturity of melatonin secretion, of two weeks or more, given that the standard deviation of the difference is 2.8 weeks[*], a sample size of 40 would enable this to be detected at the 1% level of statistical significance with over 90% power. To allow for greater variability in clock gene expression we will aim to recruit a sample size of 50 babies.

[*] Temperature
8 – 16 = 95% CI
4SD = 8
SD = 2

SD (temp) = SD (melatonin)
SD (diff) = $\sqrt{2^2 + 2^2} = \sqrt{8} = 2.8$

Chapter 3

Results

3 Results

3.1 Recruited subjects and participant families

In total thirty five infants and their families were recruited into the study. There was 100% retention with no participating families withdrawing from the project. In most cases the monitoring visits commenced at 6 weeks of age.

3.1.1 Infant Recruitment

Sixty three mothers were approached for an expression of interest to take part in the study. All approaches were made on the postnatal ward of the tertiary university hospital where the research team were based, at the Leicester Royal Infirmary. The recruitment period spanned 16 months from February 2007 to June 2008.

Mothers were selected at random from a ward based list, in consultation with midwifery staff. For each recruitment period (of which there were four), on average, 15 mothers at a time were approached during a five day period – from which a maximum of eight could be enrolled into the study at any one time due to equipment availability and time. The four separate recruitment periods ran in series with the four periods within which monitoring took place.

An introduction was made to all mothers on the postnatal ward in the first few days after the delivery of their baby. This was for an expression of interest only. An attempt at phone contact was made 2-4 weeks later with mum's permission. Recruitment visits were then arranged for mothers wanting to participate.

From the sixty three mothers approached for an expression of interest on the ward:

3 (5%) mothers declined the information pack about the study and did not want any further contact.

11 (17%) mothers stated they would make contact with the university if they wished any further information. They were given information packs but no telephone contact was made with them. No mothers made further contact with the researcher.

49 (78%) mothers agreed for further telephone contact to be made.

Telephone contact was successfully made in all cases except two.

37 mothers agreed for recruitment visits of which

35 (95%) mothers agreed to the study.

From the original number of mothers approached for expression of interest 55% (35/63) agreed to participate. The high recruitment rate may be due to the personal introduction on the ward rather than a random mail shot. Of those who had recruitment visits 95% (35/37) agreed to the study.

All 35 (100%) families completed the study. Parents cited 'wanting to help other babies' as a common reason for taking part in the study. An added bonus was a fortnightly home visit from a paediatrician, fortnightly baby weights and a ready source of medical advice. 34 out of 35 mothers would volunteer for more research.

Similar studies involving physiological measurements in infants have had recruitment rates of 25% to 30% (Murray 1996; Jackson 2003; Atkinson 1995), carried out in a

predominantly affluent areas of the city and county. One study in a deprived area had 71% of mothers agree to participate from an inner city area. It should be noted that in these earlier studies a different method of recruitment using a negative response reply. In these studies mothers were selected from a child health records list and sent letters of invitation to participate with a fixed visit time already prearranged. Mothers were then expected to call the university to cancel the appointment if they did not want to participate.

The retention rate in the Murray study was 23%. In this study it was 100%. This is despite initial anxiety by members of various panels and committees that mothers would not want to or be able to participate in a study of this type. Families were very motivated and very keen to help.

The mothers who took part in the study were significantly older (maternal age SD) than those who declined (mothers of participant infants 31.23 (1.114) years c.f. mothers who declined study 26.84 (1.239) years) p=0.005. There is missing data from the mothers who declined (n=3). The mothers cited lack of time, the equipment, or partner objection as the most common reasons for declining to take part.

3.1.2 Summary of demographic data

One in three infants in the study (11/35) was resident in Leicester City centre. The rest were recruited from the surrounding area (Leicestershire, Rutland, Northamptonshire). The demographic data for the infants who participated in this study are given below with special reference to infant characteristics, social class of the family and key aspects of infant care such as method of feeding (Table 3.1).

Table 3.1 - Description of demographic data for infants and families

	Count (%)	95 %CI[1]
Gender		
Male	15 (43%)	(26 to 60)
Female	20 (57%)	
Birth weight g, mean (SD)	3515 (644)	
Gestation weeks, mean (SD)	39.6 (1.35)	
Feeding		
Breast fed	29 (83%)	(66 to 93)
Bottle fed	6 (17%)	
Social class[2]		
I or II	16 (46%)	(29 to 63)
III or IV	5 (14%)	(5 to 30)
V or VI	13 (37%)	(21to 55)
VII or VIII	1 (3%)	(0.07 to 14)
Smoking status		
Non smoking	19 (54%)	
Smoking	16 (46%)	(29 to 63)
Birth Order, mean (SD)	1.7 (0.94)	
Age weeks, mean (SD)[3]	10.2 (2.86)	
Weight g, mean (SD)[4]	5577 (973)	

Smoking status refers to that of the household (either parent or both parents smoked)

1 95% CI for the proportion (for continuous variables) % (categorical variables).
2 Social class was based the reported occupation of the highest earner in the household.
3 Measurement taken at time of monitoring visit.
4 Measurement taken at time of monitoring visit.

Birth history and past medical history:

29 pregnancies were uneventful (82%). Six mothers had obstetric problems (one large for dates foetus on antenatal scans, one case of maternal hypertension, one known maternal carrier group B streptococcus, one maternal confirmed diagnosis of urinary tract infection, one case of antenatal polyhydramnios and one maternal report of reduced foetal movements).

Four (11%) mothers had a history of previous miscarriage/termination of pregnancy.

Just over half of the mothers (51%) of participant infants had difficulties during labour: (4 foetal distress, 1 placental abruption, 3 breech presentations which resulted in two emergency caesarean sections and one ventouse delivery, 2 face to pubes presentation, 5 cases of failure to progress which resulted in 2 instrumental deliveries and 3 emergency caesarean sections, 1 prolonged second stage labour, and 1 case of maternal eclampsia). One infant was delivered with the cord was around the neck (this mother had forceps delivery). There was one failed induction, and one meconium aspiration. Nine (26%) deliveries had associated foetal distress.

The method of delivery was roughly split into thirds. Thirteen mothers had normal vaginal deliveries (37%). Thirteen had emergency caesarean sections (37%). The remaining nine mothers had instrumental (ventouse/forceps) which made up 26% of the total.

Apgar scores at 5 minutes ranged from 7 to 10 (mean 9).

No infants were admitted to the special care baby unit.

Twenty nine (83%) mothers chose breast feeding at delivery while six (17%) mothers chose to bottle feed.

During the first 6 weeks of life, six (17%) infants were taken to their general practice or health visitor clinic with medical concerns: 2 with thrush, 2 viral infection, and 2 with snuffles. One infant had conjunctivitis. Two were investigated for prolonged jaundice. One infant was hospitalized with bronchiolitis.

Three families (9%) had a history of Sudden Infant Death Syndrome/ Apparent Life Threatening Event (SIDS/ALTE).

All infants had Caucasian parents, except one participant who may be described as dual heritage or Caucasian (maternal ethnicity Caucasian/paternal Caucasian-Afro Caribbean).

3.1.3 Socioeconomic demographics of participant families

Lifestyles, housing conditions and socioeconomic background of the participant families varied within the group. There was a predominance of higher social groups (57% - 20 families class I-III). 42% (15 families) were in the lower social classes (IV-IX). Only 2% were from a household were both partners where unemployed. This is not fully reflective of the unemployment rates in this region. The unemployment rate in Leicester city is 11.5% and the county rate is 5.2% compared with the national average of 6.9% (ONS 2009).

The social class of each family was determined by using the classification as assigned to the highest wage earner in the family (irrespective of whether this was mother or father) using government classifications from the Office for National Statistics (ONS 2009).

Social class 1 - six (17%) families - higher manager/professional

Social class 2 - ten (29%) families - lower manger/professional

Social class 3 - four (11%) families- intermediate

Social class 4 – one (2%) family – small employer / account worker

Social class 5 - no families - lower craft/supervisory

Social class 6 - eight (22%) - semi routine workers

Social class 7 -five (14%) families - routine workers

Social class 8 - one (2%) family was unemployed.

Only 2 families (6%) were resident in a flat, the rest lived in houses of various types. 6 (17%) of houses were detached properties, 17 (49%) semi detached, 10 (29%) were terraced houses. 69% of all housing (24/35) was owner occupied, leaving 11 (31%) rental accommodation. 4 (11% of total) of these houses/flats were rented from the council. 94% (33 properties) had full gas central heating.

The average pre tax total household income (SD) was £39 532 (£18K) with a range of £10 000 to £80 000. Maternal income pre birth of baby was £17 772 (range £0 to £40 000) whereas paternal income averaged at £22 795 (range 0 to £60 000). All participant mothers completed the questions related to finance.

The Corney score (SD) was 1.88 (1.67). It is a measure of the number of adverse life events experienced by the families. The minimum score was 0/10 and the maximum score was 6/10.

The average Edinburgh Postnatal Depression Score (SD) for the mothers was 5.32 (6.202); range 0 to 25. A score of 13/30 or greater indicates possible depression. Three (8%) mothers required intervention or were clinically depressed. One mother of three children, at the same time as coping with the birth of her newborn, had to cope with the death of her older child during the family's participation in the study. He had been ill with chronic illness and severe disability from birth. She elected to continue in the study despite her bereavement. One mother had a history of depression and was going through a possible separation with her husband. The third mother had a significant number of adverse life events including unemployment, bereavement of

her best friend losing her battle with cancer, relationship problems and poor housing. Referrals were made to the mothers' GP/health visitor with maternal consent.

The average social support score was 24.47 (6.66); range 7 to 36, indicating high levels of support within the families enrolled in the study. The maximum achievable score is 36.

Parental details

The average (SD) maternal age was 31.2 (6.5). The youngest mum was 18 years old. The oldest was 43 years old at the time of enrolment into the study. Paternal ages ranged from 21 to 53 years old (mean (SD) 33. 9 (8.3)).

46% of infants lived in smoking households. In these homes mothers smoked on average 3.3 (4.3) cigarettes while fathers smoked 9.5 (8.0); ranges 1 to 10 no. of cigarettes smoked maternal, 1 to 20 cigarettes smoked paternal.

All mothers lived with their current partner. There were no single mothers in the study.

The Automated Morningness-Eveningness Questionnaire (A-MEQ) was conducted on mothers of participant infants. The average score (SD) was 54.1 (10.88); range 27 to 75. A person scoring between 59 and 86, is classified as a type 1 individual morning type; scores 16 to 41 is type 2 evening type; scores from 42 to 58 is intermediate type 3. This is available on line from the Centre of Environmental Therapeutics[5].

[5] http://www.cet.org/en/index.html?/en/Questionnaire.html

Sleep environment of infants

All mothers reported placing their infant supine routinely during night time sleep. The most common sleep surface was a Moses basket (19 infants which equals 54%). Just under half of the group were placed in cots (42.9%). Most commonly infants (80%) infants were in the same room as their parents. One infant co slept (slept in parental bed). Seven (20%) slept in their own room. Wrappings were light to moderate with an average total tog (SD) value (clothing and bedding combined) of 7.19. (2.454). The lowest night time temperature in the rooms were the infants slept (SD) was 19.36 (1.95) ^{o}C. The highest average temperature (SD) was 22.24 (1.99) ^{o}C.

Sleeping bag use changed as babies got older with infant sleeping bags becoming more common. Thirteen infants (37%) used a sleeping bag whilst twenty two infants had no routine use.

3.2 Summary of Data Collected

147 temperature reading were made. 136 were included in the analysis, which made an average of 4 per infant. The data logger failure resulted in six temperature readings being discarded. A further 3 sets were not used due to probe dislodgement. A total of 466 urine samples were collected. 20 were insufficient for cortisol analysis. 4 were not suitable for melatonin estimation. 138 actigraphy recordings were taken. Of these 135 were analysed. In total 599 samples were processed and analysed for determination of peripheral gene expression.

There were 19 visits rescheduled, mainly by request of the family. One mother remained in the study despite her older son dying of chronic illness during the study.

Summary

1. This study had higher rates of recruitment and retention when compared with similar studies.

2. 55% of mother approached for an expression of interest in the study consented to their infants taking part in the study. 95% of families who had recruitment visits agreed to take part. There was 100% retention.

3. There were high rates of breast feeding (73%), over half of the families were non smokers (54%) and there was a majority in high income bracket (Social class I to III) at 57% recruited into this study. .

4. There are low rates of postnatal depression and a low prevalence of indicators of social deprivation in the participant families, when compared to the general population.

5. There were high levels of social support and low numbers of adverse life events at the time of enrolment to the study.

Chapter 4

Results

4 Results

4.1 Core body temperature monitoring

This chapter examines the development of the overnight rectal temperature pattern in the participants enrolled in the investigation of circadian rhythms in human infants. The infants were monitored once a fortnight in order to describe the timing of maturation of a temperature rhythm. In this study weekly recordings were not undertaken in an attempt to simplify the protocol and maximise recruitment and retention of participant families. Weekly readings would however have allowed a more accurate estimation of age of temperature maturity.

One hundred and forty seven temperature readings were taken. One hundred and thirty six temperature recordings were included for analysis in this study. Three recordings were discarded due to the probe becoming dislodged. The Grant squirrel data logger locked out due to battery failure on seven occasions. One recording was discarded as it was less than four hours in length.

On average each infant was monitored on 4.2 occasions between the ages of 5 and 18 weeks.

The Grant squirrel data logger sampled core rectal temperature, skin temperature (probe attached to infant shin) and room temperature at one minute intervals during the overnight recording from 2 hours prior to sleep onset up to a maximum of 8 hours after sleep onset. Single temperature readings at ½ hourly intervals were extracted

from the continuous minute data between bedtime and four hours after bedtime. For each given night of monitoring, nine temperature readings were used to calculate the maximum and minimum temperature achieved as previously conducted in similar studies of infant physiology (Jackson *et al.*, 2004).

4.2 Summary of development of core body temperature during night time sleep

Figure 4.1 shows the change in minimum overnight core temperature for the participant infants from 5 to 16 weeks of age. There is a gradual decline. This decline is characterised as being more abrupt and occurring in two stages with a period of transition when the change in minimum core body temperature achieved during night time sleep is related to the infants' developmental age rather than postnatal age (Figure 4.2).

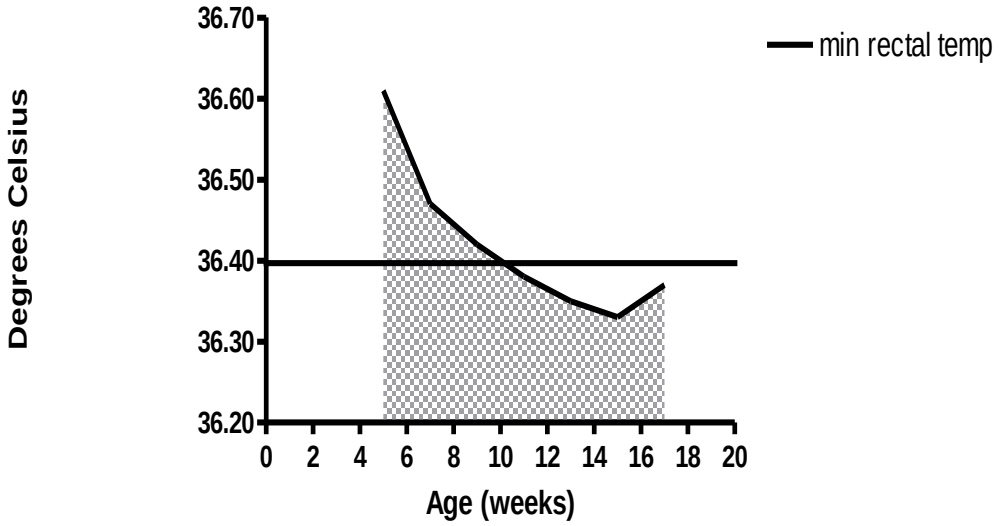

Figure 4.1 - Gradual decline in minimum rectal temperature plotted against postnatal age in 35 infants over 12 weeks period.

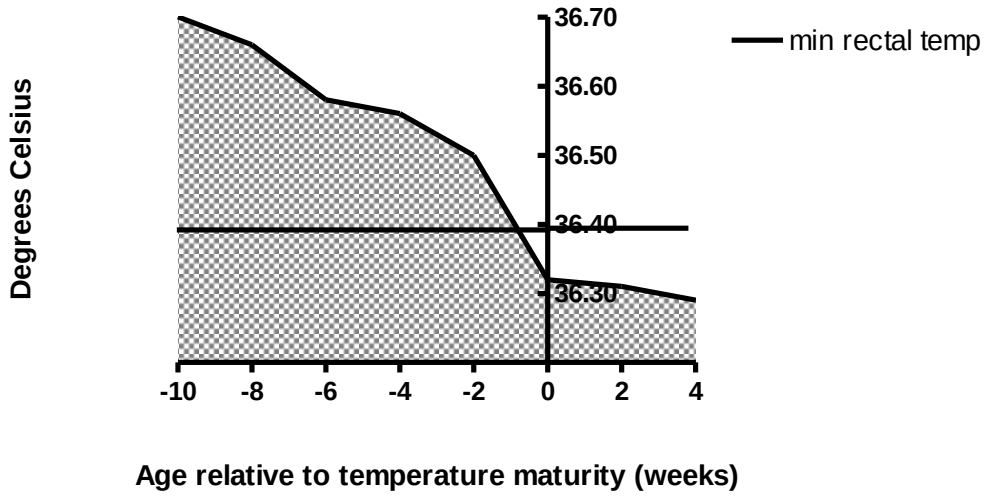

Figure 4.2 - Decline in minimum rectal temperature in 35 infants over 12 weeks period from 10 weeks prior to development of achievement of mature physiological pattern in deep core temperature during night time sleep.

4.3 Changes in core body temperature with sleep relative to postnatal age

The age related change in longitudinal core body temperature was clearly demonstrated in this cohort of infants. Figure 4.3 demonstrates the pattern of the development of overnight rectal temperature from 6 weeks to 18 weeks of age. The graph shows a significant change with age for all babies. The plot shows infant data that was available for that week of measurement.

5-7 weeks

Initially the core body temperature (SD) of all 35 infants averages at around 37.21 (0.49) ^{o}C at one hour prior to bedtime for the time period 5-7 weeks. Prior to time of confirmed onset of sleep, the deep body temperature already demonstrates a rapid fall. The fall in night time temperature continues with sleep onset and it reaches its maximum within one and a half hours to a value of 36.74 (0.46) ^{o}C. Over the next hours of night time sleep, there is a general trend towards a gradual rise in the measured deep body temperature, with a final temperature of 36.85 (0.44) ^{o}C at the time recording was stopped.

8-10 weeks

The mean waking temperature at 8-10 weeks is 37.12 (0.53) ^{o}C. This drops to a minimum of 36.57 (0.40) ^{o}C. The maximum drop occurs again at 1.5 hours. The gradient of the fall is deeper and reaches it maximum velocity after the onset of sleep. There is a gradual increase towards time of waking to 36.82 (0.50) ^{o}C.

11-13 weeks

There is no significant change in the mean temperature one hour prior to sleep onset which remains unaltered from previous week, at 37.17 (0.54) ^{o}C. The depth of the fall after onset of sleep reaches the level at which the temperature can be described as having reached that of developing maturation with minimum core night time temperature of 36.50 (0.46) ^{o}C. This occurred within the first four hours of sleep at 2.5 hours. The final recorded temperature at 8 hours post sleep onset was 36.77 (0.45) ^{o}C.

14-16 weeks

Temperature while awake measured one hour prior to sleep averages at 37.24 (0.46) ^{o}C. The temperature at 14-16 weeks drops to 36.41 (0.42) ^{o}C with an increase to a lower level of 36.70 (0.43) ^{o}C. The mean recordings over the night period remain at a lower level below 36.5 ^{o}C for a longer period which may be reflective of deeper sleep states being associated with a lower core night time core body temperature. The average readings at 14-16 weeks of age do not differ greatly from those at 11-13 weeks of age, the age range when temperature maturation occurs.

17-18 weeks

Fewer readings were taken at the later age range of 17-18 weeks as very few infants had not fully demonstrated temperature rhythm maturity at this stage. The temperature fell from 37.12 (0.72) ^{o}C to 36.53 (0.24) ^{o}C within 1.5 hours. The slightly higher nadir may be accounted for by a very small sample size and infant

episode of illness which causes a temporary rise in core temperature even whilst in the prodromal phase (Jackson *et al.*, 1994).

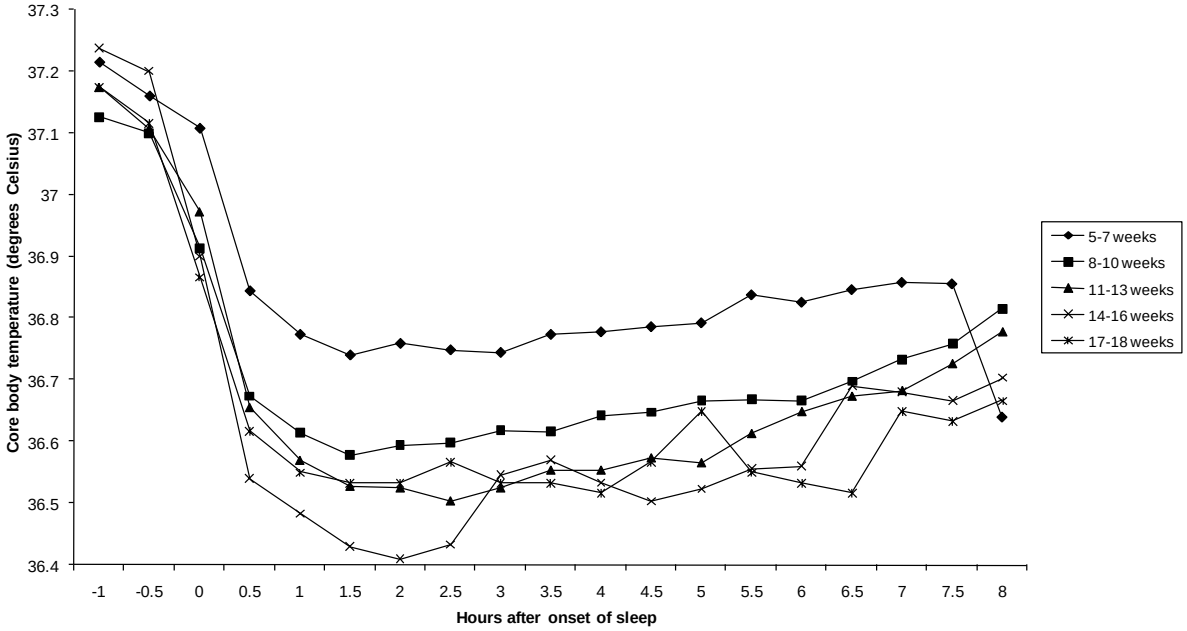

Figure 4.3 - Overnight core body temperature of 35 infants, mean for 5 to 18 weeks of age

Timing of maturation of temperature pattern

Figure 4.4 illustrates the timing if the emergence of a mature "adult like" temperature pattern as defined by the demonstration of fall in core body temperature during night time sleep to a minimum value of 36.5^{o}C within the first 4 hours of sleep. Maturation occurred during a range of early developers from as early as 7 weeks to developers as late as 18 weeks of age. A quarter of all cases (10 infants) achieved temperature maturity by 10 weeks of age (see introduction section 1.5.3). However, the mean age (SD) for the group was 11.06 (0.46) weeks.

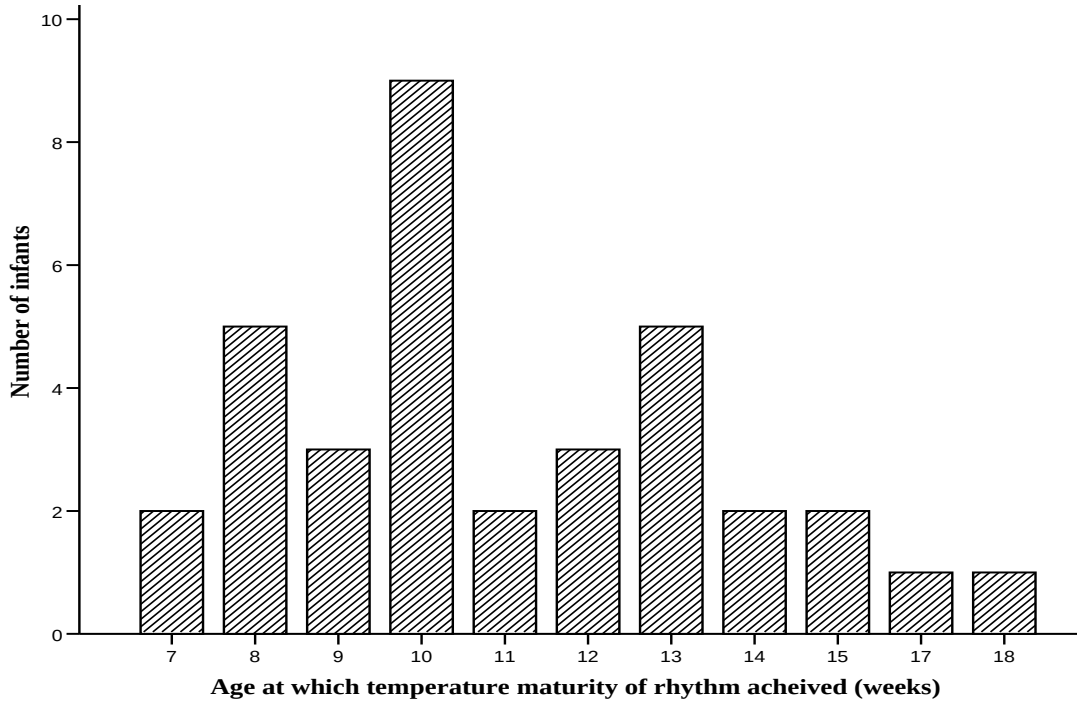

Figure 4.4 - Distribution of age of attainment of an adult mature temperature pattern in 35 infants.

Minimum rectal temperature

The minimum rectal temperature during the first four hours of night time sleep gradual falls in the infant group. Figure 4.5 shows a minimum temperature of 36.65^{o}C at 5 weeks of age on average for the group. During weeks 10 to 12 the minimum core body temperature is able to drop consistently below the critical level of 36.5^{o}C to 36.37^{o}C, which shows infant physiological maturity in terms of temperature. This minimum level once achieved remains for subsequent weeks 13 to 18, in the infant group.

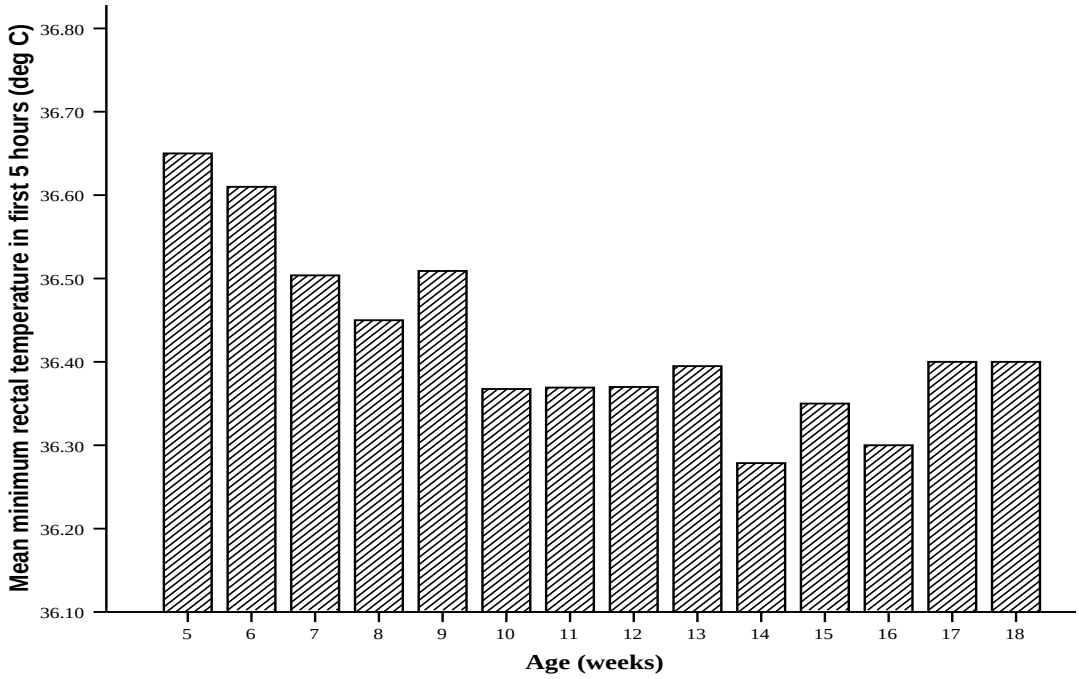

Figure 4.5 - Average minimum rectal temperature during overnight sleep in the study group of infants displaying the gradual change with postnatal age.

Data for minimum infant rectal temperature were normalised in order to take in account the week maturation of temperature patterns occur, rather than plotted against postnatal age of the infant. In this study, stage -10 represents the average minimum rectal temperature data for infants measured 10 weeks prior to the development of their temperature rhythm. Stage 0 represents data measured at the week of maturation. Stage +4 represents data measured 4 weeks after maturation. When the data are presented in this way the difference in pre and post maturation is more clearly seen (Figure 4.6). In the 10 weeks prior to maturation the minimum temperature does not fall below 36.5^{o}C. At maturation the mean minimum rectal temperature falls to 36.3 ^{o}C and remains well below 36.5 ^{o}C in subsequent weeks. Overall there is a significant drop in minimal rectal temperature with each week the infant moves towards maturity and there is no significant change once maturity has occurred. There is a significant difference in the value of minimum rectal temperature pre and post maturity states ($p<0.001$).

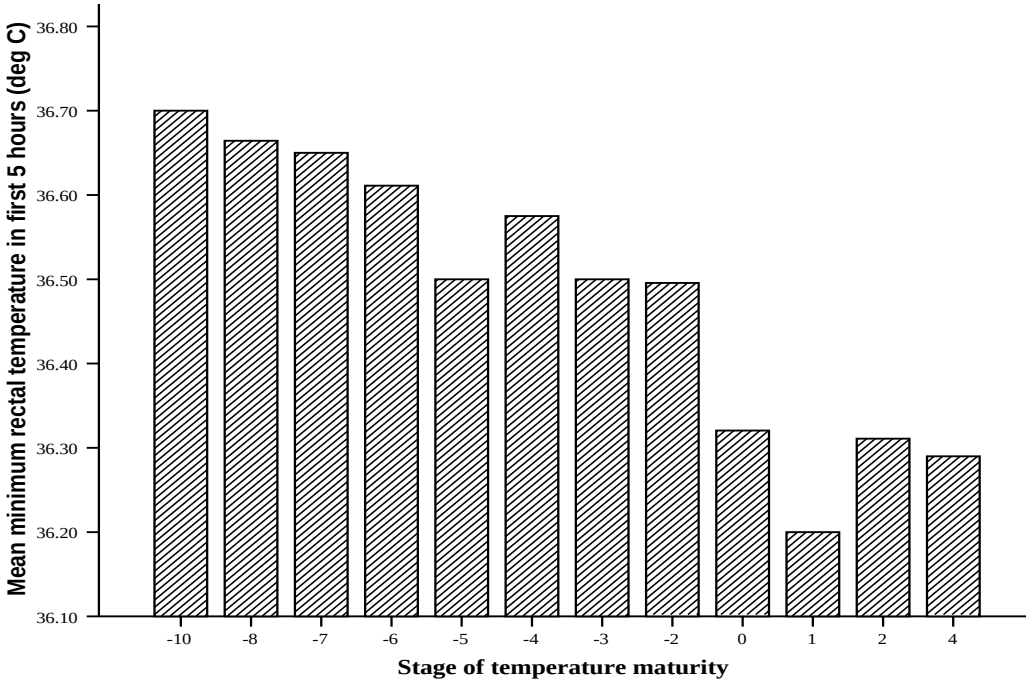

Figure 4.6 - Average minimum rectal temperature during overnight sleep in the study group of infants displaying the definite marked change from immature temperature state to mature temperature state.

4.3.1 Thermal Environment during night time sleep for infants

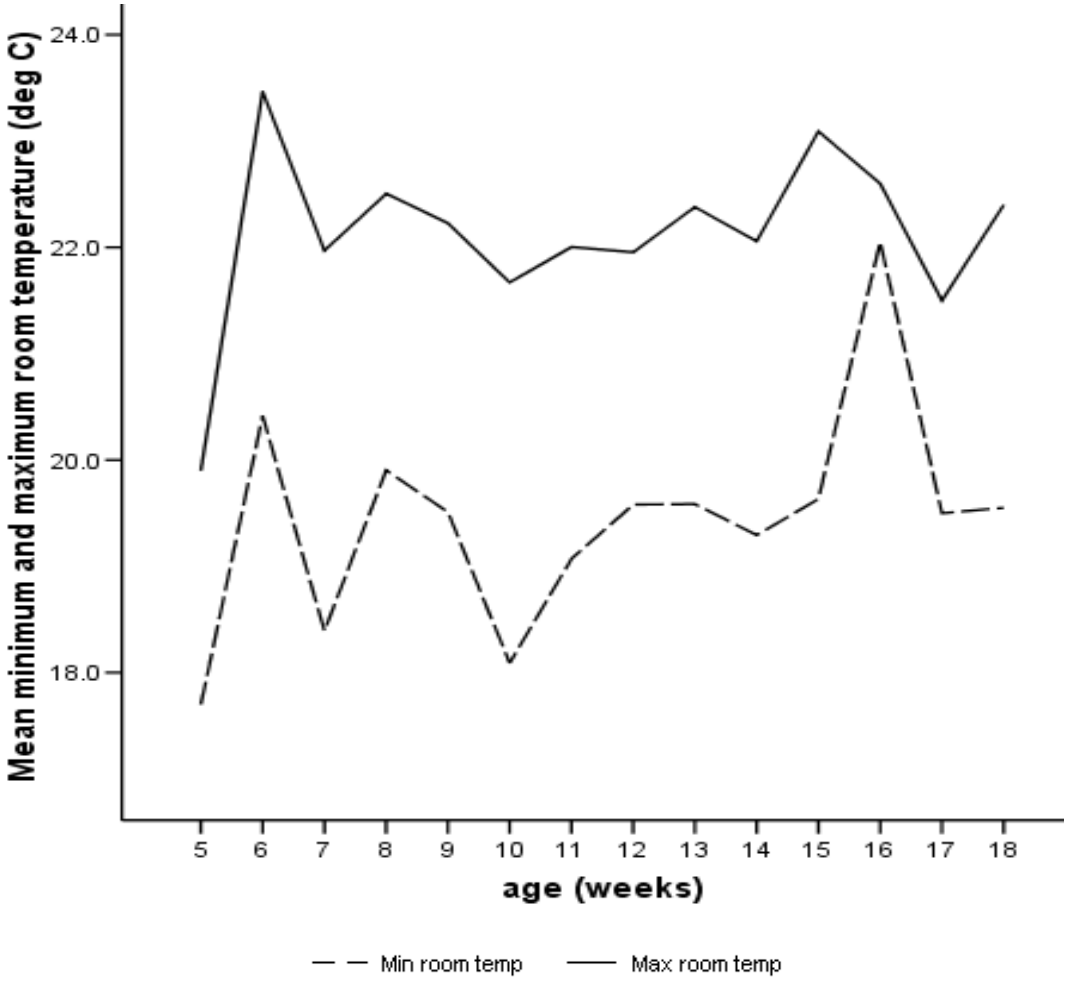

Figure 4.7 - Changes in mean minimum and maximum room temperature with postnatal age in the rooms where the infants were resident during night time sleep.

Figure 4.7 shows the changes in the thermal environment in which the infants were cared for and placed for their night time sleep during the period of the study from 5 weeks to 18 weeks of age, for the group on average. There was no significant difference in minimum or maximum rectal temperature (SD) during the night period before and after the achievement of temperature maturity (minimum room temperature: pre maturity 19.12 (0.272) ^{o}C post maturity 19.56 (0.202) ^{o}C p=0.264; maximum room temperature: pre maturity 22.03 (0.258) ^{o}C post maturity 22.47 (0.233) ^{o}C p = 0.195).

Summary

1. All infants achieved a minimum rectal temperature of below 36.5 ^{0}C at a given age which can be defined as the age that a mature temperature pattern occurs (Fig 4.2).

2. The average age (SD) of temperature maturity was 11.06 (0.46) weeks. Temperature maturity for the group of infants as a whole occurred between 7 and 18 weeks, with the majority of babies achieving maturity by 10-11 weeks of age.

3. There was no further significant fall in minimum rectal temperature after the age of temperature maturity.

4. The infants in this study developed physiological temperature maturity in a similar way to those described previously.

5. There was no significant difference in the thermal environment in which the infants had their night time sleep before and after temperature maturity. The minimum and maximum room temperatures were similar.

Chapter 5

Result

5 Results

DESCRIPTION OF THE URINARY EXCRETION OF MELATONIN

5.1 Analysis of urinary melatonin in infants

Night and day time urine samples were collected for melatonin estimation, using the metabolite 6-sulphatoxymelatonin as a proxy measure of melatonin secretion. There is strong correlation between urinary melatonin and 6-sulphatoxymelatonin (Bojkowski *et al.*, 1987). In order to take into account the rate of renal excretion of the metabolite, the melatonin results are expressed as a ratio with $mg.ml^{-1}$ creatinine as the denominator. Sampling times were restricted to an evening and morning sample selected to reduce the interruption of the normal infant care routine for participant families. In addition the timing of the urine sampling had to fit into the overall study monitoring schedule which also involved the care giver taking six hourly buccal swabs from the infant on two consecutive days at weekly intervals, completion of sleep-activity diary, wearing of Actiwatch device (for mother and infant) and fortnightly overnight infant temperature monitoring.

Samples were collected at or as close as possible to midnight (night time sample) and midday (day time sample) on the subsequent day for one to two days at weekly intervals. The infants ranged from 5 to 17 weeks of age during the period of data collection. The change of night time and day time secretion of melatonin (using the metabolite 6-sulphatoxymelatonin as a proxy measure) with post natal age was examined. Cosinor analysis methods were not used to examine/define the emergence of a circadian rhythm due to the frequency of samples (12 hourly sampling rather than

4 or 6 hourly samples. Circadian rhythm analysis instead was defined in terms of differences between day night values.

A total of 466 urine samples were collected from 34 infants recruited into the study. 236 (51%) were evening samples. The remaining 230 (49%) samples were collected as close to midday as possible. 4 samples (0.9 %) were thought to be insufficient for melatonin analysis. The adequacy of volume of urine collected for melatonin estimation was improved after an alternative method of urine collection (urine pads) was added to the study protocol, following feedback from mothers of participant infants in the first monitoring phase, regarding the use of U-bag in the nappies. All samples were assayed in duplicate and a mean value used.

Ideally to determine melatonin diurnal rhythmicity, multiple samples at numerous time points throughout the 24 hour period should have been taken to calculate melatonin on set and off set time accurately.

In the analysis of the data, looking for age related changes in infant melatonin secretion, night-day time pairs of 6-sulphatoxymelatonin were selected on a weekly basis for each infant. If 12 hourly samples were taken on two consecutive days within the same postnatal week, the second night-day pair was excluded from the analysis to allow consistent analysis without the need for repeated measures analysis methods. Only paired data were used.

The secretion of melatonin both during night and day time was positively skewed. Therefore $\log_{10}(x)$ values were used when required, for statistical analysis. Raw data are presented for graphical interpretation.

For the purpose of this thesis, 6-sulphatoxymelatonin (MT6s) will be referred to in short hand as melatonin, as the MT6s assay was used as a proxy for melatonin estimation.

For ease of illustration, some charts have had error bars omitted.

5.1.1 Changes to melatonin secretion relative to postnatal age

Figure 5.1 shows the increase in night time melatonin with postnatal age (mean values represented are for raw data for ease of interpretation). Random effects modelling, using logged data, demonstrated a significant rise ($p<0.001$), with a week on week rise of 21% for night time secretion (95% CI: 13% to 29%).

The random effects model for day time melatonin secretion (using logged data) showed no associated increase in day time log melatonin secretion with postnatal age. The average weekly increase of 5% was not found to be significant; $p=0.158$ (95% CI: -2% to 13%). Figure 5.2 shows the change of daytime secretion with age. Mean values are calculated using raw data.

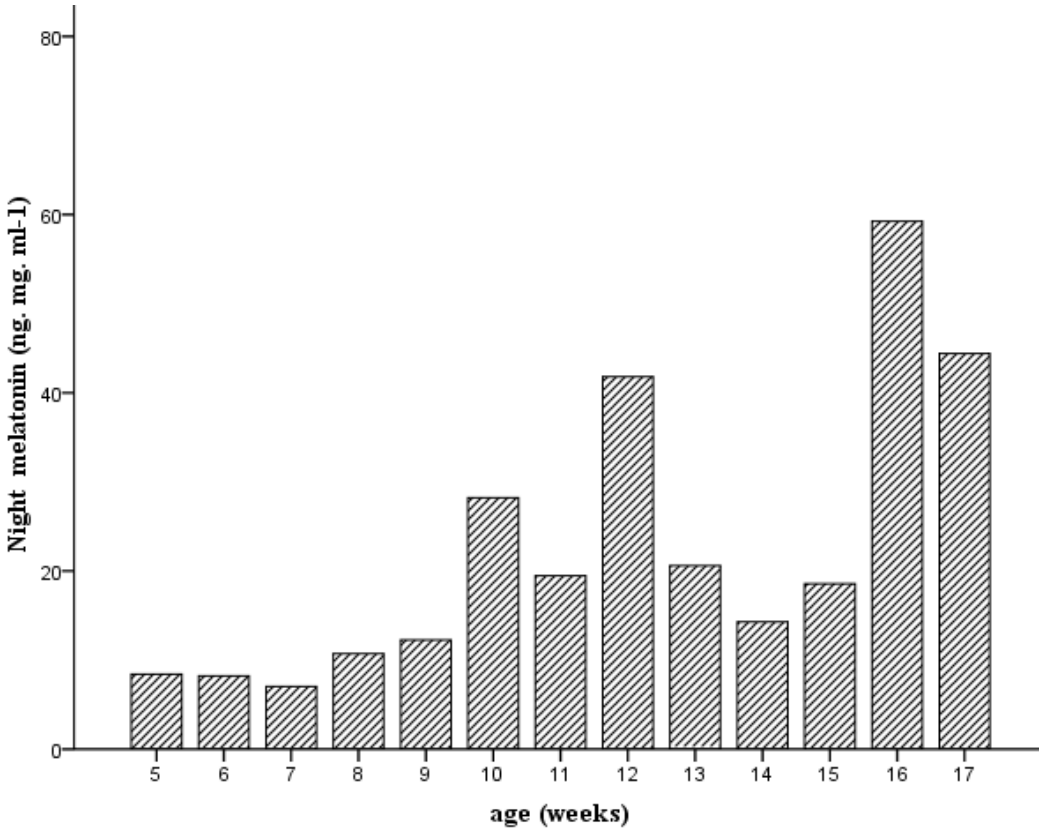

Figure 5.1 - Distribution of mean night time melatonin secretion (unlogged values) in 34 infants with postnatal age.

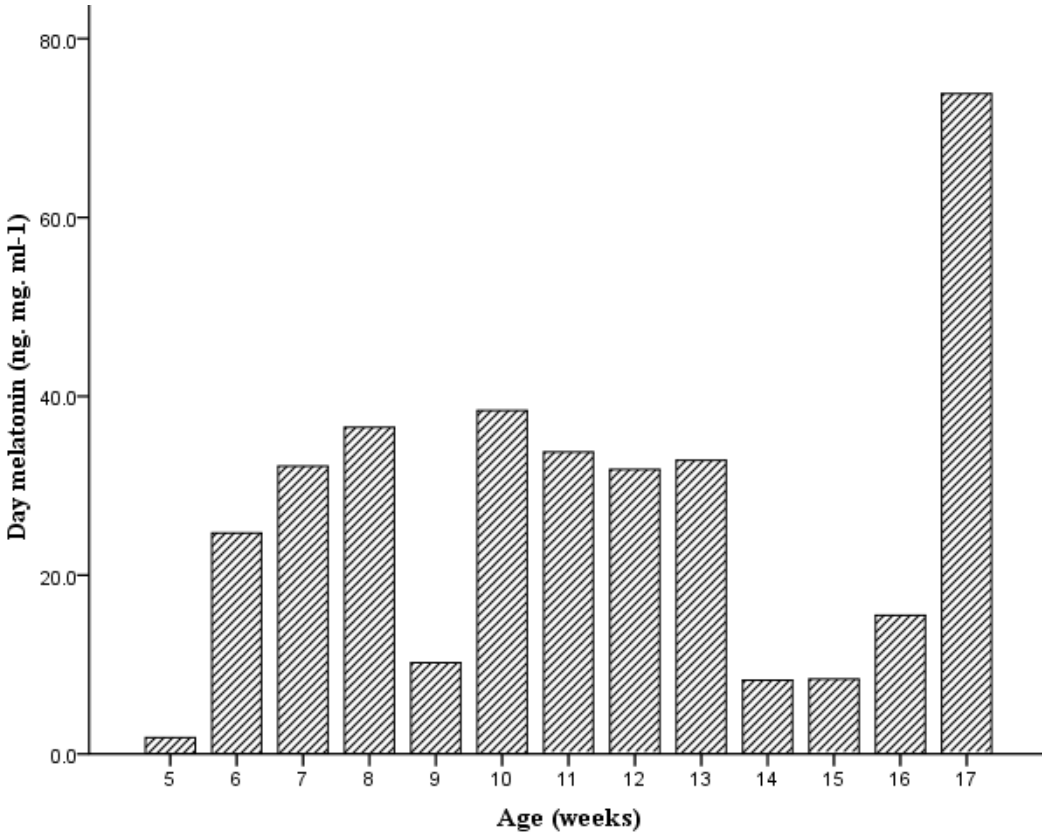

Figure 5.2 - Distribution of mean day time melatonin secretion (unlogged values) in 34 infants with postnatal age.

5.1.2 Emergence of diurnal rhythm for melatonin secretion

A diurnal pattern of melatonin secretion was characterised by the age when greater than fifty percent of the total melatonin was secreted at night time (Sivan & Laudon, 2001).

Apart from an initial peak at 5 weeks of age, the percentage of total melatonin produced at night remains below fifty percent mark for weeks 6 to 8. More melatonin is secreted at night compared to day from 9 weeks onwards. The persistence of the night time dominance between 9 and 17 weeks of age is summarised in table 5.1.

Units melatonin measured in ng. mg. ml^{-1}

Age (weeks)	Mean (%)	N	Min. (%)	Max. (%)	Std. Deviation (%)	Std. Error of Mean (%)
5	76.85	2	69.71	84.00	10.105	7.145
6	45.51	6	4.42	70.15	25.629	10.463
7	41.05	16	1.40	82.02	20.298	5.075
8	44.06	20	.68	91.30	29.545	6.606
9	55.30	17	11.40	88.25	22.628	5.488
10	52.48	25	2.59	98.19	26.278	5.256
11	51.79	17	2.99	98.19	23.918	5.801
12	52.06	20	5.42	94.81	30.283	6.771
13	51.27	12	12.31	90.70	26.664	7.697
14	57.80	8	21.06	98.20	26.278	9.291
15	59.73	5	24.96	90.84	26.062	11.655
16	59.15	4	20.00	91.45	36.415	18.208
17	55.48	4	22.61	81.82	29.291	14.645
Total	51.11	156	.68	98.20	26.025	2.0834

Table 5.1 – Distribution of night time (unlogged values) melatonin secretion as a percentage of total melatonin (night plus day melatonin) for 34 infants for each week of age studied

There is wide distribution in the percentage of night time melatonin produced by the infants as a group for each week of age studied (See figure 5.3).

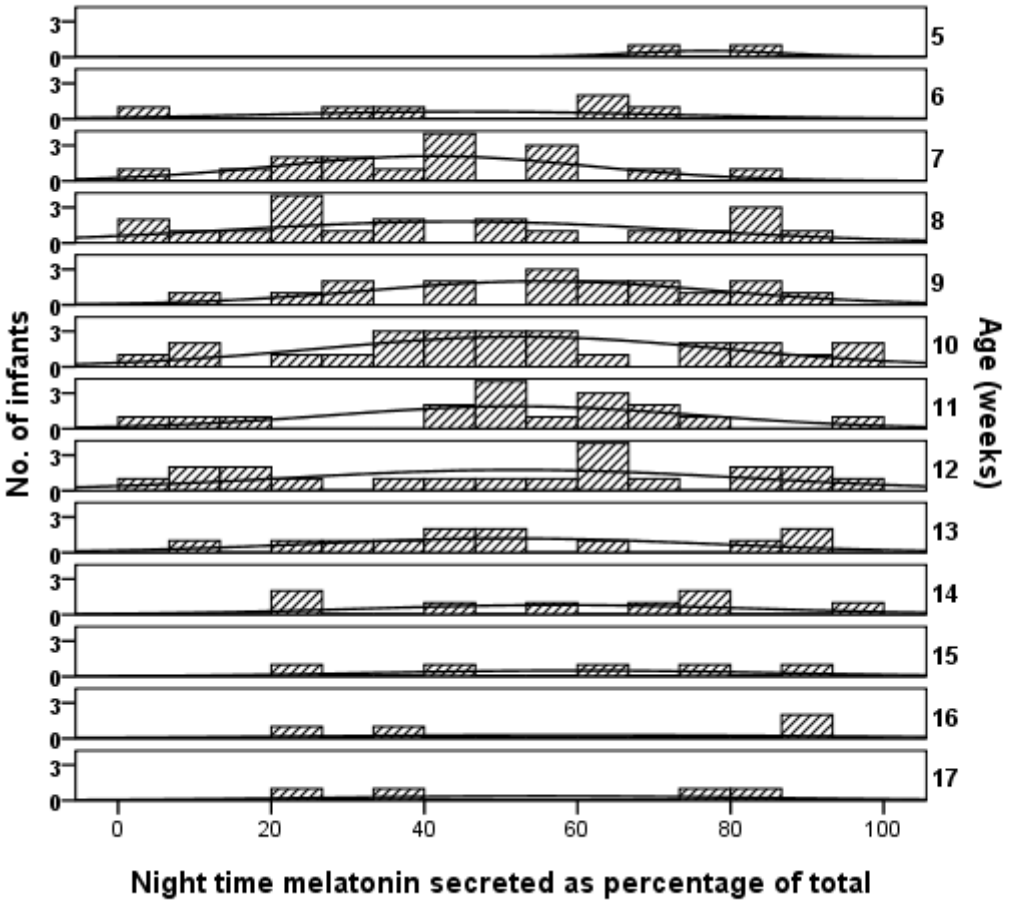

Figure 5.3 - Histogram showing distribution of night time (raw data) melatonin as percentage of total melatonin secreted for 35 infants for each age group studied in weeks

5.1.3 Melatonin secretion relative to temperature maturation stage

Figure 5.4 and Figure 5.5 demonstrate the changes in night and day time melatonin relative to the stage of maturation of core body temperature during sleep (as described in section 4.3). Night melatonin shows again an increase with maturing of temperature state while there is no change with the corresponding day time values. Week zero is the reference week that temperature maturity was achieved in all infants. All other weeks of measurements have been corrected, for example week -2 is two weeks prior to temperature maturity was achieved, week +2 is two weeks after temperature maturity was achieved. It should be taken into account when reviewing this graphical presentation of the data that there are very few infants measured at - 10 weeks and + 8 weeks maturation, which creates the appearance of a sharp increase. The steepness of the visual slope may be misleading.

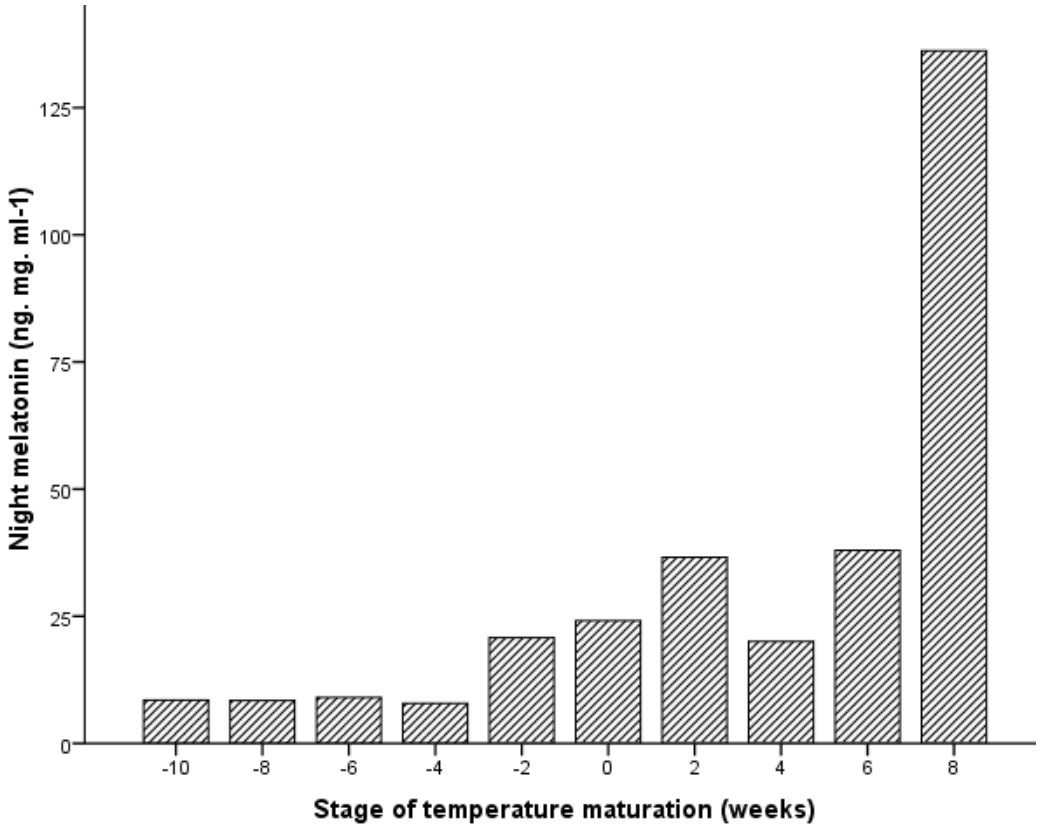

Figure 5.4 - Changes in night time excretion of melatonin (unlogged values) between 10 weeks prior and 4 weeks after the development of a mature temperature pattern.

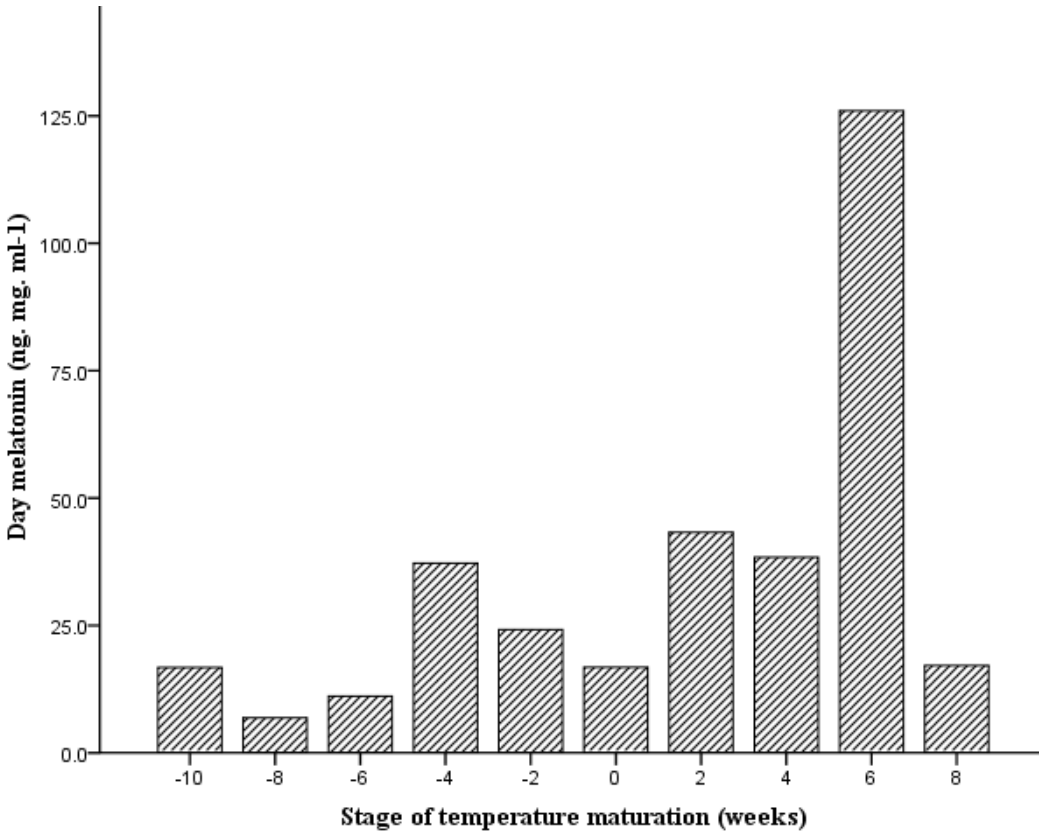

Figure 5.5 - Changes in day time excretion of melatonin (unlogged values) between 10 weeks prior and 4 weeks after the development of a mature temperature pattern.

Night-day difference in melatonin secretion prior to temperature maturation

There is no difference in median night and day values for melatonin in the infants measured in the weeks prior to their development of a mature circadian rhythm for temperature (weeks 5 to 10 weeks). The median night time level is 9.207 $ng.mg.ml^{-1}$ and the day value mean is 8.378 $ng.mg.ml^{-1}$ before temperature maturity achieved (p=0.374; Wilcoxon signed ranks).

Night-day difference in melatonin secretion following temperature maturation

Median value for night melatonin is higher than the day time value after temperature maturity is achieved (weeks 11 to 17). The median night time level is 17.433 $ng.mg.ml^{-1}$ and the day value is 11.629 $ng.mg.ml^{-1}$. This does not reach statistical significance (p = 0.452; Wilcoxon signed ranks).

Changes in night time melatonin production in relation to temperature maturity

Night time melatonin (SD) after temperature maturity is significantly higher than secretion prior to temperature maturity. It increases from a median value of 9.207 to 17.433 ng. mg. ml^{-1} with temperature maturity (p=0.001; Mann U Whitney).

Changes in day melatonin production in relation to temperature maturity

There is no corresponding change in day time melatonin secretion with temperature maturation. Pre temperature maturation levels of secretion (SD) are 8.378 and median values are 11.630 ng. mg. ml^{-1} once core body temperature has matured (p=0.289; Mann U Whitney).

5.1.4 Relationship between night and day time log melatonin

There is a direct correlation between night time (logMT6s_1) and day time log (logMT6s_2) melatonin ($R = 0.212$; $p=0.008$).

Summary

1. There is an increase in night time melatonin with postnatal age (Fig 5.1).

2. There is no overall associated increase in day time log melatonin with postnatal age (Fig 5.2).

3. Night time melatonin production increases significantly once physiological maturity has been achieved in terms of core body temperature control.

4. There is no corresponding change in day time melatonin production when temperature maturity is achieved.

Chapter 6

Results

6 Results

DESCRIPTION OF THE URINARY EXCRETION OF CORTISOL

6.1 Analysis of urinary cortisol

Night time and morning cortisol samples (from urine) were obtained from all infants in the study. This chapter presents the results of the analysis of these. As was the case for melatonin, only two sampling times for every 24 hour period were measured. This was to try to maintain simplicity of the monitoring schedule as several physiological parameters as well as the measurement of gene expression in the infant were being undertaken concurrently. All results are expressed as mg/ml creatinine to correct for renal excretion.

Samples were collected at or as close to midnight (night time samples) and midday (day time samples) on the subsequent day over a 24 to 48 hour period at weekly intervals. The infants ranged from 5 to 17 weeks of age during the period of data collection. The change of night time and day time secretion of cortisol with postnatal age was examined. Cosinor analysis was not applied to cortisol data.

A total of 466 urine samples were collected from 34 infants recruited into the study. Twenty samples were insufficient for cortisol analysis. Three methods for urine collection were used: U-bag collection system, clean catch and urine pads in the nappies. All samples were assayed in duplicate and a mean value used.

Cortisol samples are reported as weekly values. If collected, a second night-day pair for the same week was excluded from the analysis, to avoid repeated measures analysis. Unpaired data were discarded.

The night and day secretion of cortisol did not follow a normal distribution. Therefore values were logged to base 10 to obtain normal distribution.

For ease of illustration, error bars have been omitted from bar charts.

6.1.1 Changes to cortisol secretion relative to postnatal age

There was no overall increase or decrease in night time cortisol secretion with postnatal age. Random effects modelling (logged values) demonstrated a non significant 1% increase per week (p=0.702; 95% CI: 4% decrease to 6% increase). The distribution of night cortisol levels (unlogged) is shown in Figure 6.1.

Figure 6.2 illustrates how the production of morning cortisol varies with postnatal age. There is no overall increase or decrease in day time cortisol values between 5 and 18 weeks of age. Random effects modelling shows the average weekly change being a 2% decrease per week (95% CI: 7% decrease to 3% increase; p=0.429), using normalised data.

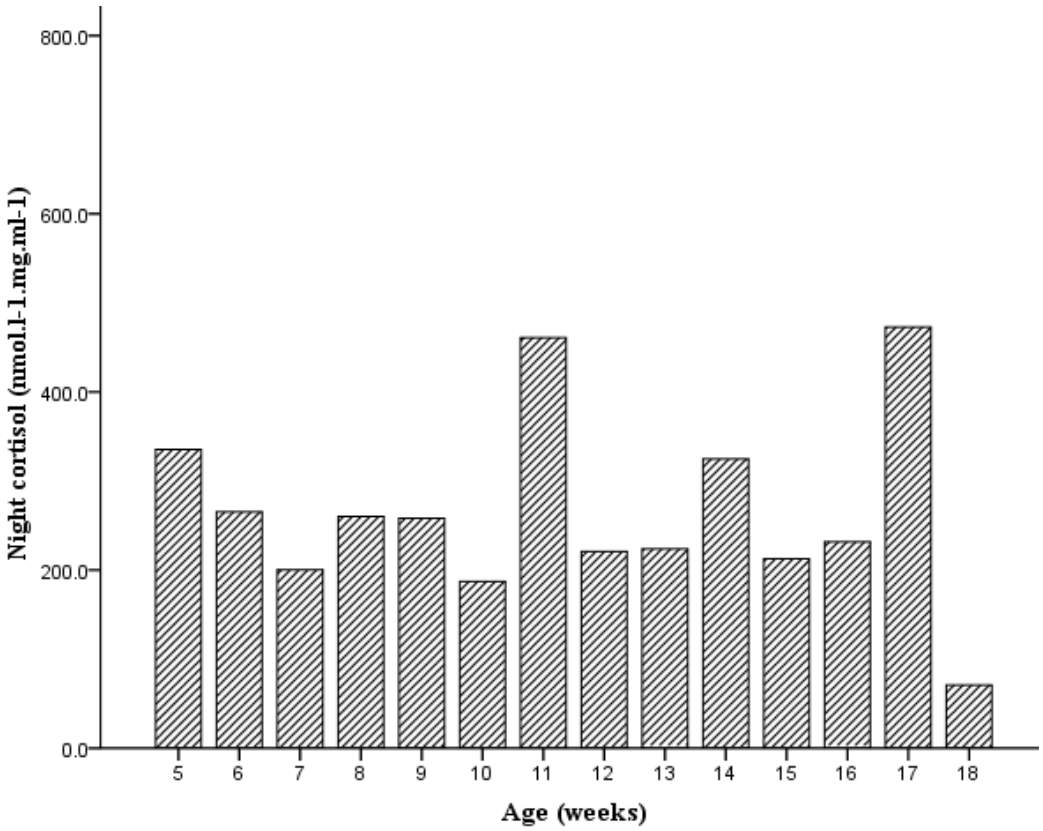

Figure 6.1 - Changes in night time excretion of cortisol (unlogged values) between 5 and 18 weeks of age in 35 infants.

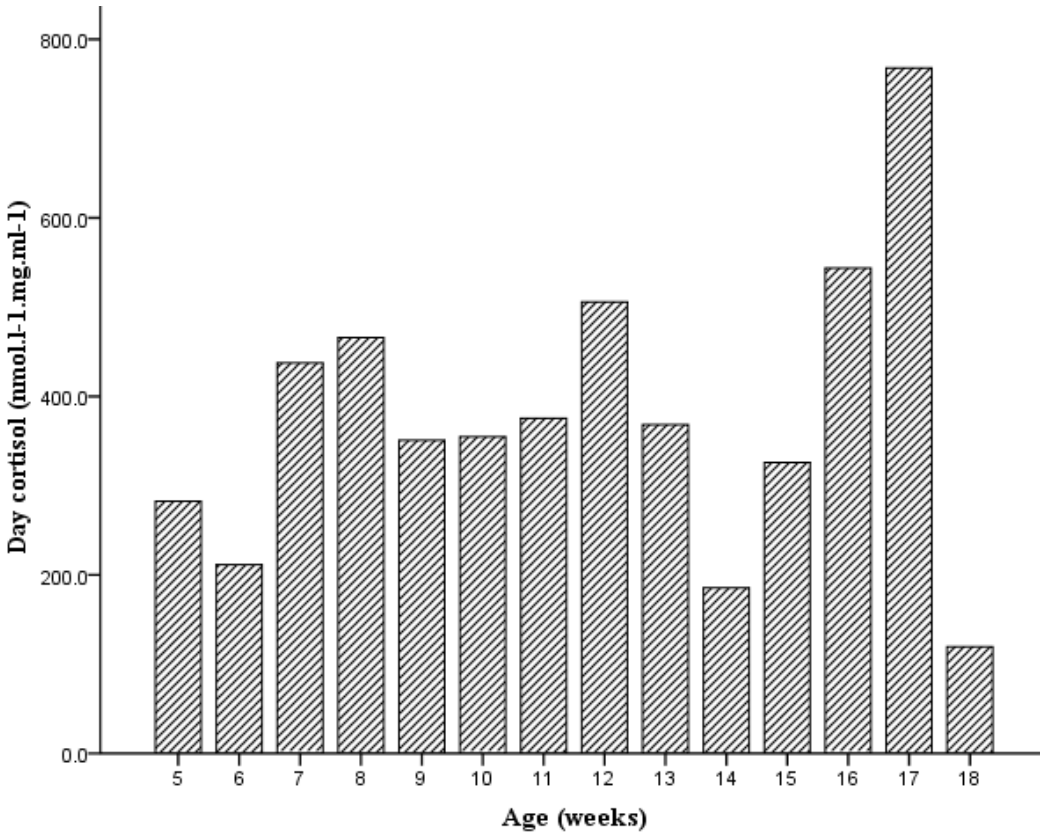

Figure 6.2 - Changes in urinary day time cortisol excretion (unlogged data) in 35 infants between 5 and 18 weeks of age.

6.1.2 Emergence of diurnal rhythm for cortisol secretion

As for melatonin, the first appearance of a diurnal pattern of cortisol secretion was characterised by the age when greater than fifty percent of the total cortisol was secreted during the morning period (Sivan & Laudon, 2001).

Apart from a value of 43% at 14 weeks of age, consistently more cortisol was produced during day hours from 6 weeks of age till 17 weeks of age (3 weeks earlier than data for night time melatonin production). These results are summarised in table 6.1.

Units cortisol measured in $nmol.l^{-1}.mg.ml^{-1}$

Age (weeks)	Mean (%)	N	Min. (%)	Max. (%)	Std. Deviation (%)	Std. Error of Mean (%)
5	45.73	1	46	46		
6	59.28	6	11	89	28.881	11.791
7	65.25	19	24	89	17.539	4.024
8	61.11	21	5	94	22.860	4.989
9	57.51	17	7	86	25.181	6.107
10	62.69	21	20	85	16.659	3.635
11	50.94	14	1	85	26.865	7.180
12	63.41	19	38	95	14.568	3.342
13	62.91	18	10	84	21.519	5.072
14	43.49	6	12	79	26.961	11.007
15	65.23	5	51	86	13.489	6.032
16	61.70	4	21	80	27.284	13.642
17	64.09	2	58	70	8.878	6.278
18	62.73	1	63	63		
Total	60.35	154	1	95	21.073	1.698

Table 6.1 - Distribution of day time (unlogged values) cortisol secretion as a percentage of total cortisol (day plus night cortisol) for 34 infants for each week of age studied

The distribution of the day time cortisol as a percentage of the total occurs over a narrower range for each week of age studied, in comparison with the melatonin data where the variation is wider (See Figure 6.3).

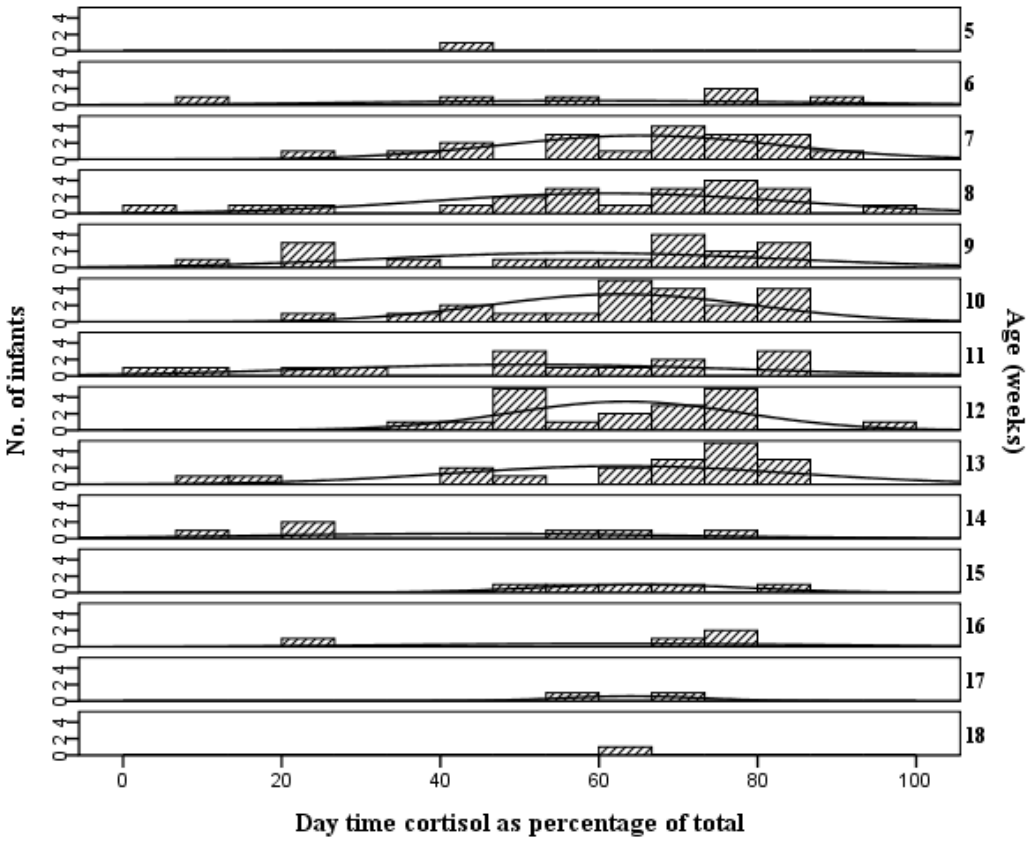

Figure 6.3 - Histogram showing distribution of day time cortisol (unlogged values) as percentage of total cortisol secreted for 35 infants for each week of age studied (in weeks)

6.1.3 Cortisol secretion relative to temperature maturation stage

Figure 6.4 & Figure 6.5 demonstrate that there is no change in cortisol secretion when night and day mean values are compared, in relation to physiological maturation of core body temperature during sleep.

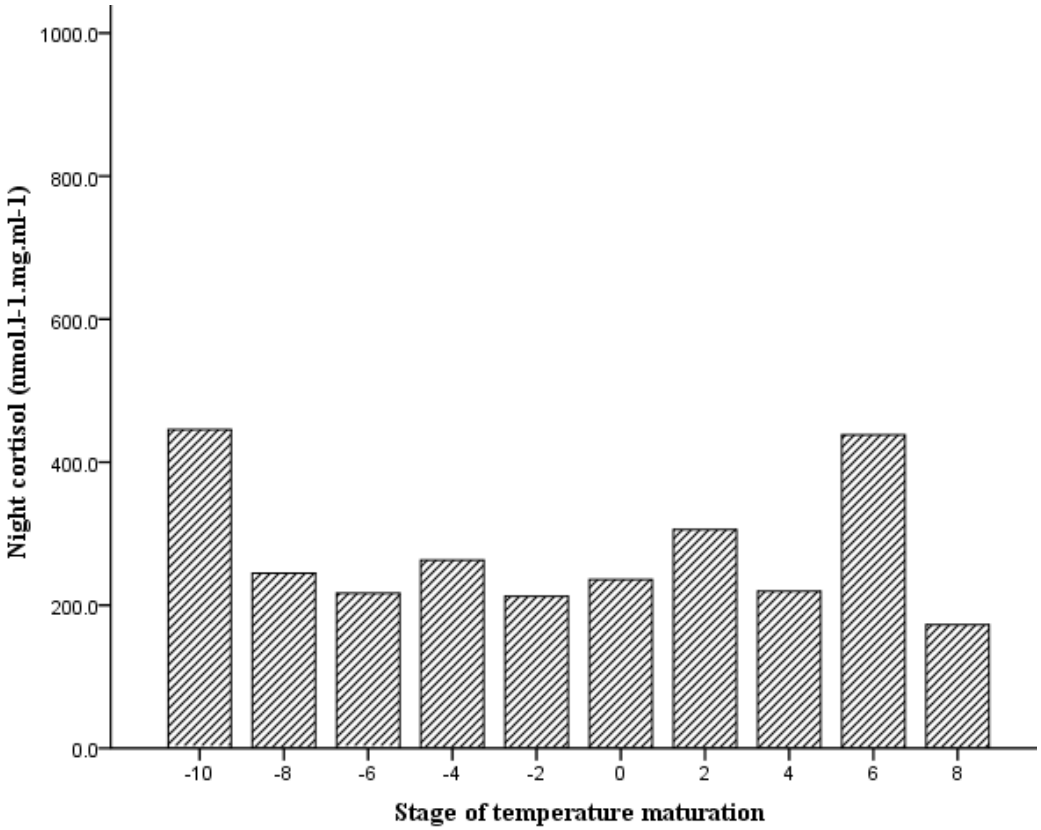

Figure 6.4 - Changes in night time excretion of cortisol between 10 weeks prior and 4 weeks after the development of a mature temperature pattern.

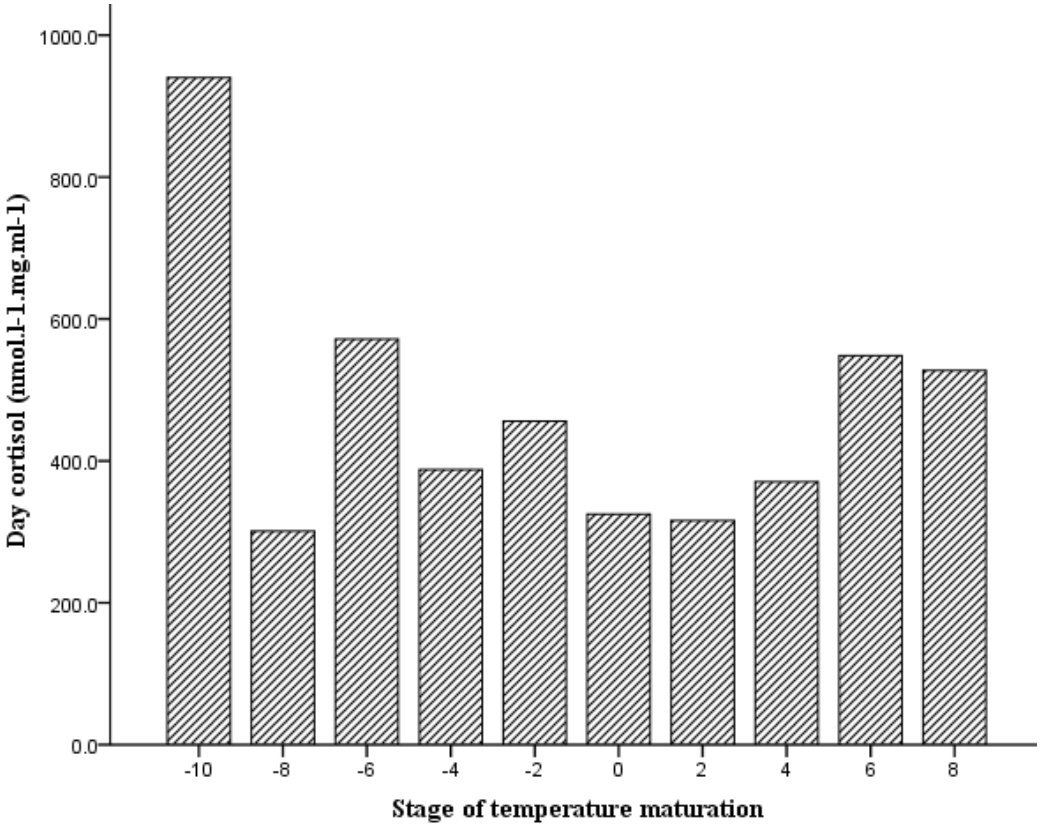

Figure 6.5 - Changes in day time excretion of cortisol between 10 weeks prior and 4 weeks after the development of a mature temperature pattern.

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Cortisol values were corrected to age of temperature maturation. At all stages of physiological development relative to core body temperature day-night cortisol secretion exceeds fifty percent of the total, indicating the emergence of a diurnal pattern of cortisol secretion from 10 weeks prior to 8 weeks post maturity (see Table 6.2).

Units cortisol measured in $nmol.l^{-1}.mg.ml^{-1}$

Rhythm stage (wks)	Mean (%)	N	Min. (%)	Max. (%)	Std. Deviation (%)	Std. Error of Mean (%)
-10	68.93	2	66	71	3.532	2.498
-8	56.09	8	18	85	22.848	8.078
-6	65.37	10	20	82	17.887	5.657
-4	59.79	27	11	94	21.270	4.093
-2	62.37	36	5	95	21.543	3.590
0	59.36	21	10	86	22.180	4.840
2	56.68	33	1	85	23.162	4.032
4	64.50	13	12	84	19.678	5.458
6	55.33	3	51	58	3.650	2.107
8	75.35	1	75	75		
Total	60.35	154	1	95	21.073	1.698

Table 6.2 - Distribution of day time (unlogged values) cortisol secretion as a percentage of total cortisol (day plus night cortisol), relative to temperature maturity, for 34 infants for each week of age studied

Day-night difference in cortisol secretion prior to temperature maturation

Cortisol secretion was significantly higher in the day time when compared to night values in the period prior to the attainment of a mature temperature circadian rhythm (weeks 5-10). Day time secretion had a median value of 370.308 while night time secretion had a median value of 180.085 $nmol.l^{-1}.mg.ml^{-1}$ (p=0.0001; Wilcoxon signed ranks).

Day-night difference in cortisol secretion following temperature maturation

Day time cortisol secretion was higher than night time secretion; once temperature maturity had been achieved (11-17 weeks); with median day values of 298.051 and night values being 172.562 $nmol.l^{-1}.mg.ml^{-1}$. This was a trend (p=0.061; Wilcoxon signed ranks).

Changes in night time cortisol production in relation to temperature maturity

Night time cortisol does not change with temperature maturation. Pre temperature maturity median value was 180.085 $nmol.l^{-1}.mg.ml^{-1}$; the post temperature maturity median value 172.562 $nmol.l^{-1}.mg.ml^{-1}$ (p=0.786; Mann U Whitney).

Changes in day time cortisol production in relation to temperature maturity

Day time cortisol secretion also remained unchanged before and after a mature temperature rhythm was achieved (p=0.250; Mann U Whitney). Pre temperature maturity, secretion had a median value of 180.085 while secretion after temperature maturation had a value of 298.051 $nmol.l^{-1}.mg.ml^{-1}$.

6.1.4 Relationship between night and day time log cortisol

There is a direct correlation between night time (cortcr_1) and day time log (cortcr_2) cortisol ($R = 0.440$; $p<0.0001$). Mean night time cortisol (SD) is 2.313 (0.435); morning cortisol (SD) averages at 2.424 (0.456).

Summary

1. There was no overall change in day or night cortisol with increase in postnatal age (Fig 6.1 & Fig 6.2).

2. Day time levels of cortisol production, from 6 to 18 weeks, were higher than evening levels.

3. Overall day and night time levels of cortisol remained steady, over the period temperature maturity was achieved.

5. There is a positive correlation between day and night time cortisol.

6. A circadian rhythm of cortisol production was present from 6 weeks of age and preceded temperature maturation.

Chapter 7

Results

7 Results

DESCRIPTION OF INFANT SLEEP

7.1 Analysis of infant sleep measurements

In this chapter sleep parameters during night time sleep are described for 35 infants. Full sleep staging by polysomnography was not conducted. Actigraphy was used to plot actigrams for each night of sleep monitored with activity-rest estimated, utilising a digitally integrated measure of gross motor activity, assessed by an internal accelerometer. 1 minute epochs were used to ensure accuracy.

Sampling of sleep measurements began at a routine infant bed time as reported by the mother/main care giver on the night of monitoring, at the same time the Grant squirrel data logger was attached to the baby.

The following sleep parameters were recorded by the Actiwatch:

Table 7.1 - Definitions of sleep parameters used by Actiwatch

Sleep parameter	Definition
Bed Time	Inputted bed time
Get Up Time	Inputted get up time
Sleep Start	The start of sleep as set by the operator or derived automatically from a marked event.
Sleep End	The end of sleep as set by the operator or derived automatically from a marked event.
Time in Bed	The difference between the get up and bed times.
Assumed Sleep	The difference between sleep end and sleep start.
Actual Sleep Time	The amount of sleep as determined by the algorithm and is equivalent to assumed sleep minus wake time.
Actual Awake Time	The amount of time spent awake as determined by the algorithm.
Actual Sleep and Wake Time %	These are displayed to the right of the Actual Sleep and Actual Wake boxes.
Sleep Efficiency	The percentage of time spent asleep whilst in bed.
Sleep Latency	The latency before sleep onset following bed time.
Number of Sleep Bouts	The actual number of episodes of sleep.
Number of Wake Bouts	The actual number of episodes of wakefulness.

Mean Length of Sleep/ Wake Bouts	These figures are determined by dividing the total duration of sleep and wake by the corresponding number of sleep and wake bouts.
Number of Minutes Immobile	The total number of minutes where a score of zero was recorded during the assumed sleep period.
Number of Minutes moving	The converse of the above being the total number of minutes where scores of greater than zero were recorded during the assumed sleep period.
Percentage of Minute Immobile and Minutes Moving	The percentage of time spent immobile or moving during the assumed sleep period.
The Number of Immobile Phases	The number of periods of continuous scores of zero being recorded in consecutive epochs.
The Number of Immobile Phases of 1 Minute	The number of immobile phases where the duration was only 1 minute.
Percentage Immobility Phases of 1 Minute	The percentage of Immobility phases of 1 minute as a proportion of the total number of Immobility phases. This value is also termed the Fragmentation Index.
Movement and Fragmentation Index	The addition of Percentage Time Spent Moving and The Percentage Immobility Phases of 1 Minute. This is used as an indicator of restlessness.
Total Activity Score	The total number of activity counts between sleep start and sleep end.
Mean Activity Score	The average value of the activity counts per epoch over the assumed sleep period.
Mean Activity Score in Active Periods	The average activity score in those epochs where scores of greater than zero were recorded during the assumed sleep period.
Average Wake Movement	The average activity score per epoch for the wake period preceding the previous night's sleep. Derived from activity counts between sleep end in the morning and sleep start of the current day.

A total of 138 actigraphy measurements were taken from 35 infants. On average each infant had (SD) 2.67 (1.338) measurements (range 1 to 6). Three actigram readings were discarded due to incomplete data collection therefore only 135 actigrams were included in analysis. Six nights of sleep were not captured using actigraphy due to unavailability of equipment.

On every given night of monitoring, there was variation in the times each infant was put to bed (determined by infant care routine and family schedule), fell asleep and woke. Fixed bed times were kept by three families following a Gina Ford infant care and sleep schedule. During the analysis of the actigraphy data using the accompanying software bed time and get up times were inputted by the operator from sleep diary entries completed by the mothers for each night of sleep monitored. The actigraphy software used a default sleep start and sleep end time of 23:00 and 8:00. The sleep start and end times therefore were manually altered using sleep diary entry. This was related to time of lights out using data from light sensor and the height of the actigraphy trace. To allow compete data capture and allow for a sufficient margin for error the analysis window was set to 60 minutes before and 60 minutes after bed and get up time.

Details of infant sleeping arrangements (bedding, tog value, location, and sleep position) were recorded and validated by direct observation by a trained researcher. The mother self reported on infants sleep pattern using a standardised sleep diary.

Infant sleep was monitored on average from 5 to 18 weeks of age.

Sleep parameters were recorded as means for the group. Changes in sleep were related to maturation of infant physiology as determined by achievement of adult pattern of core body temperature during night time sleep.

7.2 Description of mean sleep parameters for 35 infants

On average infants were put to bed at around 20:42 hours according to sleep diary. This was confirmed by actigraphy with sleep start at 20:40 in the evening. The latest sleep start for an individual baby was 00:19 by actigraphy. Mothers recorded on sleep diaries that infants woke at a mean time of 07:20 hours. On average sleep ended at 07:16 in the morning. The earliest sleep end for an individual infant was at 03:55 hours by actigraphy.

On average the infants slept for 7.18 (SD =1.20) hours per night, with minimum duration 3.45 hours and maximum 10.52 hours. During the period from sleep onset to the end of night time sleep 2 hours 3 minutes were spent awake (range 3 minutes to 5 hours 9 minute; SD = 1 hour 7 minutes). Average sleep latency (SD) was 11 (15) minutes (range 0 minutes to 1 hour 23 minutes). The infants spent 78.3% of their time in bed actually asleep during the night time period. Sleep efficiency (SD) averaged at 75.3 (10.98) %. The most sleep efficient infants had a score of 97.8% with the minimum being 49.4%.

The mean fragmentation index (SD) was 74.85 (35.42) with the score ranging between 22.3 and 201.4. The infant mean activity score (SD) during periods of wakefulness overnight was 32.69 (21.39). The minimum activity score was 2.89 and maximum

104.23. Total activity throughout the night (including periods of wakefulness) was 19349 (range 12 to 60488; SD = 13547). 40.4% of the total sleep time was spent moving.

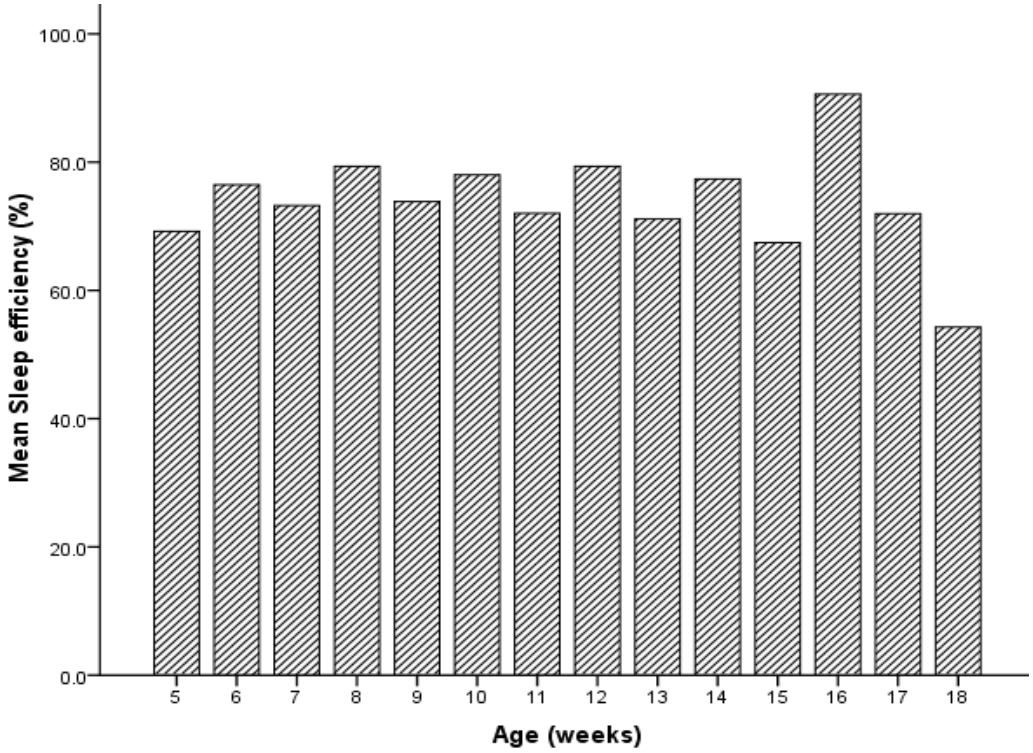
The number of immobile minutes (SD) averaged at 359.7 (118.73), with a range of 0 to 554 minutes. The number of immobile phases of 1 minute duration ranged from 0 to 420 (mean 31.07 (SD = 37.595).

There were on average 39.54 sleep bouts per night lasting 16 minutes 43 seconds (SD 18 minutes 47 seconds; range 4 minutes 5 seconds to 3 hours, 10 minutes and 34 seconds) and 36.81 wake bouts lasting 3 minutes (SD 1 minute 26 seconds; range 50 seconds to 9 minutes to 30 seconds).

7.3 Development of infant sleep relative to temperature maturation stage

7.3.1 Care giver dependent infant sleep parameters

There was no change in infant sleep efficiency (SD) after a mature temperature rhythm was established 76.59 (10.401) vs. 73.89 (11.516)%; p=0.174. Sleep duration remained unchanged (total number of hours asleep (SD) before mature temperature rhythm achieved was 7 hours 10 minutes (1:24 hours); this became 7 hours 25 minutes (1:15 hours) once temperature maturity was achieved; p = 0.268. Sleep latency remained unchanged (pre and post temperature rhythm latency = 11 mins; p=0.997). The percentage time spent asleep did not alter. The infants spent on average 2 hours 10 minutes (0:08 hours) awake during the night time period before overnight temperature matured. After maturity 1 hour 58 minutes (0:07 hours) were spent awake (p=0.308).The above aspects of sleep may be described as 'mother' dependent factors. For example infant sleep latency is determined in this case by the time the baby was put to sleep by mother and time of lights out. This differs from use of actigraphy in adults where sleep latency maybe more accurately elicited as bedtime is determined by the actiwatch wearer not the carer). As the sleep data were not collected using full polysomnography; assumptions are made by validating observer and actigraphy software which may affect results. Therefore the parameters thought most likely to be affected or influenced by maternal behaviour in relation to the infant's bed routine will be referred to as care giver dependent sleep parameters. Summary graphs are shown in Fig 7.1 to Fig 7.4.



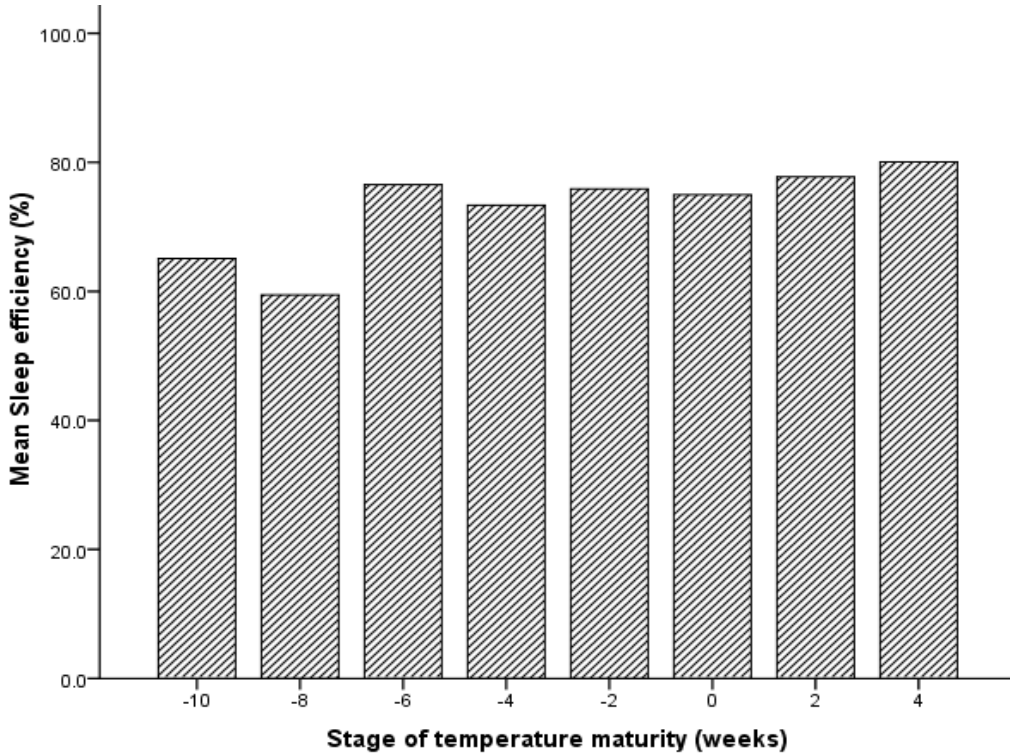


Figure 7.1 - Changes in infant sleep efficiency with age and in the weeks prior and after development of temperature maturity

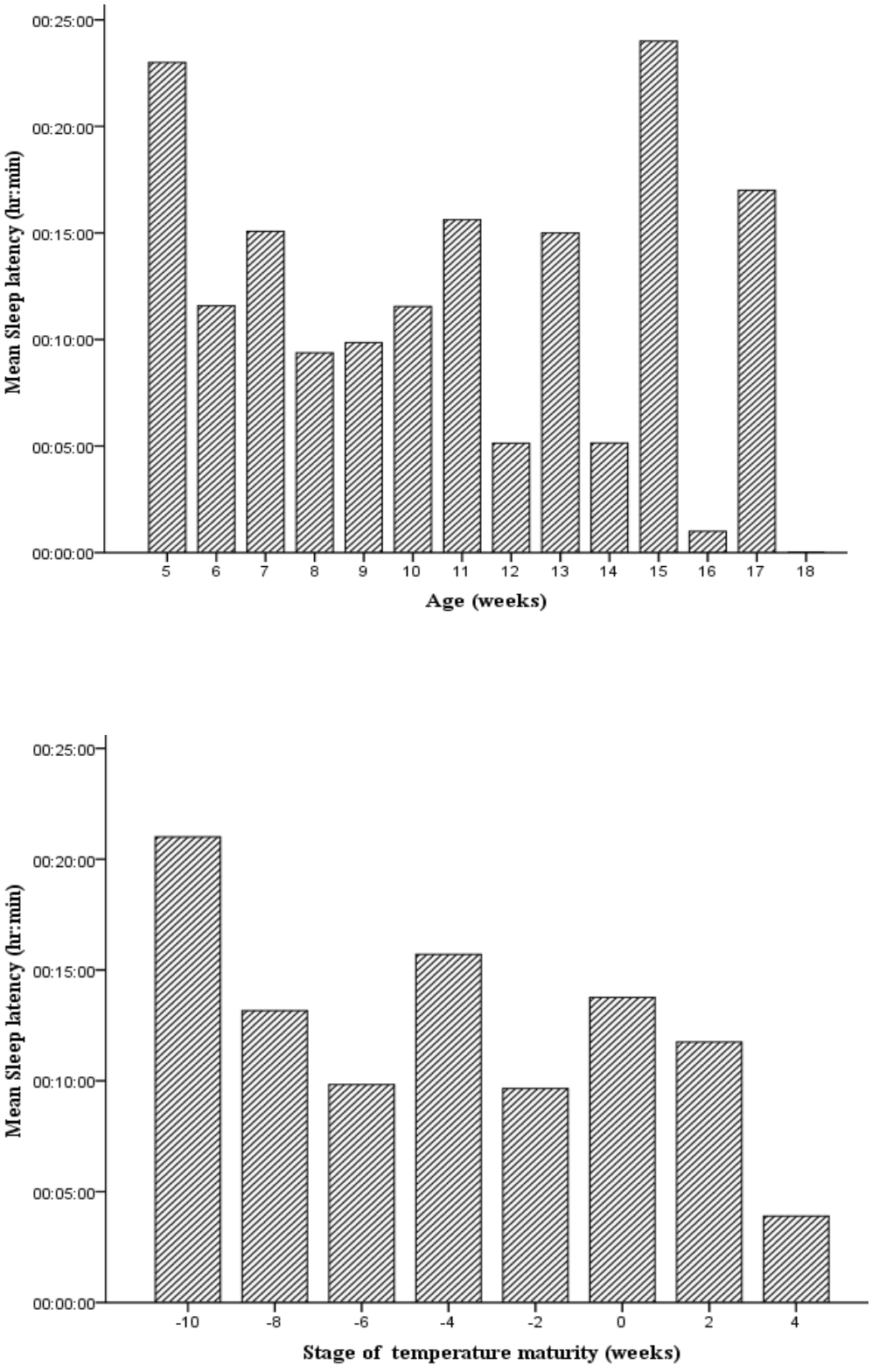


Figure 7.2 - Changes in infant sleep latency with age and in the weeks prior and after development of temperature maturity

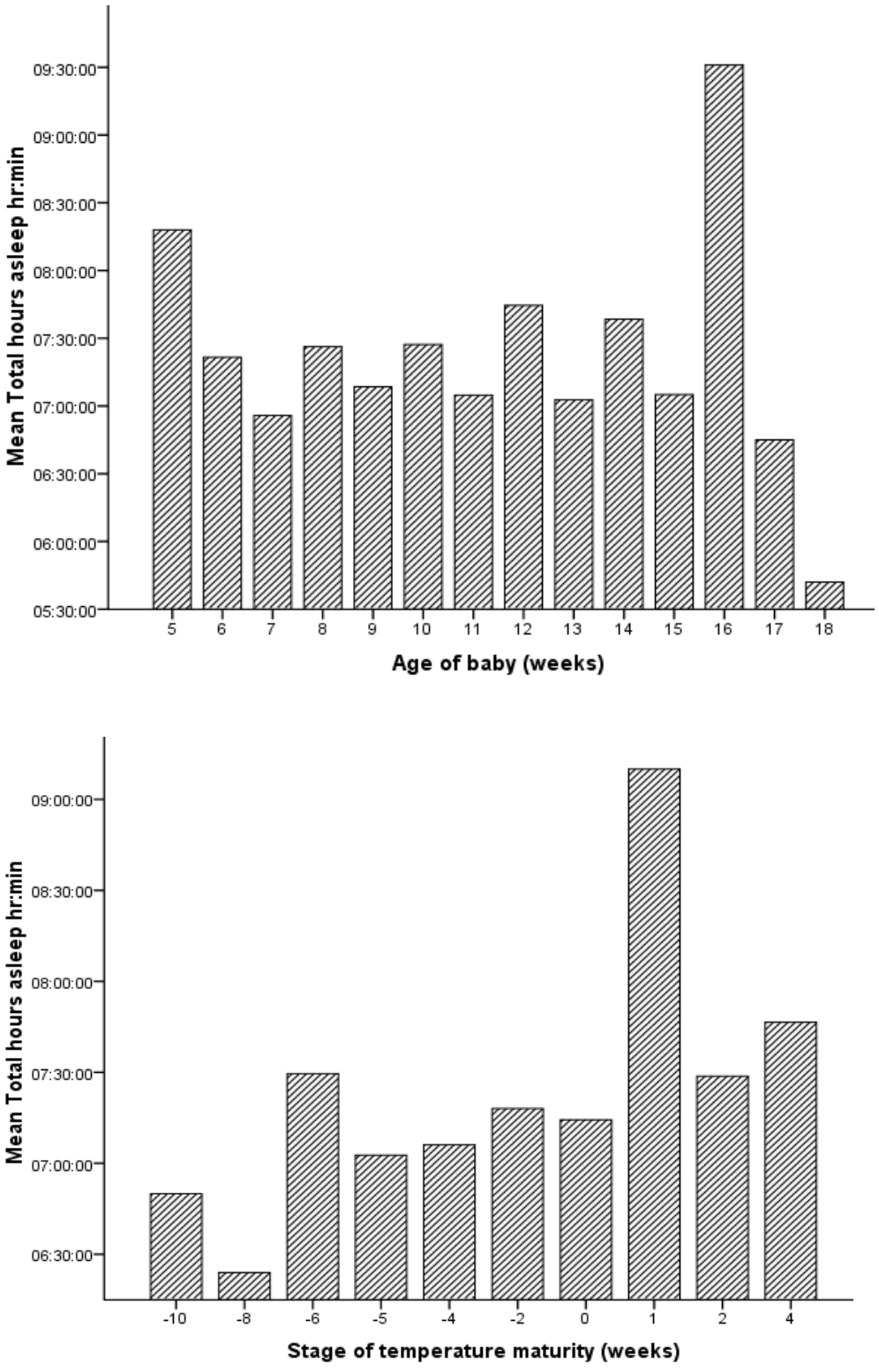
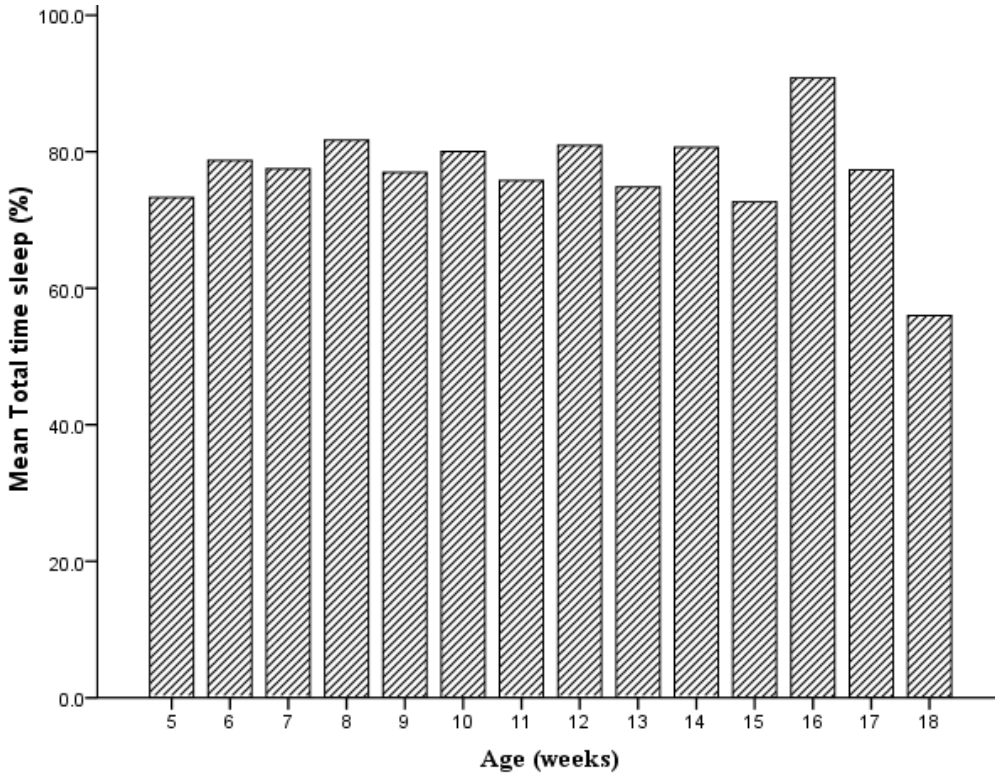


Figure 7.3 - Changes in total sleep duration with age and in the weeks prior and after development of temperature maturity for infant sleep



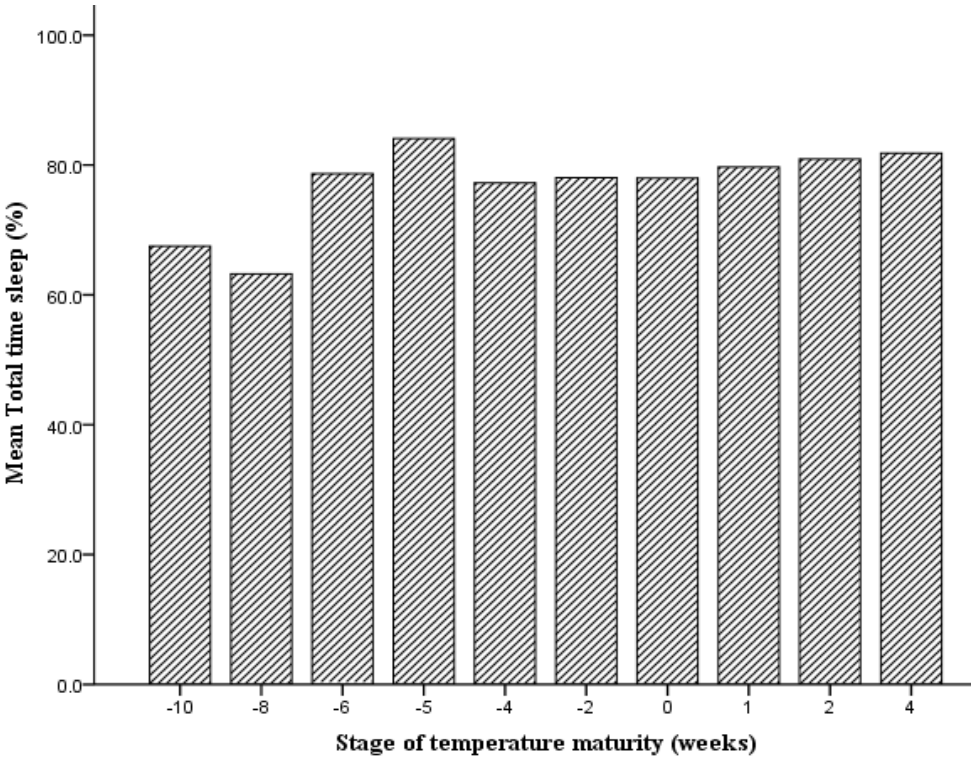


Figure 7.4 - Changes in percentage of infant sleep with age and in the weeks prior and after development of temperature maturity

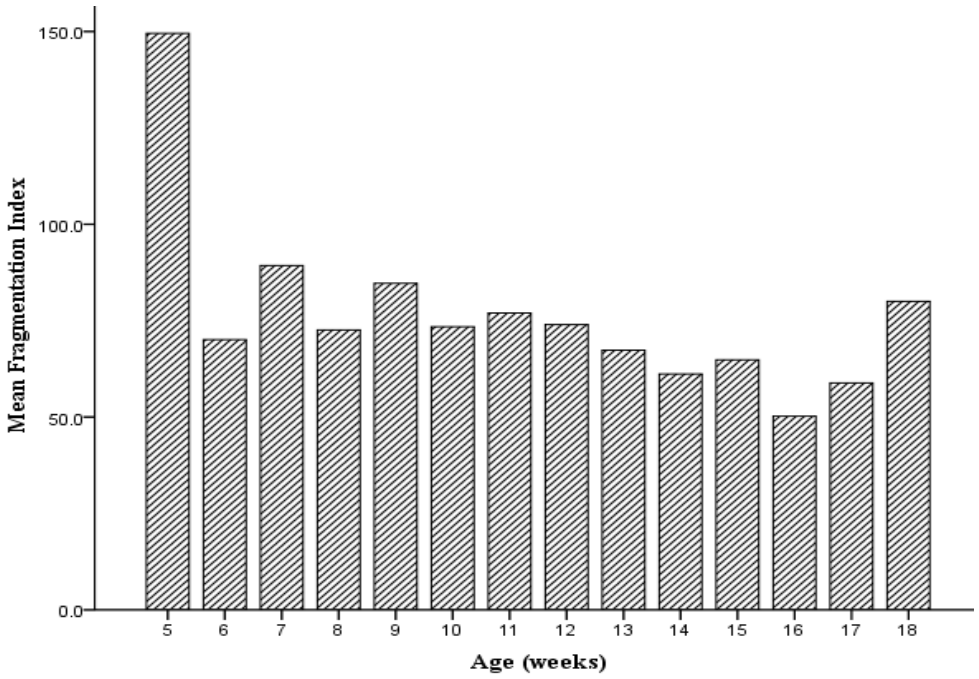
7.3.2 Non care giver dependent infant sleep parameters

Changes were seen in other sleep parameters. These measurements were not influenced or affected by infant care practices dictated by main care giver such as time infant placed in routine place of sleep; lights out. They are infant dependent and may more closely reflect intrinsic developmental change in infant sleep.

Fragmentation index (SD), which relates to a measure of restlessness during night time sleep, dropped significantly from 82.31 (39.78) to 68.24 (29.80); p = 0.021 (CI -26.006 to -2.414), when comparison was made between pre and post temperature rhythm development. A similar fall was seen in the number of minutes moving (SD) during night time sleep (pre-temperature maturity no. minutes moving is 225.952 (130.372); whilst after mature temperature maturity achieved no. minutes moving falls to 181.366 (92.119); p = 0.023; 95% CI -82.838 to -6.333.

The total number of minutes immobile during night time sleep period (SD) increased from 334.81 (133.461) minutes to 381.80 (99.779) minutes; p=0.027 (95% CI 6.998 to 86.998). There was an obvious reduction in the number of immobile phases of 1 minute duration (SD.) It fell from 75.19 (35.74) to 69.90 (21.98) (p=0.027; 95% CI -25.51 to -0.054). A trend was observed for the overall percentage (SD) of immobility phases of 1 minute duration falling from 41.79 (20.74) to 36.24 (14.92) % (p = 0.706 95% CI -11.669 to 0.5825).

As the infants' age increased, the number of immobile phases of 1 minute duration fell proportionally by an average 5.0% per week (P=0.005 95% CI: 1.5% to 8.3%). Summary graphs for these parameters are shown below in figures 7.5 to 7.11.



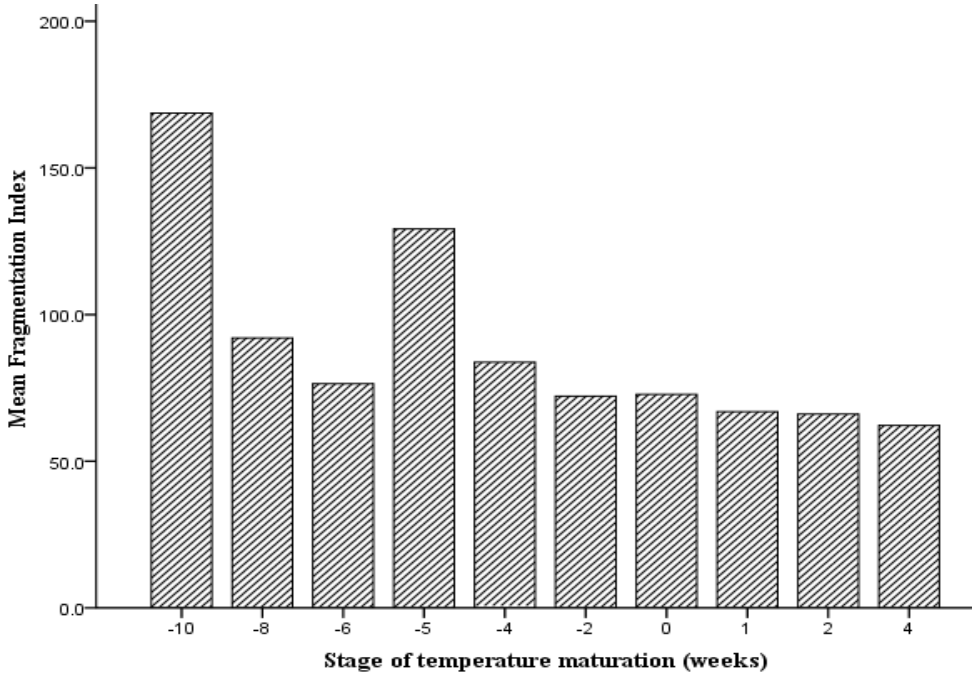


Figure 7.5 - Changes in infant fragmentation index with age and in the weeks prior and after development of temperature maturity

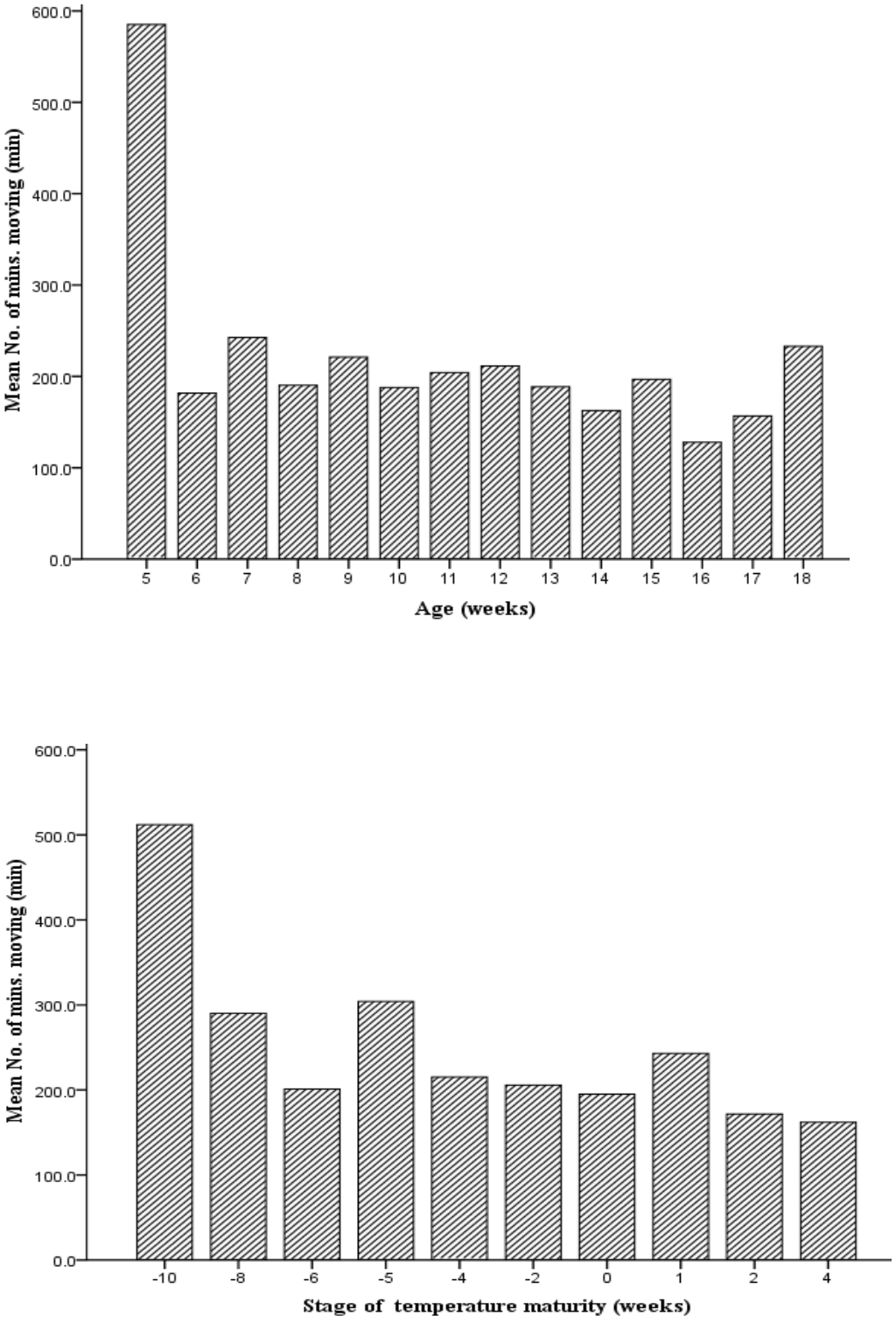


Figure 7.6 - Changes in number of minutes moving during night time sleep for infants with age and in the weeks prior and after development of temperature maturity

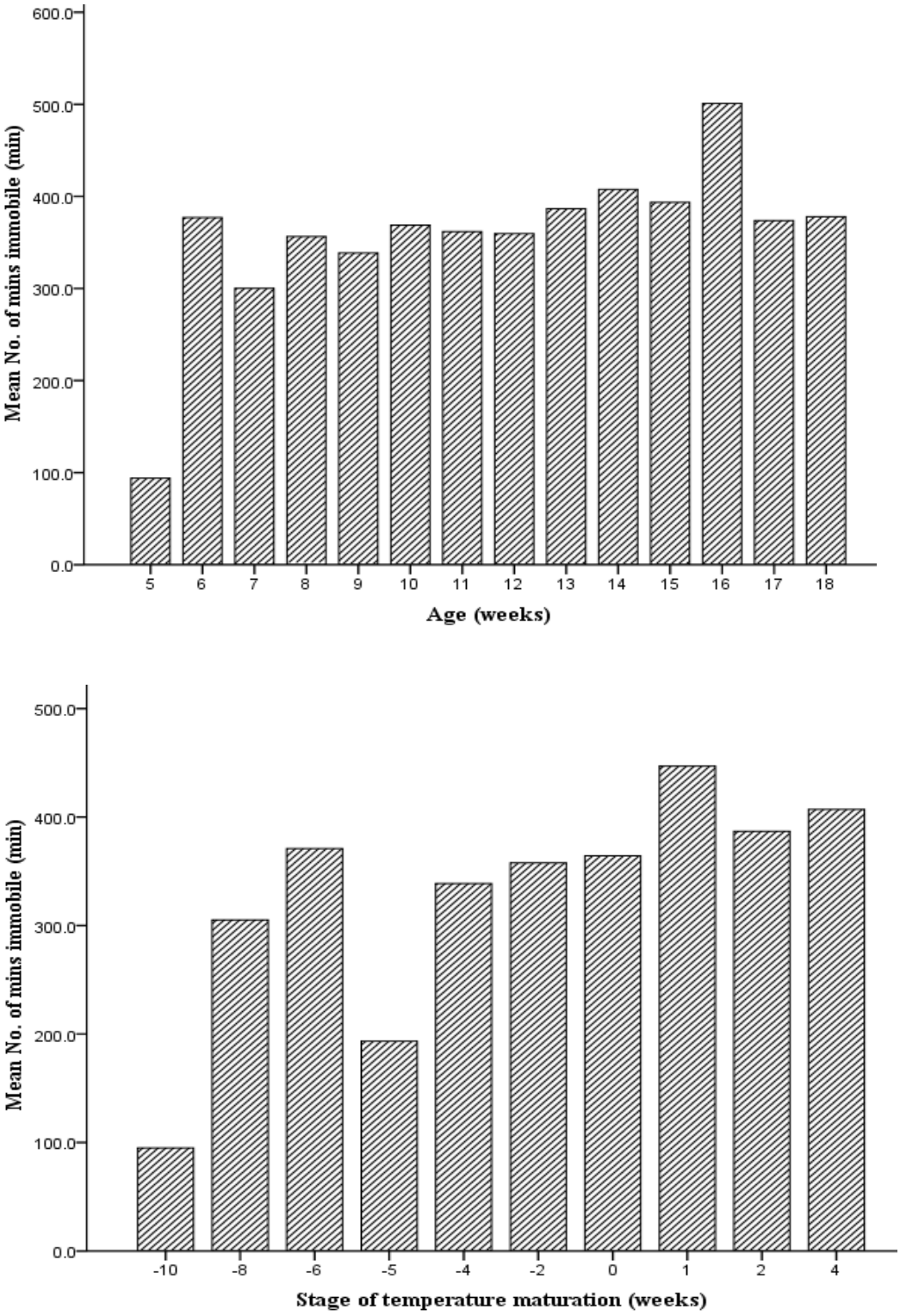


Figure 7.7 - Changes in number of minutes immobile during night time sleep for infants with age and in the weeks prior and after development of temperature maturity

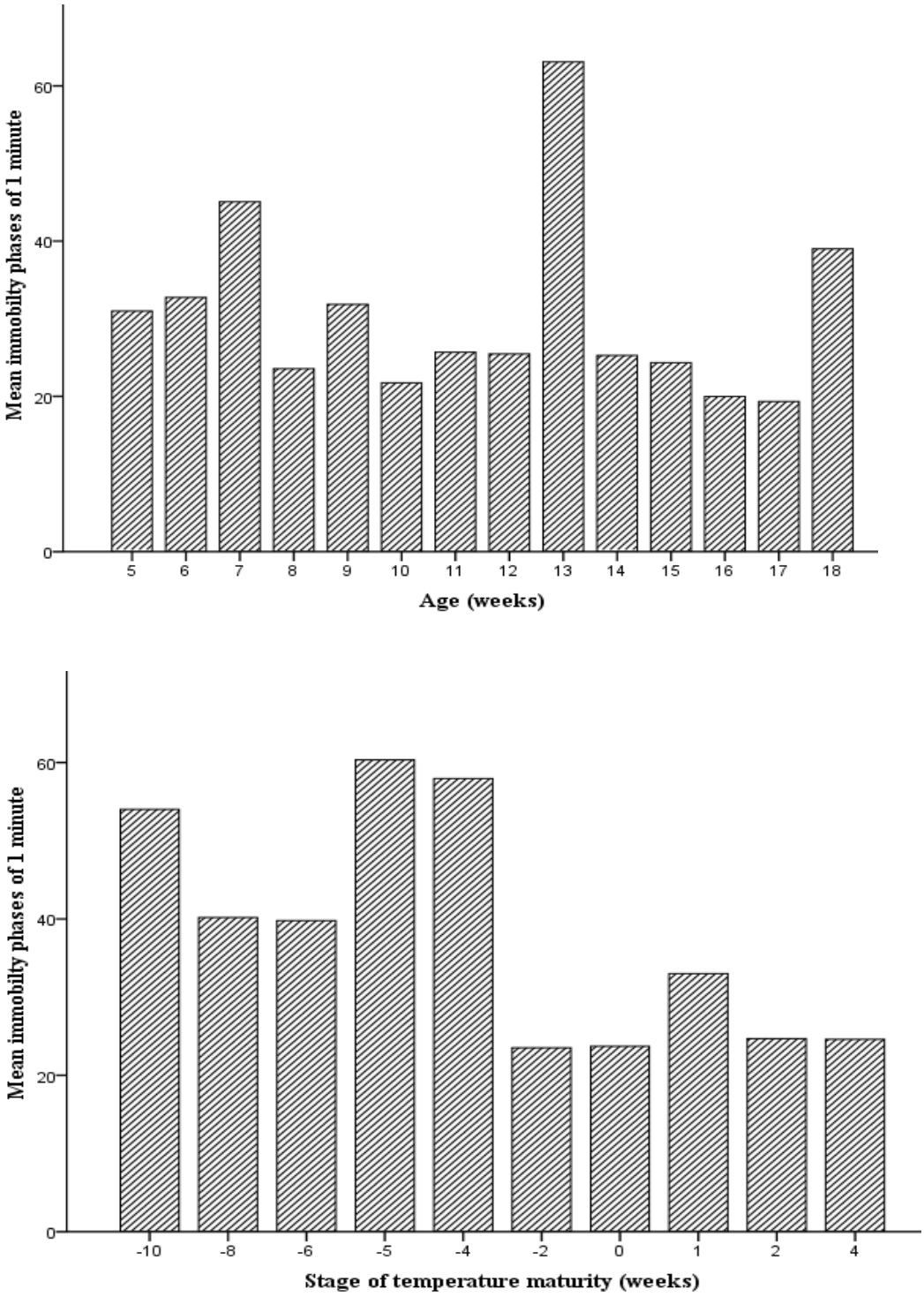
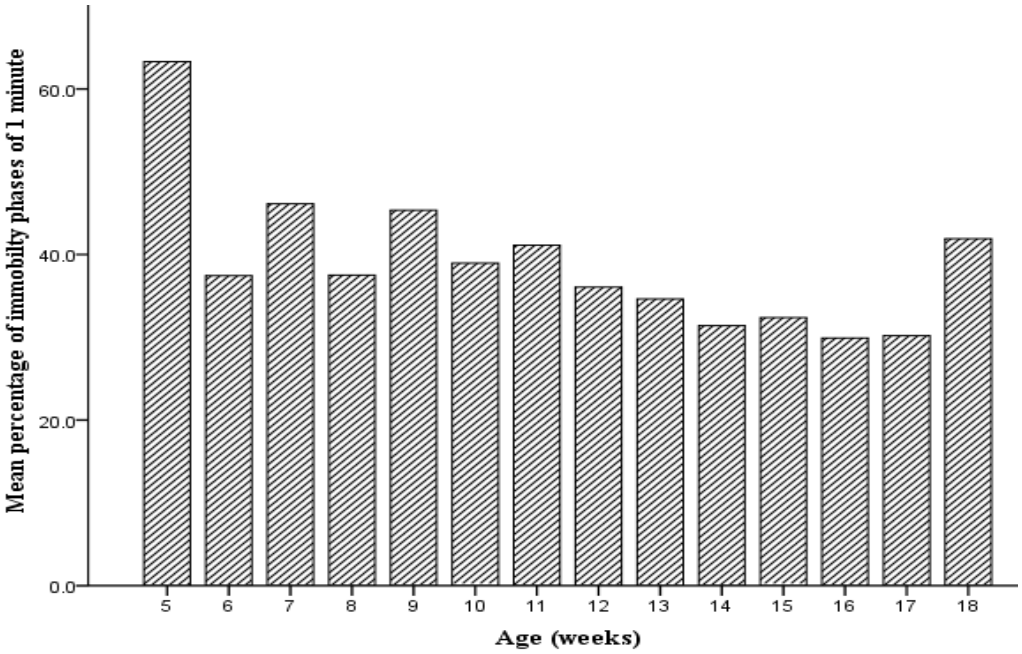


Figure 7.8 - Changes in the number of 1 minute immobile phases during night time sleep for infants with age and in the weeks prior and after development of temperature maturity



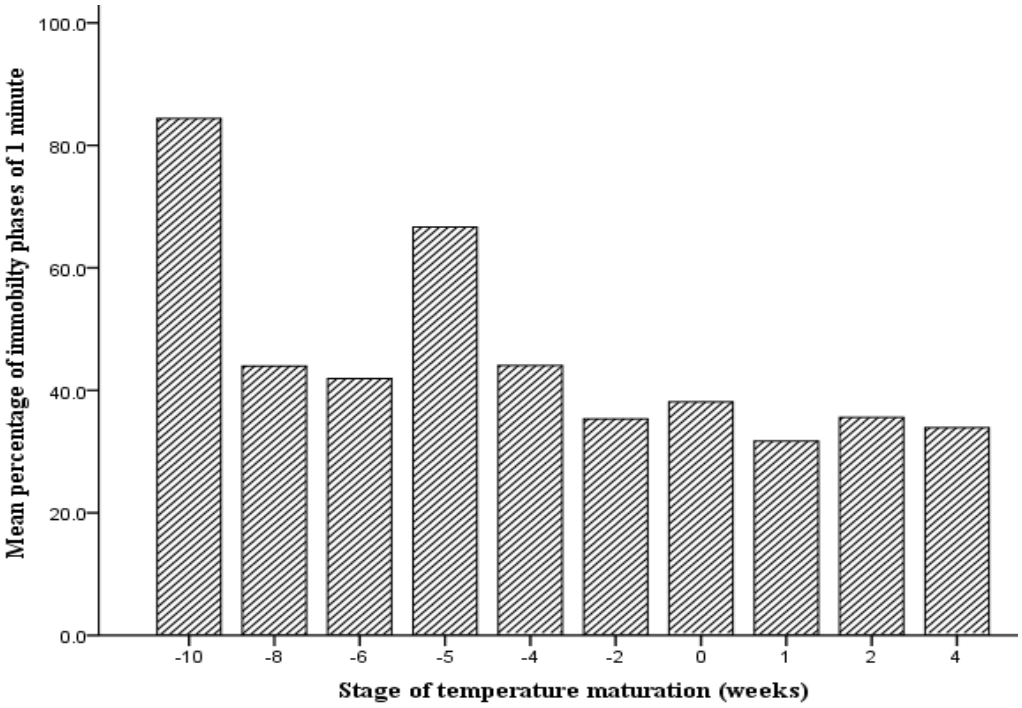


Figure 7.9 - Changes in the percentage of 1 minute immobile phases during night time sleep for infants with age and in the weeks prior and after development of temperature maturity

Overall sleep efficiency is highly correlated with Fragmentation Index (a measure of restlessness) for both infants and mothers (Figure 7.10). In general, the higher the sleep efficiency, the less restless the infant or mother (Pearson coefficient $R = -0.467$ $p<0.001$).

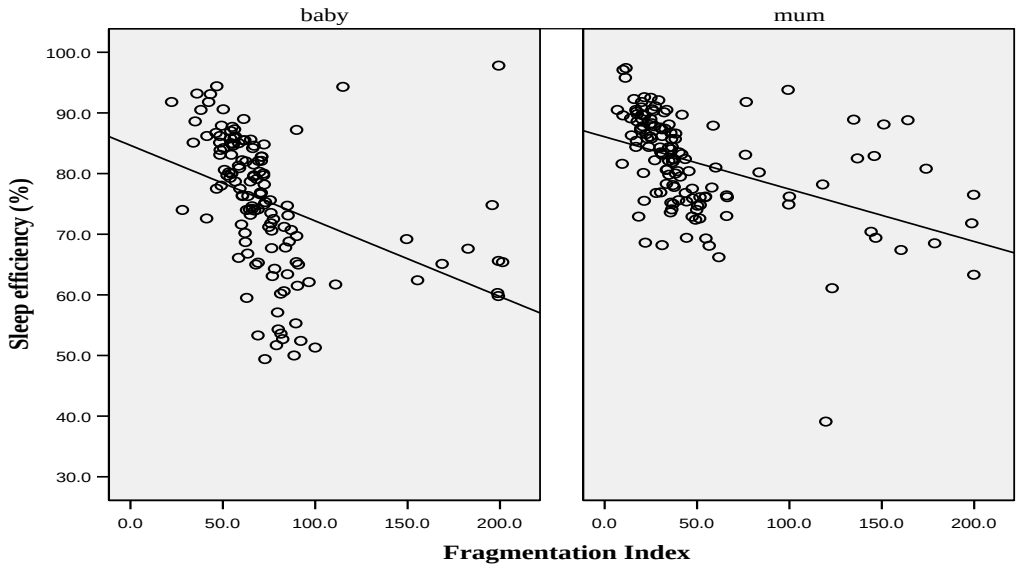


Figure 7.10 - Comparison of correlation between average sleep efficiency and fragmentation index between 35 infants and their mothers.

Summary

1. Infants slept on average 7.18 hours per night. They had an average fragmentation index score of 74.8 and mean sleep efficiency of 75.3%.

2. Sleep efficiency, sleep latency and total sleep duration were unchanged in the infant group before and after the development of a mature overnight core body temperature rhythm (Fig 7.1, Fig 7.2 & Fig 7.3).

3. Fragmentation index and the total number of minutes moving during night time sleep decreased as the infant overnight core body temperature rhythm matured (Fig 7.5 & Fig 7.6).

6. There was an observed increase in the total of minutes immobile during night time sleep as infant temperature maturity was achieved (Fig 7.7).

7. The number and percentage of immobile phases of 1 minute duration fell from pre temperature rhythm maturity to post mature in infant group (Fig 7.8 & Fig 7.9).

8. Infant and maternal sleep efficiency as well as fragmentation index are highly correlated during the night time sleep period.

Chapter 8

Results

8 Results

DESCRIPTION OF THE EXPRESSION OF PERIPHERAL CLOCK GENES IN HUMAN INFANTS

8.1 Analysis of buccal swabs in infants

This chapter describes the results from the initial analysis of peripheral gene data obtained from the buccal swabs collected by the participant mothers from their infants. Six hourly swabs were taken on two consecutive days (over 48 hours) on a weekly basis throughout the period of the study. The samples were taken concurrently with sleep and deep core body temperature measurements as well as collection of urine for melatonin estimation and cortisol, using a standard monitoring schedule. Buccal cell sampling was continued for four weeks after and including the week a mature temperature rhythm was noted in the infant in most cases.

Of the thirty five infants, one family had no access to refrigeration and freezer facilities within the home setting, so no samples were able to be taken and safely stored. One mother opted out of buccal swab collection due to personal preference. In total the remaining thirty three infants had buccal swabs collected for peripheral gene estimation. Of these, one infant had all samples excluded due to illegible labelling. The remaining ten infants had insufficient samples or incomplete data sets and were therefore excluded. Samples for any week of measurement had to include a minimum of 4 consecutive six hourly samples to be included in the analysis.

Twenty two infants had a total of five hundred and ninety nine samples processed, which are reported on in this results section.

Considering only those weeks of measurement where at least four consecutive samples were taken, the 22 infants with any peripheral clock gene marker measured were sampled on average during 4.2 separate weeks, and during each week at an average 6.6 time points.

At the time the study was set up, sampling times for collection of buccal swabs were selected as follows - 18:00 hours, 12:00 midnight, 06:00 hours, 12:00 midday. Mothers kept to these times as closely as possible. However to encourage maximum participation and compliance with the study protocol, mothers were permitted to alter collection times to fit around their family schedule on that day. The aim was to minimise disruption to the household routine. No adjustments were made to infant care practice for light exposure (Lux), routine, meal times or sleep times.

The infants ranged from 5 to 18 weeks of age during the study.

When the initial study protocol was devised, the genes of interest selected for investigation in the study were Rev-er-beta and PER2 which are central circadian clock genes. Due to the problems with RNA yield, and the use of very technically challenging procedures, compounded by the high risk of sample contamination, the original central clock genes of interest Rev and PER2 could not be isolated. Multiple adjustments to protocol and trials of several primers and techniques had to be undertaken before any data were obtained.

H3f3b was selected as an alternative. It is a common cycling gene, found in peripheral tissue, that it was postulated was controlled by circadian clock genes (Storch *et al.*, 2007, Oster *et al.*, 2006). It was chosen as it was hoped that it would be found to reflect gene activity and oscillations in the SCN in human infants. The selection of this gene helped minimise but did not eliminate the difficulties of work of this kind.

H3f3b is a member of the H3 histone gene family. Histone proteins are involved in chromatin structure in the DNA double helix or the assembly of nucleosomes. It has been observed to cycle in mouse liver and kidney tissue using microarray data (Maxson *et al.*, 1983, Mootha *et al.*, 2003). It is known to express in human epithelial cells.

GAPDH is a common easy to identify gene. It varies little in multiple experimental conditions and therefore is used in qPCR experiments quite frequently as a house keeping gene or marker or control (Zhong & Simons, 1999).

All samples were analysed in triplicate and a mean value reported.

8.2 Description of peripheral gene expression in human infants

Peripheral gene expression has not previously been described longitudinally in human infants or an attempt made to relate it to physiological development.

Once experimentation was complete and samples selected for each infant were processed, the graph plots from the triplicate ct values obtained in qPCR reactions were examined.

Initial visual inspection of data and analysis of gene data

The data were first examined and an attempt made to determine whether there was indeed an intuitive cycle of peripheral gene expression that fit any recognisable pattern. On initial inspection of the data it became evident that a proportion of the infants investigated had an observable, recognisable, repeated cycle by visual inspection, of expression of H3f3b gene. This cycle would not be evident on all weeks of investigation during the study. In nearly all cases there would initially, in the samples taken at the earliest age, be no detectable pattern with random ct values which had no regular peaks or troughs. When, for each infant, the series of 24 or 48 hour plots were placed sequentially week by week, a distinguishable and identifiable pattern appeared at a particular week of sampling. The week at which an identifiable pattern seemed to appear was noted for each infant. The investigator analysing the data were blinded and unaware of the postnatal age of the infant or the age of physiological maturation for each infant studied. The sample week was then related to the actual postnatal age of the infant to determine at what age the infant had demonstrable maximum daily variation in peripheral gene expression. A distinct

pattern appeared to emerge with increasing postnatal age and occurred around the time of the development of a mature temperature rhythm. These findings were reconfirmed by a second independent investigator who was again, at the time of analysis, blinded to the physiological data. The cycle did not always persist with all subsequent measurements.

For illustrative purposes, raw data from two infants will be described to demonstrate the initial visual inspection/pilot analysis of peripheral gene data.

8.2.1 Description of peripheral gene data from first case infant

Analysis was initially carried out on infant 16004. This infant was the longest participant in the study. She was recruited at 6 weeks of age and was monitored till 18 weeks of age (12 week duration in study). The buccal swab collection was the most complete and the samples were the most accurately timed and labelled.

The H3f3b:GAPDH ct value at 19:30 hours on day one at 14 weeks of age was 0.9555. This remains at similar level at 24:00 at 0.9561. At 08:30 hours the ratio was 0.9211 and at 12:30 it registered as 0.9306. The second day of readings; for the same week were as follows: 18:00 hours 0.9152; 01:30 hours 0.8924; 06:00hours 0.9303 and at 12:30 hours 0.9120.

At 15 weeks of age the H3f3b:GAP ratio did not follow a particular pattern of expression. At 18:30 hours it was 0.887; at 23:30 hours it was 0.9980; at 06:30 it was 0.9307 and at 14:00 it measured at 1.0146. The second day readings were as follows:

18:30 hours 0.9927; 01:00 ours 1.0154; 09:00 hours it was 0.9936 and 13:30 hours it read 1.0186.

A possible pattern started to emerge at postnatal week 16 for infant 16004. At the beginning of the series of readings for that week the evening reading at 19:30 was 0.9480; a 04:00am reading was 0.9882. The 08:00 am reading was 0.9779 and the 12:00 midday reading 0.9370. On day two 18:00 hours reading was 0.9692; the 2am reading was 0.9513; 07:00 hours it rose to 0.9820 and was 0.9503 at 13:00 hours. The lowest recordings were the evening recordings (18:00 between and 19:00) and at mid day (12:00/13:00). The highest ratios were between 04:00 and 08:00 hours.

A pattern further emerged at week 18 of age. In the evening at 18:00 the ratio was 0.9866. At 2:00 am it rose to 0.9999. At 07:00 it was 0.9979. The reading for the H3f3b/GAPDH ratio fell to 0.8661 at 11:00 hours. On the second consecutive day in week 18 at 18:00 the ratio was 0.9490; at 02:00 hours 0.9957; at 07:30 0.9914 and 0.9622 at 12:00 hours. Again the lowest recordings were the evening and lunch time samples. The peaks occurred early hours of the morning between 02:00 and 08:00 hours.

8.2.2 Description of peripheral gene data from second case infant

Infant 16003 had the second most complete data set for peripheral gene expression. The longitudinally weekly measurements are summarised below (see table 8.1).

Table 8.1 - H3f3b/GAPDH ratio clock gene values for infant 16003 taken on weeks 8, 10 -15; six hourly samples taken over 48 hours per week of measurement, where available.

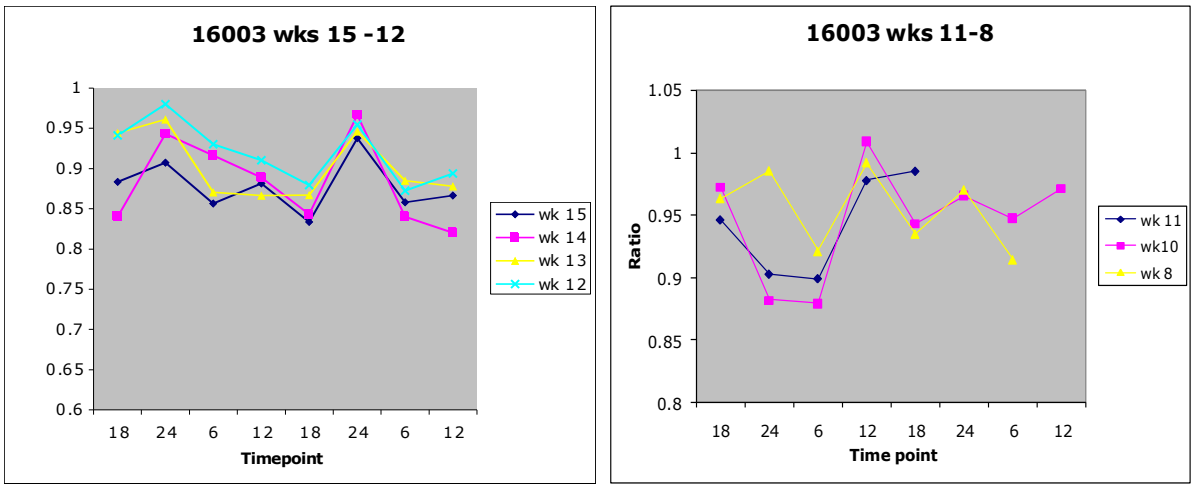
	Day 1				Day 2			
Age	18:00 h	24:00 h	06:00 h	12:00 h	18:00 h	24:00 h	06:00 h	12:00 h
8 weeks	0.9632	0.9852	0.9210	0.9918	0.9348	0.9700	0.9144	*
10 weeks	0.9721	0.8819	0.8792	1.009	0.9431	0.9653	0.9473	0.9711
11 weeks	0.9460	0.9024	0.8987	0.9775	0.9851	*	*	*
12 weeks	0.9413	0.9802	0.9304	0.9104	0.8793	0.9545	0.8772	0.8937
13 weeks	0.9441	0.9611	0.8703	0.8666	0.8671	0.9461	0.8868	0.8794
14 weeks†	0.9641	*	0.9360	0.9293	0.9482	0.9636	0.9424	0.889
15 weeks	0.8336	0.9074	0.8567	0.8815	0.8334	0.9380	0.8582	*

**Missing data*

† Altered time schedule for samples 20:00hrs; 08:00hrs; 12:00hrs; 15:00hrs; 20:00hrs; 08:00hrs; 12:00hrs at 14 weeks age

This infant appeared to have two distinct cycles of peripheral gene expression (figure 8.2). The patterns differed according to whether the samples were taken in the weeks before of after temperature maturation. The mean of the triplicate ct values for each week were initially plotted separately. The plots were then superimposed. The plots drawn for the weeks before temperature maturation had occurred (weeks 8 to 11) showed possible high ct values early afternoon or mid evening with lows at 6 am.

The weeks after temperature maturation occurred the phase shifted with highest values recorded at midnight with trough at 6pm. This cycle appeared more robust. It should be noted that all ‘gene’ analysis was carried out by an observer who was blinded to the results of the temperature maturity in infants.



**** No adjustments were made to infant care practice for light exposure (Lux), routine, meal or sleep times.***

Figure 8.1 - The 48 hours H3f3b/GAP ratio gene profile of infant 16003 taken on seven separate weeks grouped into post temperature maturation weeks (weeks 12 – 15) and pre maturation weeks (weeks 8 – 11).

Further peripheral gene graphical representation

Following the initial visual inspection, the peripheral circadian gene data were analysed in two further stages:

i) Fitting a diurnal pattern to the observed data, separately for each week at which each infant was sampled, and calculating the amplitude as a measure of the strength of the diurnal relationship.

ii) Modelling the amplitude of the diurnal pattern of peripheral gene expression against the infant's age (in weeks), in order to assess whether there is a 'natural' change-point that might be interpreted as the age of 'gene expression' maturity and also, more generally, to examine changes in amplitude with age.

More detailed data analysis was done by fitting the data points for each infant to a sinusoidal curve. The diurnal pattern chosen to represent gene expression was the sine-wave (or cosine wave) with a pre defined period of 24 hours (Bjarnason *et al.*, 2001). This is a routine manner used for reporting clock gene expression.

The diurnal sine-wave pattern that was fitted - separately for each infant - contained three parameters, to allow for i) the mean value of expression, ii) the amplitude of the diurnal variation, and iii) the phase of the diurnal variation, corresponding to the time of day at which the sinusoidal pattern estimated the maximum level of expression to occur. The estimated amplitude for any infant at any given week was taken as the primary measure of 'goodness of fit' of the data to a sinusoidal pattern as it represents estimated strength of the diurnal relationship in natural units of gene expression. The secondary measure of goodness of fit used in this study is the 'R squared' statistic conventionally used with linear regression, which may be interpreted as the

proportion of the total variance of the observed data that is 'explained' by the fitting pattern. For the 93 infant weeks that were sampled the mean amplitude was 0.0288 with maximum and minimum values 0.0035 and 0.1092 respectively.

The resultant graphs for infants 16003 and 16004 are shown for direct comparison (Fig 8.3.). Data on all infants is included in appendix 4. Graphs were drawn using R statistical package （R version 2.7.2 2008 The R Foundation for Statistical Computing).

For clarity a description of the symbols used in the gene plots and a key are given below:

Separate graphs have been prepared for each observed week of each infant showing the level of expression plotted against time, with two curves superimposed: i) the fitted sinusoidal curve for that week's data, as an unbroken line and ii) the fitted sinusoidal curve from the week (for the same infant) that has the best fit, corresponding to the greatest amplitude of the pattern, as a broken line. In other words, the week with greatest amplitude of gene expression was superimposed (represented by broken line) onto each week of individual measurement (represented by unbroken line) for ease of comparison. For each infant, therefore, the plot for that week with the best fit does not have a second curve superimposed. Each of these graphs is annotated with the amplitude, hour of maximum fitted level, the R-squared statistic, and the P-value from that individual linear regression

Raw data points are represented by open circles. The best fit curves for the raw data are represented by an unbroken continuous line. The curve for the week with the best sinusoidal curve for all weeks of measurement is represented using a broken line on these weeks. The week of best fit for gene expression is shown the week the open circles and sine wave curve have the closest fit. On this week there is a solitary is continuous unbroken line representing line of best fit.

Sine wave curves had the following standardised values calculated.

$$Amp = \frac{\text{distance between maximum and minimum point of sine wave}}{2}$$

$$Rsq = \text{measure of goodness of best fit } \left(R^2\right)$$

Peak hour gives the clock time that maximum amplitude for gene expression was achieved.

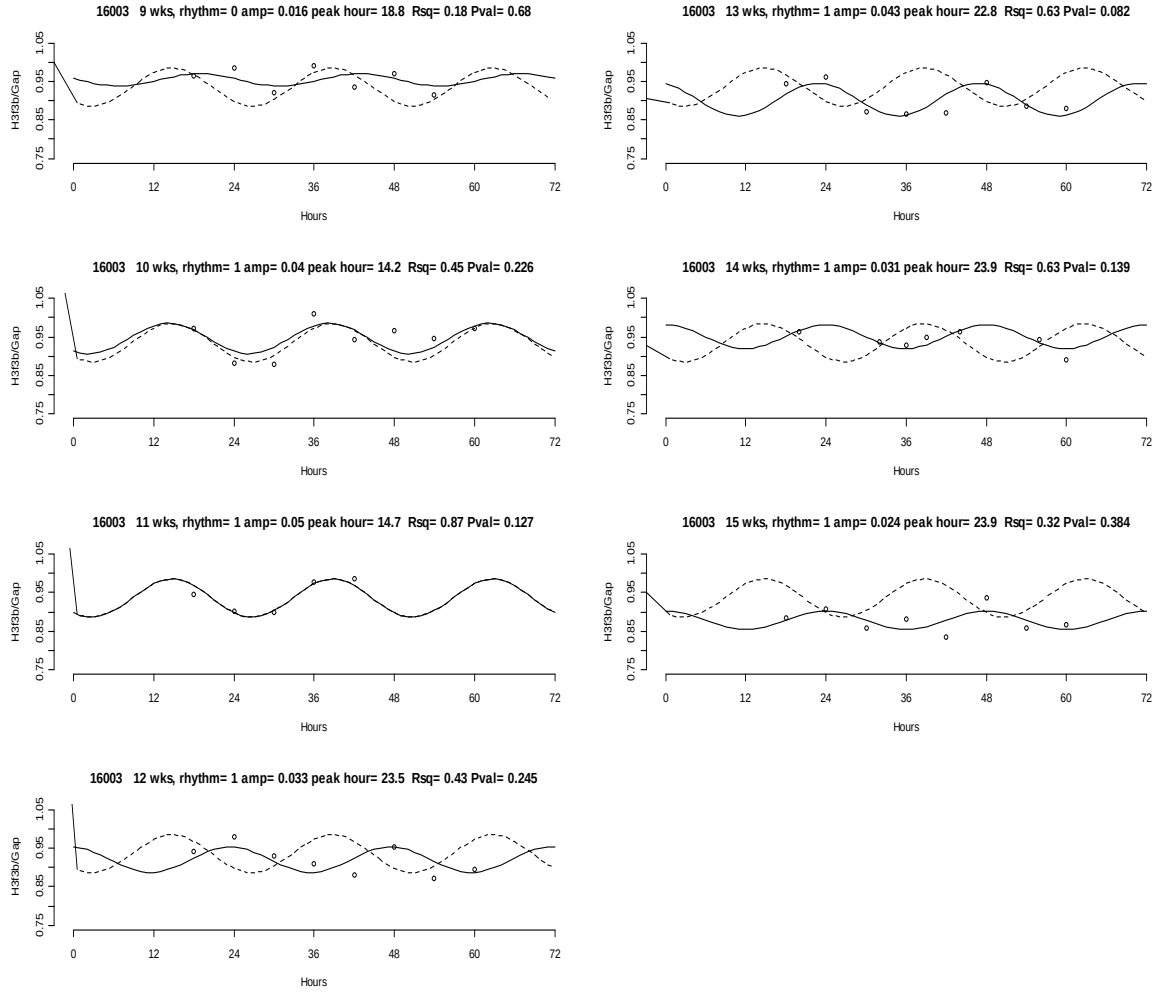
The week of best fit had the highest values for amplitude (Amp) and R^2.

For infant 16003 the week of best fit for clock gene expression is week 11 (week of highest amplitude and with corresponding R^2 values).

For infant 16004 this occurs at week 18.

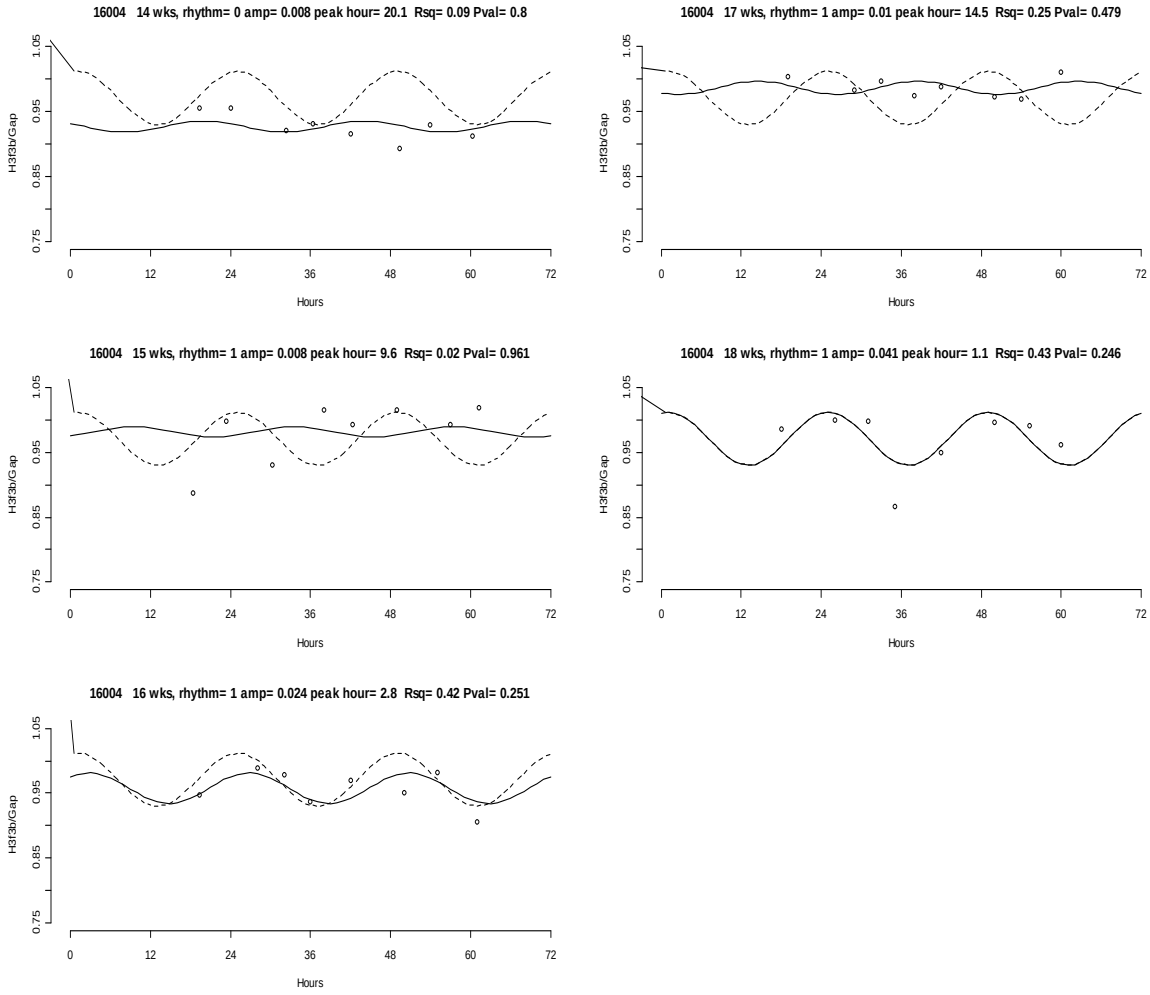
H3f3b appears to cycle at some weeks of measurements for most infants (see appendix 4). The cycle was not present in all weeks. On visual inspection for a percentage of the infants, the initial weeks had no cycle and the depth of amplitude

deepened with subsequent measurements. This may in some way be linked to aspects of infant physiology. Inevitably, the cycle did not always persist.



Legend indicates infant i.d., postnatal age in weeks, amp which is amplitude of clock gene expression, peak hour which is time of highest amplitude over 24 hour period, Rsq which is R squared a measure of goodness of fit to sinusoidal curve.

Figure 8.2 - H3f3b/GAP peripheral gene profile for infants 16003 showing lines of best fit to sinusoidal curve.



Legend indicates infant i.d., postnatal age in weeks, amp which is amplitude of clock gene expression, peak hour which is time of highest amplitude over 24 hour period, Rsq which is R squared a measure of goodness of fit to sinusoidal curve.

Figure 8.3 - H3f3b/GAP peripheral gene profile for infants 16004 showing lines of best fit to sinusoidal curve.

8.3 Detailed gene expression analysis

Summary of amplitude and R squared values for gene expression in human infants

A linear modelling analysis assuming a single diurnal pattern for all infants at all weeks measured, but with a different mean level of expression for each infant, showed a highly significant diurnal rhythm with 24-hour period ($p<0.001$), with an overall estimated amplitude of 0.0126 (on the same scale as the H3f3b/GAP levels) and an overall estimated time of day of maximum expression at 21:39 hrs. The variation in the mean level of expression between the infants was also highly significant ($p<0.001$).

Including the ages of the infants at each week did not significantly improve the model ($p=0.089$), but there was a non-significant trend of increasing H3f3b/GAP expression of 0.0041 with each extra week of age.

The data were examined to look for overall trends in peripheral gene expression for all 22 infants as a group rather than individually. This was done by random effects modelling. Amplitude of gene expression was used together with the corresponding R squared parameter as a measure of goodness of fit to sinusoidal curve. There were no significant trends with age found.

Gene amplitude had no overall average trend with age ($p=0.867$). There was an average weekly increase of -0.002 per week, in the same units as circadian gene expression is assessed (95% CI: 0.002 decrease to 0.002 increase). Circadian gene 'R squared' also has a non-significant average trend with age

(P=0.389), the average weekly decrease was 0.015 per week, where the scale goes from 1=complete linear fit to 0=appears random (95% CI: 0.050 decrease to 0.020 increase).

Distribution of gene amplitude

On average for all infants, the timing of the highest peak in amplitude of clock gene expression was between 20:00 and 24:00 (midnight) at 21:30 hours.

Summary

1. H3f3b was selected as gene of interest (peripheral cycling gene) with GAPDH as housekeeping gene.

2. The initial circadian clock genes of interest (Rev and PER2) were not used due to RNA yield.

3. H3f3b was shown to have a diurnal pattern of cycling in human epithelial cells with peak values during the evening period.

4. Mean gene expression varied from infant to infant.

5. There is a suggestion that the pattern of peripheral gene expression changes with postnatal age.

Chapter 9

Results

9 Results

FACTORS AFFECTING SLEEP AND PHYSIOLOGICAL MATURATION IN HUMAN INFANTS

9.1 Factors affecting physiological maturation in human infants

There are several factors which have been identified by previous studies to influence and delay the achievement of physiological development in infants. Overnight core body temperature was used as a marker of maturity (Lodemore *et al.*, 1991). Bottle feeding, exposure to passive smoking, poor weight gain, were shown to delay maturation of temperature control whilst supine sleep position, an increase in infant age and birth weight advanced it (Petersen *et al.*, 1991, Lodemore *et al.*, 1992, Tuffnell *et al.*, 1995). Ethnicity, changes in infant care practices (Petersen & Wailoo, 1994), socioeconomic circumstances (Wailoo *et al.*, 2003), and exposure to an infection (Rawson *et al.*, 1990) have also been shown to have a deleterious effect. Many of these factors overlap with known risk factors for SIDS.

A selection of the above described factors, were examined for their effect on the different physiological measurements that were investigated in this study (i.e. sleep, melatonin and cortisol secretion and night time deep core body temperature) in this group of participant infants. For all of the four measurements included in this section, factors directly related to the infant, as well as those related to certain aspects of child care practice and the socioeconomic status of the family were examined in univariable linear models in which they were treated as separate predictor variables.

Additional factors that were thought to be of interest were analysed for their affect on the pattern of infant sleep and development of night time melatonin secretion only.

9.1.1 Factors affecting infant night time sleep

In practice, infant sleep depends on age, environment and childcare practices. Maternal influences are significant because of proximity and mother's ability to alter the baby's environment. Her timetable and routine care practices have a profound effect on infant aspects of infant sleep. Biorhythms, absent at birth in babies, develop at varying rates postnatally. In this study the hypothesis that internal infant biorhythms may have a contributory effect on the development of night time sleep was examined.

Several factors were investigated to ascertain their influence on aspects of infant sleep in particular sleep efficiency. Factors intrinsic and extrinsic to the baby were examined for their effect on infant sleep. The factors fell into several main categories - those related to:

Sleep environment factors

- Infant sleeping bag use
- Room of infant sleep (own room vs. parental room)
- Type of bed (Moses basket vs. cot)
- Total tog (amount of wrapping/bedding)
- Exposure to tobacco smoke

Care and infant related factors

- Gender
- Feeding method
- Infant weight
- Illness

Maternal factors

- Edinburgh postnatal depression score
- Sleep score & type (Auto MEQ)
- Maternal age

Social factors

- Life events score
- Social class
- History of SIDS

Physiological factor

- age baby achieved mature temperature biorhythm

The infant sleep efficiency was advanced by the age of achievement of a mature rhythm ($p=0.004$). Also infants had increased sleep efficiency if they slept in the same room as their care giver ($p=0.015$). Sleep efficiency at the week of temperature maturity only was included in the model.

Table 9.1 - Univariate linear model for infant sleep efficiency with place of sleep and age of physiological maturity as predictor values.

Dependent variable: infant sleep efficiency (%)

Covariate	β/coefficient	95 % Confidence Interval	*p* value
Room where baby slept	-9.00	-16 to -1.8	0.015
Age rhythm achieved	-1.57	-2.618 to-0.529	0.004

No other factors examined were able to predict a change in infant sleep efficiency.

9.1.2 Factors affecting overnight deep body temperature in infants

Having a young mother was found to delay the age at which temperature maturity occurred in the infant (Table 9.2). For every year increase in maternal age there is a 0.15 week increase in age of maturity for achievement of temperature maturity.

Table 9.2 - Effect of maternal age on age of achievement of physiological development of mature infant core body temperature

Dependent variable: Age at which temperature maturity achieved (weeks)

Covariate	B*/coefficient	95 % Confidence Interval	*p* value
Maternal age, years	0.15	0.015 to 0.290	0.03

**β represents slope of regression line measured in weeks of maturity/maternal age (years)*

In addition, living in social housing, which may be used as a proxy for low social status (social versus private housing), caused a marked delay in temperature maturity (Table 7.2). There is a minimum difference in age of maturity between the infants in social housing and private accommodation of 3 weeks.

Table 9.3 - Effect of family occupancy in social housing on age of achievement of physiological maturity of infant temperature

Dependent variable: Age at which temperature maturity achieved (weeks)

Covariate	β/coefficient	95 % Confidence Interval	*p* value
Social housing	3.04	0.230 to 5.851	0.035

Other factors previously found to cause a delay in maturation of overnight core body temperature in larger studies did show any affect in this study (low socioeconomic class, male gender, bottle feeding, exposure to passive smoking, first birth order, low birth weight) using linear regression modelling with significance level $p>0.05$. This may be due to sample size.

9.1.3 Factors affecting the secretion of night time melatonin

Night time melatonin increased with postnatal age in the infants studied. The data were converted to a logarithm transformed to obtain approximately normal distribution.

For the purpose of this section, in order to examine the factors affecting the secretion of melatonin, the data were confined to night time urine samples obtained after temperature maturation had occurred for each infant.

The factors were divided into those which were directly related to each infant, such as gender and birth weight and factors which varied for each infant during night sleep such as place of sleep, time of sleep onset.

Description of constant factors on night time melatonin secretion

Singly, several factors which were examined for their effect on night time melatonin secretion showed no independent effect. They included

- Gender
- Birth order
- Weight
- Social class
- Maternal age
- Feeding method

There was an observed trend for an episode of illness during the week of melatonin estimation to decrease melatonin secretion (Table 9.4).

Night time melatonin values when well have a 2.3 ratio increase than when unwell. Being from the poorest social class also had a deleterious effect on melatonin (Table 9.5).

Table 9.4 - Effect of infant episode of illness in postnatal period on night time melatonin secretion

Dependent variable: Night time melatonin secretion after temperature maturity achieved (weeks)

Covariate	**Ratio of increase**	**95 % Confidence Interval**	***p* value**
well vs. illness episode	2.30	0.98 to 5.41	0.055

Table 9.5 - Effect of socioeconomic status of family on night time melatonin secretion of infants

Dependent variable: Night time melatonin secretion after temperature maturity achieved

Covariate	**Ratio of increase***	**95 % Confidence Interval**	***p* value**
Socioeconomic categories			
Class 1	0.691	0.266 to 1.799	
Class 2	0.726	0.294 to 1.0791	0.432
Class 3†			0.470
Class 4	5.188	0.942 to 28.183	
Class 5†			0.058
Class 6	0.216	0.078 to 0.601	
Class 7*			0.005

*Class 7 used as reference group
†No cases fall into this category
NB ratio increase factor < 1 is actually represents a decrease

Description of variable factors on night time melatonin secretion

The following describes the factors which varied according to changes in the infant care routine for that night or linked to the sleep environment of the infant analysed for their effect on night time melatonin secretion.

For factors which changed throughout the period of the study as the infant got older; such as use of sleeping bag or room/place of night time sleep, the mode category was selected (most common for that infant during study period). For example, an infant placed in its own room for 3 out of 4 nights of study was coded as having slept most commonly in its own room as opposed to room sharing with his parents.

Factors related to infant care practice which varied with night of sleep:

- Routine use of infant sleeping bag
- Routine room for night time sleep (Own room/parents room/other)
- Routine bed/sleeping surface (Moses basket/Cot/Cosleeping)

Factors directly related to infant sleep/ sleep parameters

- Time of sleep start
- Sleep latency
- Sleep efficiency
- Fragmentation index
- Activity score (during nigh time sleep)
- Percentage total sleep

The factors related to sleep environment (use of infant sleeping bag, room and routine sleeping surface/bed) had no independent on night time melatonin secretion.

A trend was observed that infants put whose sleep period stared earlier had higher night time melatonin levels. Infants who had shorter sleep latency also had a tendency to secrete higher melatonin (Table 9.6). This was non-significant. There were no trends observed for other sleep parameters investigated (see list on previous page).

Table 9.6 - Effect of measured infant sleep parameters on night time melatonin secretion

Dependent variable: Night time melatonin secretion in ng. mg. ml^{-1}after temperature maturity achieved

Covariate	Ratio of increase	95 % Confidence Interval	*p* value
Sleep onset (mins)	0.173	0.024 to 1.276	0.083
Sleep latency (mins)	1.35×10^{-18}	0.266 to 1.799	0.093

Seasonality

Night time melatonin secretion varied as follows with season melatonin estimation was taken:

	Log night time melatonin/Cr ratio (ng. mg. ml^{-1}) (antilog)
Spring	1.19 (15.49)
Summer	1.21 (16.22)
Autumn	1.38 (23.98)
Winter	1.05 (11.22)

For the combined seasons there was no difference between night time melatonin secretion in spring and summer when compared with autumn and winter values for this group:

The spring/summer combined (SD) mean is 15.96 (2.578) ng. mg. ml^{-1} . The autumn/winter combined (SD) mean is 18.11(2.648) ng/ mg/ ml^{-1} ($p>0.05$ 95% CI -0.386 to 0.275 ng. mg. ml^{-1}).

9.1.4 Factors affecting cortisol secretion in infants

The same factors related to the infant, care practices and socioeconomic circumstances examined for melatonin were examined for their effect on morning cortisol secretion. They were treated as separate predictor variables in univariable analysis.

No factors examined were found to have an effect on morning cortisol excretion.

Summary:

1. A mature physiological overnight core body temperature increased infant sleep.

2. Room sharing with carer increased infant sleep efficiency.

3. Sleep efficiency was unchanged within different social classes represented in the study.

4. A trend was observed that having an episode of illness and being from a low social economic class had a deleterious effect on night time melatonin secretion.

5. There was a tendency for infants who had earlier sleep times and shorter sleep latency to have higher night time melatonin secretion.

6. In this cohort having a young mother and living in council housing had deleterious effect on the development of mature temperature rhythm.

7. No factors were predictors of changes in morning cortisol.

Chapter 10

Results

10 Results

10.1 Circadian rhythms – investigation of temporal relationships

In this study several parameters were examined concurrently and longitudinal measurements made, from just before 6 to 18 weeks of age, in the cohort of human infants enrolled. The length of time each infant remained in the study was determined by the age each infant achieved physiological maturity characterised by the maximum fall in overnight core body temperature (Lodemore *et al.*, 1991, Wailoo *et al.*, 1989). Once physiological maturity had been demonstrated, this was confirmed by a further core body temperature reading (taken a fortnight later) if possible. The study protocol for urine collection (for melatonin/cortisol estimation), sleep characterisation by actigraphy and buccal cell collection (for pattern of peripheral clock gene expression) continued till the study end point.

Definitions

The previously described definition of physiological maturation of deep overnight body temperature with sleep has been well described (Lodemore, 1991).

Definitions encompassing age related changes in the patterns of the other parameters (melatonin secretion and cortisol secretion, infant night time sleep and peripheral clock gene expression) were developed during the investigation period for this study.

These definitions do not include an exact age of maturation of a robust circadian rhythm in each area (other than for temperature maturation). This study was limited

due to small sample size and limits on data collection within the home setting. For tighter definitions for age of circadian maturity in melatonin secretion, cortisol secretion, maturity of infant sleep and age circadian gene expression matures occurs, more detailed, larger home based studies plus research performed under lab conditions need to be conducted.

Core body temperature physiological maturity is said to be achieved when an infant is able to drop core body temperature to a minimum of 36.4 C during the first four hours of night time sleep (Lodemore *et al.*, 1991).

The beginning of an emergence of a diurnal rhythm of melatonin excretion may be illustrated by demonstration of a day night difference in melatonin secretion for each infant. This was defined when more than fifty percent of total melatonin was secreted during the night time period, or when night time secretion exceeded day time levels (Sivan *et al.*, 2001). The initial appearance of diurnal rhythmicity was defined when the first peak in night time melatonin occurred.

Cortisol data were analysed in a similar fashion. The timing of a diurnal pattern of cortisol secretion was defined as the week the morning cortisol secretion was more than fifty percent of the total cortisol production. This characterised the first cortisol peak and the first suggestion appearance of diurnal rhythmicity.

The pattern of an age related change in peripheral gene expression was characterised by examining values of amplitude and R squared. The age when there is a combined rise in amplitude of gene expression to above 0.05 and goodness of fit to sine wave

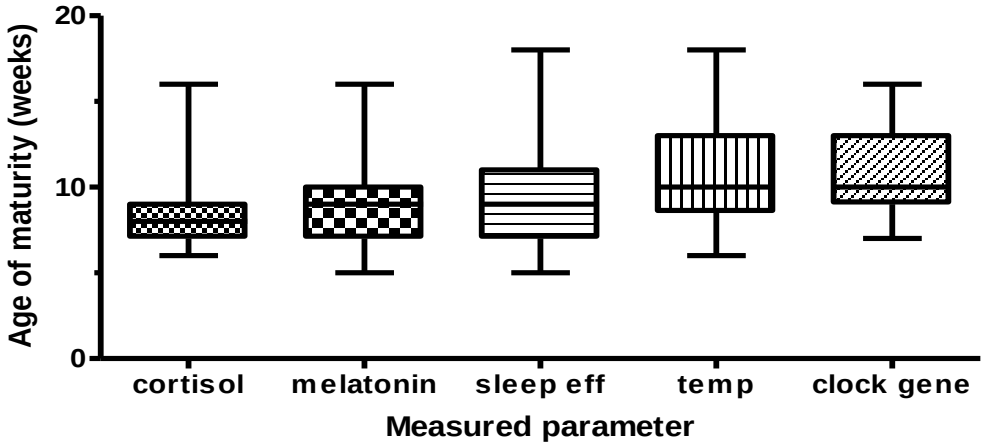
value of above 0.5 for R squared was recorded as the week a mature, robust clock gene pattern appeared.

Sleep was investigated in two ways. The initial analysis involved reporting the week the maximum sleep efficiency of the infant was achieved.

Further analysis replaced week of maximum sleep efficiency with the week the infant had the maximum number of immobile phases of 1 minute and the maximum percentage of 1 minute immobile minutes. This was felt to be more sensitive and reflect more closely intrinsic infant sleep rather than sleep efficiency in which the calculation included external factors such as the time infants are placed to bed by the care givers.

Using mean values, the timing of emergence of first maximum surge in morning cortisol secretion occurred first at 8.17 weeks of age ($p=0.0015$). Night time melatonin had first appearance of diurnal variation and at 9.10 weeks. Maximum infant sleep efficiency occurred simultaneously at 9.40 weeks of age ($p=0.9585$). Temperature maturation occurred next at 10.79 weeks ($p=0.0156$). Timing of maximum amplitude of peripheral clock gene expression and timing of week of best fit to sinusoidal curve wave form was at 10.86 weeks.

These temporal relationships are illustrated in Figure 10.1.



**Cortisol vs melatonin p = 0.0015

Figure 10.1 - Temporal relationship of parameters measured in 35 human infants from 5 to 18 weeks of age. Mean values for age of maturation of circadian rhythm in overnight temperature control during sleep, evening surge in melatonin secretion, morning surge in cortisol secretion, timing of maximum sleep efficiency and maximum peripheral clock gene expression (by amplitude and R2 goodness of fit).

Table 10.1 - Temporal relationship of parameters measured in 35 human infants from 5 to 18 weeks of age. Mean values for age of maturation of circadian rhythm in overnight temperature control during sleep, evening peak in melatonin secretion, morning peak in cortisol secretion, timing of maximum sleep efficiency and maximum peripheral clock gene expression (by amplitude and R2 goodness of fit).

	Cortisol	Melatonin	Sleep	Temperature	Clock gene
Mean age (weeks)	8.17	9.10	9.40	10.79	10.86
Number of infants	35	30	35	34	21
SD (weeks)	2.065	2.187	0.509	2.869	2.308
SEM (weeks)	0.349	0.399	3.012	0.492	0.504
Minimum (weeks)	6	5	6	6	7
Maximum (weeks)	16	16	16	18	16

When the week that the infant had minimum number and percentage of immobile phases of 1 minute duration, was examined, the infants were found to have most settled sleep period at 11.05 weeks of age (age with maximum number of immobile minutes during night time sleep) and 11.31 weeks of age (age at which minimum number of immobile phases during night time sleep).

Summary:

1. The first appearance of a diurnal pattern of cortisol secretion with day values exceeding night occurs at 8.17 weeks.

2. The first appearance of a diurnal pattern of melatonin secretion with night values exceeding day values is at 9.10 weeks.

3. There is a maximum level of sleep efficiency at 9.40 weeks of age.

4. Temperature physiological maturity occurs at 10.79 weeks.

5. Peripheral clock gene expression peaks at 10.86 weeks of age in the infants.

6. Maximum restful sleep occurred at around 11 weeks of age. This is characterised by the age when infant had minimum number and percentage of immobile phases of 1 minute during night time sleep.

Chapter 11

Discussion

11 Discussion

The main objective of this study was to describe the normal age related physiological changes in human infants within the first four postnatal months. Changes in overnight core body temperature were selected as a primary measure, as it has a well described circadian pattern and can be used as a marker of physiological maturity in infants.

The study also investigated the emergence of day-night rhythms in other physiological systems; cortisol, the main output of the hypothalamic-pituitary-axis; melatonin; the chemical messenger of the central body clock and a main determinant of sleep; peripheral gene expression and the main outcome of interest for the mothers: infant sleep.

The questions posed in the study were as follows:

- What is the pattern of physiological development in human infants in overnight core body temperature during sleep during the first four postnatal months and what is the nature of the change (gradual versus abrupt)?
- Is there a pattern of change of cortisol secretion in human infants in the early postnatal period and how does it relate to maturation of body temperature?
- Is there a pattern in the secretion of melatonin with increase in postnatal age and how does it relate to physiological maturation state in terms of core body temperature?
- What are the characteristics changes in infant night time sleep as babies get older and is there any relationship with physiological development using overnight core body temperature as a marker of maturity?

- What if any are the factors which have an effect on the development of core body temperature maturation, secretion of cortisol and melatonin and the development of infant sleep?
- Is there any suggestion of ordering/timing of physiological events in human infants in the first postnatal months?
- Can the genetic output of the clock be detected and measured in human infants from buccal swabs and are there any longitudinal changes with age?

All of the work was conducted within the home setting. No attempts were made to control any external environmental factors (lighting, noise, care settings) and infants were studied while families followed their normal schedule of care with no changes in their routine (sleep time, feeding, and activity). The parents (predominantly mothers) acted as the main research assistants.

First, the discussion will examine how well the above stated questions were answered. The development of a pattern of core body temperature maturation will be discussed. This will be followed by a discussion of the description of the patterns of secretion of cortisol and melatonin and how infant sleep changes with age. Thirdly an examination of the pilot data for the peripheral cycling genes from infant buccal swabs and the significance of that data will follow. Next an exploratory description of the relationship between the data collected for different physiological (temperature and hormonal) behavioural (infant sleep) and genetic (peripheral cycling gene) parameters will be discussed.

The discussion will then seek to critique the selection of methods used in the study, in order to justify the data collection undergone.

Finally the implications of the work, particularly in terms of developmental physiology and areas of future work will be outlined.

Each of the seven questions posed in the introduction of this study are addressed in turn in this section of the discussion.

11.1 Physiological development - age related changes in core body temperature during night time sleep

What is the pattern of physiological development in human infants in terms of age related change in overnight core body temperature during sleep and what is the nature of the change (gradual versus abrupt)?

In this study, the cohort of infants investigated demonstrated the characteristic change in deep body temperature during sleep with age that has been previously well described in the literature (Lodemore *et al.*, 1991, Wailoo *et al.*, 1989). The infants in the initial weeks, exhibited a small drop in core body temperature just before the onset of sleep, during the first four hours of the night time temperature recording. This was followed by a gradual rise in temperature towards day time values by the end of the night. The depth of the drop in core temperature within the first four hours of sleep deepened with increasing postnatal age. This process has been described by researchers, involving the infant moving from neonatal physiology, to an infant state of *pre-maturation* in terms of physiological development (Jackson, 2000, Atkinson, 1995). The infants are said to exhibit a *mature* rhythm (similar to that of an adult) at a particular and specific age, which can be determined when the maximal fall occurs in deep body temperature during sleep to below 36.5^0C. This occurs on average at around 3 months of age. No further fall occurs with increase age, denoting stability of the circadian rhythm (unless infant is ill or vaccinated).

The overnight temperature readings for the infants studied in this project showed the characteristic changes in core body temperature with sleep as described above, with a fall in minimum temperature recorded within the first four hours of sleep with age (see results section 4.2). These data were analysed to see whether there was a gradual

or abrupt change in minimum core temperature during night time sleep over the period of time the infants were monitored. There was evidence that the change was an abrupt one when minimum core body temperature was plotted against developmental age (figure 4.2). This suggests a 'state' change from immaturity to maturity as has been shown in previous work (Atkinson, 1995) rather than just a slow steady decline in night time core body temperature over time. This may signal an important and definitive point in the maturation process with the possible conferring of stability. It raises the question whether other physiological systems in the body mature at the same time point or is there a critical period of development over which many physiological systems mature in a sequential and ordered manner; one following the other. If this is the case, what determines the order? Is this order universal to all infants? What controls the timing and sequence? What is the consequence of a delay occurring in the maturation of any aspects of physiology? Is there any way of influencing the timing or sequence?

Previous work has shown wide variation in the age at which temperature maturity occurs. Within the normal population this may range from 7 weeks to 18 weeks (or later in certain cases where specific factors are at play). The infants studied by Lodemore matured on average at 13 weeks of age (Lodemore *et al.*, 1991). The average age in this study in terms of temperature was 11 weeks. The earlier age of development of temperature maturity in the cohort of infants in this study may be partially explained by the higher prevalence in this study of higher social economic status families as social class is known to influence age of maturation (Wailoo *et al.*, 2003).

There are other proven factors which are known to affect physiological maturation and may have affected the age of temperature maturity in this cohort. Male gender, being bottle fed, young maternal age, being first born, exposure to passive smoking, and being IUGR cause a delay in age of maturation (Jackson *et al.*, 2004, Lodemore *et al.*, 1992, Petersen & Wailoo, 1994, Tuffnell *et al.*, 1995). In this study the infants were more likely to be breast fed (more than 70%), have older mothers and half of the group were non-smokers (see results 3.1.1 & 3.1.2). A more detailed exploration of these will be found later in this chapter (see section 11.5).

For the purpose of this study the age of temperature maturation was taken to be the first age there was a fall in deep body temperature during the first four hours of sleep to below 36.5^0C as described earlier. On two occasions there was a transient reversal back to the pre-maturation pattern. This was thought to be related to exposure to illness. Although there can be this occasional temporary reversal of the infant's temperature pattern, it is still thought to be a robust measure of physiological development (Jackson *et al.*, 2001, Rawson *et al.*, 1990).

In summary core body temperature during sleep provides a reliable and reproducible measure to help place infants into different categories of physiological maturity. The changes which occur are abrupt and denote a state change. For a more complete understanding of complexity of infant development it is necessary to investigate other aspects of physiology concomitantly and explore the relationships between each parameter and how they interlink.

11.2 Pattern of melatonin secretion and how it relates to age and temperature maturation

Is there a pattern in the secretion of melatonin with increase in postnatal age and how does it relate to physiological maturation state in terms of core body temperature?

Urine was collected for melatonin estimation using 6-sulphaoxymelatonin as a proxy for melatonin production. Night-day samples were paired and analysed for emergence of diurnal rhythms.

There was an appreciable rise in night time melatonin with increase in postnatal age in the group of infants studied. There was a 21% rise week on week for logged melatonin values by random effects modelling ($p<0.001$). There was no corresponding rise in day time melatonin ($p>0.05$) results section 5.1.1. This constitutes the main result from the melatonin data and was calculated using random effects modelling (see section 2.11 methods).

It was also found that a higher proportion (greater than fifty percent - using methods reported by Sivan et al, 2001) of melatonin was produced at night from nine weeks of age onwards (see results section 5.1.2) in this cohort (Sivan *et al.*, 2001). There was a wide variation in the percentage secretion at night for each week of age the infants were monitored. These results (table 5.1) along with the bar charts in this section gave an initial visual impression of changes in melatonin during infancy. The visual impression that the infant group increased night time melatonin secretion with age was supported by the random effects modelling (this examined trends with age and did not examine diurnal rhythmicity). This modelling overcame the particular

drawbacks of the spread of data - at the earlier and later age ranges the number of cases monitored at that point were far fewer (e.g. N=2 at 5 weeks and N = 4 at 17 weeks). In addition, there were repeated measures as mentioned above (in this instance for the graphs plotted against developmental age, there were two week bands which would have included repeated measures from the same infant within the same bar). Also each infant was measured at different age intervals and these intervals were not consistent. These limitations should be taken into consideration when interpreting this section of the results, although the random effects modelling do substantiate and confirm the finding that night time melatonin increases with postnatal age. A larger study with a more fixed sampling schedule would reduce some of these limitations.

Using the differences in the median values, there was a peak in night melatonin once physiological maturity was achieved (in terms of overnight core body temperature). Evening melatonin (SD) prior to temperature maturity had a median value of 9.207 ng. mg. ml^{-1}. This level rose to 17.433 ng. mg. ml^{-1}, once temperature maturity was achieved (p=0.001; Mann U Whitney). There was no change in morning melatonin levels with temperature rhythm development (see results section 5.1.3).

The above findings seem to point to changes in night time melatonin in newborn babies as they develop and grow which may have clinical significance. This may have direct effects on infant sleep (see section 11.4).

Melatonin is widely used for sleep promotion in the context of paediatric medicine, especially in children with developmental delay (Dodge & Wilson, 2001, Phillips & Appleton, 2004) but with limited evidence of its effectiveness and efficacy. Melatonin use in children and adolescents warrants further investigation, not only in this context but in light of this study's findings, also in the infant group. This will allow a fuller understanding of its role in developmental physiology and potential therapeutic uses which may have broad and far reaching applications and implications.

11.3 Pattern of cortisol secretion and how it relates to age and temperature maturation

Is there a pattern of change with age of cortisol secretion in human infants in the postnatal period and how does it relate the change to development of body temperature?

Infants included in this study had urine samples collected by their parents, during the period of monitoring, for cortisol estimation. This was to determine the pattern of cortisol secretion in the group, with particular interest in the emergence of a diurnal rhythm.

Using a comparable definition to that used in melatonin analysis, the emergence of a diurnal rhythm was taken as the week more than fifty percent of total cortisol was produced during the day (Sivan *et al* 2001). Day time cortisol secretion exceeded night time secretion from 6 weeks of age onwards (see results 6.1.2). The percentage day/total cortisol was on average sixty percent for all weeks measured. Similar caveats apply for the interpretation of this section of the results, as for melatonin (see discussion section 11.2). The graphs (figures 6.1, 6.2, 6.4 and 6.5) as well as the data

presented in table 6.1 do not have a constant number of measurements at each week of age and the same infant may be represented within the same developmental age bracket (figures 6.4 and 6.5) more than once. However, again, the random effects modelling supports the visual impression from the graphs that there is no sustained rise or fall in cortisol levels with age over the period measured.

The levels of cortisol secretion remained steady throughout the study period. Random effects modelling showed no overall net increase or decrease in cortisol with age for day or night time values ($p>0.05$). This contrasts with the data for melatonin which showed evidence of increase with age for night time secretion. It may be interpreted that cortisol levels rose over a period prior to when monitoring commenced and the initial rise would have been demonstrated if sampling had been commenced earlier. Alternatively the measurements of cortisol obtained may be a baseline and if further samples were taken at older age ranges, there may have been a greater change in cortisol at a later age that was not demonstrated in this study. A long term follow up study may be needed to confirm this.

For the comparison of median values of cortisol secretion day and night, there was a recognisable diurnal pattern of cortisol secretion (day cortisol greater than night cortisol levels) in the weeks before temperature maturation occurred. Day cortisol peaked at 370 $nmol.l^{-1}.mg.ml^{-1}$ whilst night values were significantly lower at 180 $nmol.l^{-1}.mg.ml^{-1}$ ($p=0.0001$; Wilcoxon signed ranks). Once temperature maturation had occurred day time cortisol continued to exceed night values (median day values

298.051 nmol.l^{-1}.mg.ml^{-1} night values 172.562 nmol.l^{-1}.mg.ml^{-1}; Trend p=0.061; Wilcoxon signed ranks).

It is interesting that there seems to be a diurnal rhythm in cortisol, which was present and sustained from early on in the time course of the study. It may be that cortisol is pivotal to maturation of other physiological systems within the body to a degree that has not previously been highlighted. High levels of cortisol early on in the life of a newborn baby may confer stability to the infant's physiology, evident by the achievement of a mature temperature pattern.

Studies have reported cortisol rhythmicity occurring from 4 weeks , 8 weeks (de Weerth *et al.*, 2003, Santiago *et al.*, 1996, Vermes *et al.*, 1980)**,** 12 weeks of age (Mantagos *et al.*, 1998, Price *et al.*, 1983), or as late as 6-9 months (Onishi *et al.*, 1983). This difference between the age of emergence of rhythmicity for cortisol in this study and in other reported studies, may be due to the fact that there is generally little agreement or consistency in the manner in which a diurnal rhythm is denoted in the different studies (de Weerth *et al.*, 2003) .

The illustrative paper by De Weerth's group, highlighted the inherent difficulty of comparing findings from different research papers. They applied three different methods for examining the emergence of a cortisol circadian rhythm as described by Price, Santiago, Antonini and Krieger, on the same data set. There was little consistency or agreement as to whether a rhythm had actually appeared when the

separate methods were applied to the same data (Krieger *et al.*, 1971, Santiago *et al.*, 1996, Price *et al.*, 1983, de Weerth *et al.*, 2003, Antonini *et al.*, 2006).

Using a single definition for circadian rhythmicity (as applied to melatonin and cortisol samples) within the same group of infants in the same study, in this research, adds strength to the findings discussed in this section, as there is comparability and consistency, which will be absent if different methods are compared from different groups of infants.

Atkinson demonstrated (Atkinson, 1995) large variability in the daily cortisol excretion with marked differences for individual infants. This may reflect normal variation due environmental cues. It is possible that outlying infants with significantly higher values were undergoing a response to inter current infection or stress (Jansen *et al.*, 2010). An inappropriate cortisol response to stress or illness may reflect immaturity of physiological and immune systems, which expose the infant to a greater degree of vulnerability (Wailoo *et al.*, 2003, Westaway *et al.*, 1995). This, together with the observation that early development of cortisol rhythmicity appears to be crucial, could prove an important and noteworthy finding for the fuller understanding of infant physiological development and subsequent susceptibility to disease and mortality should development be delayed.

This study has highlighted some interesting findings in light of previously published work of other authors. The role of cortisol and development of its rhythmicity warrants further study.

11.4 Pattern of sleep and how it relates to age and temperature maturation

What are the characteristic changes in infant night time sleep with increase in postnatal age and is there any relationship with physiological development using overnight core body temperature as a marker of maturity?

In previous work (Lodemore *et al.*, 1991, Jackson *et al.*, 2004, Wailoo *et al.*, 2003, Tuffnell *et al.*, 1995, Atkinson, 1995, Petersen *et al.*, 1994), sleep diaries were completed prospectively, with the mother self-reporting 'on behalf of the infant'. This data may be open to a margin of error and be subjective. To obtain more objective sleep data, infant actigraphy was used. This was paired with actigraphy from each mother. Maternal data are not reported on here.

On average the infants slept 7.18 hours per night (range 3.45 hours to 10.52 hours). The infants were estimated as being awake for 2 hours 3 minutes during the night period. The average sleep latency was calculated as 11 minutes (range 0 minutes to 1 hour 23 minutes) and sleep efficiency 75.3%. These mean values were obtained for all infants over the entire period of the study with all ages included. The reporting of mean values for sleep, does not give specific information regarding the development or maturation of infant sleep, so alternative means were considered during this study. Changes in the sleep were examined in relation to temperature maturation. The first measures of infant sleep reported on showed no change with temperature maturation. Sleep efficiency remained unchanged in the infants group. Sleep duration was 7 hours 10 minutes prior to timing of temperature maturation. After temperature maturation this increased to 7 hours 25 minutes. The difference was not statistically

significant. Sleep latency was constant at 11 minutes. Time awake during the night time period of monitoring averaged at 2 hours 10 minutes pre temperature rhythm and 1 hour 58 minutes after.

Measures such as sleep efficiency and sleep latency have inherent problems with interpretation when an actiwatch is used on a baby. Sleep efficiency may have a false low reading if the mother put the infant to bed at a set time but the infant lay awake for an hour and a half (as occurred in one instance). Likewise sleep latency may have a false high reading. It may be that commonly used sleep measures that report on 'aggregate' aspects of sleep such as sleep efficiency, sleep latency, percentage total sleep may actually be more a reflection of maternal rather than infant behaviour. These actiwatch measures are more difficult to corroborate as they rely mainly on mother self-recording what she perceives as infant bedtime and this may not be the actual time the infant fell asleep although it may be the time baby was put to bed. Possibly more conscientious mums will have more accurate supporting sleep diary entries.

A second group of sleep parameters were analysed for changes in relation to timing of temperature maturation. These were thought to reflect infant factors more closely. Fragmentation index, a measure of restlessness, fell from 82.31 to 68. The number of minutes spent in motion during night time sleep dropped from 225 to 181 minutes. Also the total number of minutes immobile rose from 334 to 381 minutes. The number of immobile phases of 1 minute (related to fragmentation index) decreased from 75 to 69. All changes were significant and may be reported as a proxy for changes in sleep state. Infants were less restless and had improved quality of sleep –

again pointing to temperature maturation being a potential pivotal event. This observation may provide a scientific basis to the anecdotes from many mothers that their babies are 'different' and sleep better once their temperature rhythm is achieved.

In this study there was an increase in night time melatonin with age. In addition infant sleep 'improved.' These changes along with others described in this study may reflect a universal developmental process, which culminates with possibly the single most significant event for the parents at this time– the baby sleeping through the night! This *biological* and *social* experience for the infant, the acquisition of a normal sleep pattern or 'knowing the difference between day and night' – as mothers put it, may symbolise an actual consolidation of different physiological systems within the body which are unseen to the naked eye (Glotzbach *et al.*, 1994, Weinert *et al.*, 1994, Claustrat *et al.*, 2005, Rivkees, 2003) It may denote a possible conferring of full stability to the infant's physiology by achievement of temperature maturation. A disruption of infant sleep may be a marker of a deeper disruption within the body or on the other hand disrupting infant sleep may in turn disturb physiology. This can be turned on its head – promoting infant sleep and the acquisition of 'sleeping through the night' with or without the use of melatonin possibly could advance or stabilise physiology and decrease inherent susceptibility.

Sleep consolidation, maybe an external feature, which is visible to carer and researcher, of the consolidation of several internal physiological parameters.

The function of sleep in development deserves further consideration in future studies. Salzarulo and Fagioli (Salzarulo & Fagioli, 1995) went some way in examining the function of sleep itself in early development. They considered the ontogenetic hypothesis that the high percentages of REM (active sleep) seen early on (see introduction section 1.8.3) is necessary to stimulate the developing brain and promote maturation of the early central nervous system. Other researchers have advised a more cautious interpretation of the role of sleep in development (Mirmiran & Someran, 1993).

11.5 Factors affecting physiological development (in temperature, melatonin, cortisol and infant sleep)

What if any are the factors which have an effect on the development of core body temperature maturation, secretion of cortisol and melatonin, the development of infant sleep?

Factors, that are known to influence the timing of maturation of temperature rhythm in infants in the first postnatal months, were examined for their effect on cortisol secretion, melatonin secretion and on sleep efficiency, as well as their effect on temperature for this group of infants. These factors overlap with those which are associated with an increase in the incidence of SIDS (see results section 9.10). Infant sleep and melatonin had additional factors related to sleep environment and care practice examined (see results section 9.1.1 & 9.1.3).

Linear regression models were used rather than simpler t test comparisons. For each physiological area only one or two factors were shown to have any effect and in the direction expected. A repeat study with higher statistical power may highlight greater numbers of factors having an effect. This data set the sample did not have much variance in most of these factors, which made analysis difficult.

Temperature maturation was delayed in infants with young mothers (see section 9.1.2 β coefficient = 0.15 weeks; 95% CI 0.015 to 0.290 weeks; p=0.03) and those living in poor social housing, with social housing having by far the greater effect (β coefficient = 3 weeks; 95% CI 0.230 to 5.851 weeks; p= 0.035).

Melatonin secretion was higher on nights infants were well and lower on nights they exhibited signs of illness (ratio of increase = 2.3 weeks; 95% CI 0.98 to 5.41 weeks; p= 0.055). It was also lower in infants from poorer families (see section 9.1.4). Night time melatonin secretion had a tendency to be higher in infants put to bed earlier and who had shorter sleep latencies. This was not significant.

No factors were shown to have an effect on cortisol secretion. This contrasts with previous work, which demonstrated a significant difference in cortisol secretion in economically deprived infants when compared to infants from affluent families (Wailoo *et al.*, 2003). It may be that in this group of infants, measurements of cortisol should have been commenced early enough in the first postnatal weeks to demonstrate an appreciable change from low cortisol levels to high levels. To investigate what factors affect cortisol production, it may be necessary to examine these during the period levels are changing (earlier in the infants' developmental process and postnatal life; or possibly even in the foetal stage) rather than when they have already possibly plateaued and stabilised.

Sleep efficiency was directly related to infants' temperature related physiological state. Infants with mature temperature rhythm were more sleep efficient (ß coefficient - 9; 95% CI -16 to -1.8; p= 0.015). Sleeping in the same room as the carer also improved sleep efficiency (-1.57; 95% CI -2.62 to 0.53; p=0.004).

In combination, several of the above results suggest that socioeconomic status or factors related to it, along with illness, may have a direct or indirect effect on physiology. This may go some way to explain why aspects of infant morbidity and mortality, including SIDS, have higher rates in poorer economic groups. This may be mediated by a difference in care routine in poorer 'chaotic' families (Wailoo *et al.*, 2003).

There is a suggestion that an infant's physiology can be influenced by the method and parent selected routine of care used to look after the baby as well as adjustments made to the environment in which the baby is looked after. This was particularly highlighted by the infant case studies included in the appendices. A chaotic less well ordered family lifestyle in some cases may have contributed to a delay in the development of circadian rhythms and more disrupted sleep (case study 2 in appendix 3). Infants looked after in a more structured fixed care setting with 'sleep promoting' practices such as darkening the room may have had earlier or a more stable rhythm development and more settled sleep (case study 1). Manipulations of lighting and darkened room conditions have been investigated in research on babies and have been used in an attempt to entrain the infant clock prior to discharge from the neonatal unit by exposure to fixed lighting regimes (Rivkees & Hao, 2000). It has also been incorporated into certain child care 'guru' advice regimes such as Gina Ford (Ford, 2002) which was used by mothers participating in this study (case study 1). Their infants' temperature profiles and accompanying physiological data were strikingly different. Temperature maturation is affected by method of feeding with bottle fed babies maturing later.

In this study sleeping in the same room as the parents improved sleep efficiency. This finding is interesting in light of a review paper by McKenna and Ball (McKenna *et al.*, 2007). They surmise that the predominant style of infant care in the Western hemisphere (separate bed, separate room, non-breast feeding) is a possible contradiction to the biological model seen in anthropological studies for providing the best environment for infant care. Ethnic groups with low rates of SIDS are observed to have care practices that promote maximum preservation of the mother-infant dyad, whilst those with higher rates have practices that value separation as a 'goal' advertently or inadvertently (Gantley, 1994, Davies & Gantley, 1994, Blackwell *et al.*, 2004). Near contact between and mother and her child may have positive effects on aspects of the baby's physiology (Winberg, 2005), which has been the basis for practices such as kangaroo care (Ludington-Hoe *et al.*, 1994). The high rates of SIDS in some regions may now, in light of the findings of this study, have a primarily physiological basis combined with a genetic predisposition and environmental factors such as infection, interacting with aspects of infant care. These observations, if validated by further study, could form the basis of simple public health messages regarding 'the science of infant care' such as providing a physiological basis for sleeping in same room as your infant in the first 6 months.

11.6 Ordering of physiological events in postnatal months

Is there any suggestion of ordering/timing of physiological events in human infants in the first postnatal months?

The reported analysis and resultant graphical representation regarding the relationships between the timing of physiological events (Table 10.1 and Figure 10.1) must be highlighted as being exploratory and is presented primarily in a visual manner in order to provide a platform for discussion, further work and investigation. These first few postnatal weeks are a time of rapid change and this change in itself could confer vulnerability to the developing infant. If this is coupled with an insult such as an episode of illness, any delay in physiological maturation could be detrimental or at its worst, fatal (Bajanowski *et al.*, 2007).

From the graphical plots it appears that diurnal pattern of cortisol emerges first at 8 weeks. This may be linked to the fact life cannot be sustained without cortisol (although survival is related to its presence in the body not necessarily the body's ability to secrete it in a diurnal fashion). The emergence of a diurnal pattern of secretion for melatonin seems to occur next at 9 weeks of age. Physiological stability maybe conferred by the achievement of a mature core temperature rhythm. This is then followed by the sinusoidal pattern of peripheral cycling genes which may reflect stability of the peripheral oscillators at a cellular level at 10 weeks of age.

Timing of sleep maturation was difficult due to the limitations of actigraphy. Maximum recorded sleep efficiency occurred early in most infants (at 9 weeks). This may be a reflection of mothers leaving infants for longer periods before the onset of sleep as they get older. More anthropological data may help in this regard. When the number of immobile phases of 1 minute as determined by actigraphy was used as an alternative (to sleep efficiency), this showed 'maturation' at 11 weeks of age – indicating less restless sleep as the baby aged and after temperature maturation. A more sophisticated method of monitoring sleep in infancy (e.g. polysomnography) would help answer this question more accurately. It would allow the actual natural changes in sleep states which occur in infancy (decrease in active sleep and increase in quiet sleep) to be tracked precisely. It is possible that maturation of infant sleep could be used as an alternative point of reference to temperature maturation.

The early maturation of a cortisol rhythm may have significance for the central clock. In vivo, cortisol 'resets' the clock (Albrecht, 2004, Balsalobre *et al.*, 2000). Perhaps an early abrupt surge in cortisol has a role in 'kick starting' the clock and the cascade of events which lead to a maturation in terms of physiology as later manifest as changes in infant behaviour as seen in consolidated sleep patterns. Other rhythms (heart rate, temperature and sleep) are thought to exhibit clear differences in timing of maturation between premature and term infants (delay by up to 4 months) (Rivkees & Hao, 2000). A small pilot study which attempted to determine the age at which maturation of the hypothalamic-pituitary-adrenal (HPA) axis occurred in premature infants suggested that cortisol rhythmicity occurs at the same postnatal age for premature and term babies irrespective of gestational age (Antonini *et al.*, 2006). If accurate this would further support the notion that cortisol rhythmicity is critical and

crucial in physiological development and maturation. However it should be noted that the study did not include extremely premature infants. It included only nine infants born between 31 and 34 weeks gestation. The HPA axis of extremely premature infants would be expected to be significantly immature and not likely to mature at a similar postnatal age to term infants.

The visual data were based on working definitions of the appearance of a diurnal pattern of secretion for the secretion of the hormones melatonin and cortisol, sleep and peripheral gene expression.

Detailed statistical analysis to determine the exact timing of the maturation of each aspect of physiology studied was not feasible due to the sample size, and the inherent difficulties comparing different types of outputs. For example it is problematic to attempt to link hormone secretion directly to gene expression; whilst comparing age of maturation of cortisol secretion with melatonin secretion by determining timing of maximal diurnal rhythmicity (as there are both humoral outputs) is more straightforward. Although there are limitations as to what can be inferred from the data, there is a possible suggestion that the physiological maturation of the different, but interrelated systems, occurs over a period of weeks, rather than simultaneously. The box and whisker plots show there is wide variability within each physiological system measured for the age at which 'maturation' took place but does not clearly give any indication of the precision of the estimate of the mean for each week of maturation. This study does, however, contribute strongly towards the argument for larger studies, to more accurately answer the question whether there is actually a

systematic, ordered developmental process that is universal and upon which environment and external factors have no effect. Alternatively might the ordering of the maturation of the different systems change if an alternative system of reference was used to define maturity, for example using sleep as a term of reference rather than core body temperature?

Further work is needed to confirm the emergence of these rhythms and elicit their relationship to each other.

11.7 Measurement of peripheral gene expression and changes with age

Can the genetic output of the clock be measured in human infants from buccal swabs and are there any longitudinal changes with age?

The longitudinal description of the ontogeny of circadian rhythmicity for clock gene expression has not been attempted in human infants. The initial genes of interest *Clock, Bmal1* and *Period* were substituted with a proxy gene *H3f3b,* expressed in peripheral buccal tissue and is thought to reflect the activity of the central clock (Maxson *et al.*, 1983). *H3f3b* codes for a histone protein and can be isolated from buccal swabs. It was not known, prior to the commencement of this project, if cells from infant swabs were suitable for genetic analysis investigating developmental gene expression. Could sufficient genetic material be isolated from infant cells to allow meaningful analysis? What techniques would allow maximum RNA extraction? Was there any evidence of cycling of the peripheral gene in human tissue from infant buccal swabs? Would there be any evidence of a link to developmental process and did the gene expression relate in any way to physiological maturation?

H3f3b was successfully extracted from infant buccal cells and shown to cycle in peripheral tissue.

A distinct pattern of peripheral cycling gene expression was identified for each infant, with a change occurring with increase in age. There was in the main, an increase in amplitude and the goodness of fit to a sinusoidal curve. It is the examination of

individual gene plots that provide an interesting novel insight into the working of the central clock and the peripheral oscillators it synchronises.

There was evidence of 24 hour diurnal variation in the expression of the peripheral genes for all infants in the group at all weeks measured using linear modelling. This was highly significant ($p<0.001$ see results section 8.3). It may be that additional cycles exist (e.g. shorter ultradian rhythms) that may have equivalent or greater significance biologically. There was variation in the mean level of expression for each infant ($p<0.001$).

Peak peripheral gene expression occurred between 8pm and midnight which may be an indicator as to how the peripheral clock links in with the central SCN.

There was no overall increase in values with age using random effects modelling ($p>0.05$).

Further work is definitely required, as this is preliminary work, however, the gene data does mark the successful culmination of concentrated hard work, sustained effort and meticulous scientific method involving researchers, in partnership with the parents of the participant infants, without whom there would be no results.

11.8 Choice of physiological measurements in human infants

Were the measurements taken an appropriate choice of for the study of physiological development in human infants?

Temperature measurements

Rectal temperature is a method of measurement of core body temperature, which has been widely published and is an accepted method for investigation within the postnatal period (Jackson *et al.*, 2004, Lodemore *et al.*, 1992, Wailoo *et al.*, 2003, Wailoo *et al.*, 1989, Messaritakis *et al.*, 1990, Brown *et al.*, 1992, Morley *et al.*, 1992). It is found to be safe, with risk of perforation by probe being quoted as low as 1 in 2 million in older studies, using mercury in glass thermometers (Morley *et al.*, 1992) which have now been superseded by use of soft flexible probes. It has been shown by Craig et al 2002 (Craig *et al.*, 2002) to have high agreement with core body temperature measurements taken with more invasive methods used in monitoring critically ill children in intensive care which would be ethically and socially unacceptable for use in research involving healthy volunteers, such as pulmonary artery site use, distal oesophagus or tympanic membrane using a thermistor probe. Other methods have not been shown to reflect core body temperature accurately (axillary or infrared tympanic membrane).

Temperature within the body is closely regulated to a very narrow range of values. This is essential for normal metabolism, and enzymatic function at a cellular level. It allows direct outcome measure of a robust physiological diurnal rhythm.

The data loggers used were robust and small enough to allow reasonably easy carriage alongside the infant during routine care within the home. The rectal probes were tolerated well by the infants. However the use of smaller loggers or alternative

temperature recording devices adapted for use in infants in future work may further improve ease of use. Examples include external skin probes placed on the hepatic/splenic area of the abdomen or ingestible caplets which are used in older children and adults. The temperature recordings although taken minute by minute were extracted half hourly from graphical plots. It may be possible to extract the minute by minute data and apply these for use in an algorithm for more detailed analysis.

Urinary cortisol and melatonin measurements

The collection method for urine samples in this study mirrored that used in clinical practice. The attachment of urine bags to the infant perineum is in the main a simple procedure to teach and straightforward. If the bag was not applied correctly this led to leakage and loss of sample. Some mothers were uncomfortable with the removal of the adhesive tape from the skin once collection was complete and asked for an alternative method. The urine pad proved a popular alternative. The collection is very straight forward and allowed for potentially larger volumes of urine. Both methods were susceptible to faecal contamination. Also the study protocol did not allow for very accurate recording of the timing of the samples which may have affected results. Mothers may have applied a bag or pad at 20:00 but may not have checked the nappy till later, for example till 22:00. The sample will then have been labelled as 22:00 however it may have been for any of the two hour period leading up to that. Frequent (e.g. hourly) sampling (blood or saliva) would have overcome this but may have compromised recruitment and retention to the study.

There are further issues regarding sampling for both melatonin and cortisol. The age sampling for cortisol secretion was commenced was 6 weeks of age in my study, to coincide with the start of temperature monitoring. To denote a more accurate timing of the appearance of a day night rhythm in cortisol secretion it may be reasonable in future work to start sampling at an earlier age. The converse would also be true. A longer monitoring period would allow elucidation of full stability of the multiple aspects of physiological maturation, as it may be the case that some rhythms appear – then reappear with increased strength, stability and subsequently develop permanence at a later age.

Both melatonin and cortisol rhythms would have been more clearly delineated if there had been an increase in the frequency of sampling (Minors, 1981). This had to be weighed up against the necessity of making the protocol simple enough to encourage maximum participation and retention. No families withdrew from the study despite concerns raised by some that mothers of newborn babies would not want to nor would be able to participate in a study of this nature. On the contrary families enjoyed contributing to a body of scientific evidence that they felt would be assisting babies other than their own.

In addition, as mentioned above, there was variation in the number of infants that were measured at each week, with smaller numbers at each end of the monitoring period. Also there were repeated measures. Standardising all timings (clock time and date) of collections of samples may have overcome this – although a less flexible protocol may have compromised recruitment and retention of participant families.

Variations in urinary cortisol and the urinary metabolite of melatonin both reflect serum values (see methods section 2.5/2.6). Salivary measurements are an alternative but the assays required for the processing of these were not available to the research team, therefore urine was used. This technique, which utilised correction for renal excretion was successfully used for measuring cortisol. This was reported by Jackson (2004), Wailoo (2003) and Atkinson (1995) (Jackson *et al.*, 2004, Wailoo *et al.*, 2003, Atkinson, 1995). The urinary levels reflect change in body hormone level not change in creatinine excretion.

Actigraphy

Actigraphy was selected for use in this study because of the advantages linked to its small size and ease of use. The families participating in the study were relied on to provide accurate timings regarding bed routine, sleep times, feeding patterns, timing of samples and other aspects of infant care. This was in addition to actually collecting samples themselves from their baby over several months and navigating the temperature monitoring equipment. Actigraphy provided objective data which supplemented parent sleep diary entries. The actiwatches did not require any involvement from the parents in set up or attachment and the data collected was subsequently straightforward to interpret and analyse. Its choice helped counter arguments that had been presented to the research team that mothers would not be able to cope with the research schedule while simultaneously looking after a young infant.

In other work by Korte sleep maturation was thought to be related to the proportion of total sleep taken during the night period only, a marker of sleep consolidation (Korte *et al.*, 2001). In this study day time sleep was not measured so we were unable to calculate this in our group of infants. Conversely 'sleep maturation' may be mapped out using actiwatches with algorithms which code sleep into active or quiet sleep. This technology was not available for this project. Instead sleep measures such as number of immobile phases were used as a proxy to examine any indication of sleep maturity instead..

Actiwatches are traditionally used to monitor longer periods of rest-activity (several days or weeks). Infants wore an actiwatch for one night only on each occasion.

Extension of this period of monitoring may be helpful for future studies. An alternative is to use a completely different sleep scoring technology (portable PSG, video behavioural observation, motion sensitive mattresses etc. – see introduction) which will allow fuller sleep staging.

Actigraphy is thought to be a valid tool in contributing to the assessment of sleep, including in infants. However it does have limitations, in that it measures motion rather than sleep itself and translates movement and non-movement into presumed wakefulness and rest. In an adult this may be easier to interpret. In the absence of simultaneous video records, it was difficult to extract movement artefact, for example if the infant was being rocked to sleep. It is difficult with absolute certainty to differentiate whether an infant was actually asleep or awake. For these reasons it may overestimate or underestimate sleep. However in this data set there was high agreement between the actigraphy and sleep diary entries. Future work comparing sleep diaries, actigraphy and more complex methods of measuring infant sleep (see introduction 1.8.4) is warranted.

Buccal swabs for peripheral gene estimation

This was novel work. There are no previous studies investigating longitudinal gene expression in human infants over the first four postnatal months concomitantly with physiological development.

Traditionally molecular biological and genetic studies have taken ‘snap shot’ views of gene expression at any given time either from central or peripheral tissue; or rhythms were monitored for several days at a maximum (Brown *et al.*, 2008) and much of this

is work performed using animal models (Welsh *et al.*, 2004, Nagoshi *et al.*, 2005). Investigating longitudinal trends over several weeks or months in this project required a robust protocol, determination on the part of the researchers and persistence and patience on the part the parents collecting the swabs. The collection of samples for RNA extraction from the infants, alongside several other measurements, over a lengthy period; made the project more labour intensive. This however did not affect the retention or compliance of the participant families, which was very high (100% retention - see results section 3.2).

The method of buccal cell collection from the infants was straight forward and simple to train mothers to do themselves without supervision from a member of the team. This study illustrates that home based sample collection; even in a molecular biology research program can be successful in small babies with a carer as main research assistant.

First, we were, after a significant investment of time, finance and effort, able to identify laboratory techniques which were reproducible and suitable for the type of samples we had. This was a tremendous feat due to the huge technical challenges faced during the processing of the samples. Multiple kits and methods were systematically trialled and tested before any results were obtained. The entire process took several months to set up and assess before any infant samples were processed. Despite this, the results remain striking.

Second, we demonstrated the gene of interest H3f3b had a distinct recognisable cycle over 24 hours. More work is required in this novel area of ontogeny of gene expression in human infants.

Further statistical analysis

There are a series of secondary analyses that will allow supplementary questions to be answered regarding the development of circadian rhythms in human infants. They involve methods similar to those employed in this thesis and further statistical analyses.

For all physiological measurements and gene expression data it may be reasonable to look for a clinically relevant 'cut off' point which would be determined by step function analysis/S curves (see below). Dichotomous analyses using repeated binary measures could then be employed for further analyses. An example may be that if a certain value for sleep efficiency is deemed to be important for neurocognitive development. One may be interested to know at what age the infants obtained this minimum value of sleep efficiency and examine what the effects at this time are on others aspects of physiological development.

How a 'cut off point' could be determined is described within the section below entitled Cortisol and Melatonin where S curves and stepwise functions are dealt with. It should be noted that these methods are relevant for the other physiological parameters, which have been measured in this study and for the gene expression data.

Cortisol and melatonin

Slope based analysis should be performed examining the rate of change or the relationship between night and day secretion of hormones relative to gestational age rather than postnatal age.

Further random effects modelling may be employed for the cortisol and melatonin data, with age relative to attaining temperature maturity. These two methods combined will help determine the robustness of the findings as to whether birth or conception should be treated as baseline events. We hope this will shed light on whether postnatal age, gestational age or the concept of an 'age of maturity' or 'developmental age' are more relevant to the processes we are examining.

As an alternative to paired testing of means, before and after temperature maturity, it is feasible to attempt to use statistical methods to determine maturity, either as an 'abrupt' state change or cut point (using a step function analysis) or a more generalised gradual period of increase such as defined by an S curve. Postnatal age could again be substituted with gestational age or developmental age. This would involve the use of non standard advanced statistical methods. A minimum of five to six consecutive measurements per infant would be required.

Sleep data

A further slope based analysis/linear analysis investigating rate of change of sleep parameters could be performed against gestational and 'developmental' age; as well as sinusoidal curve modelling.

Gene expression data

The above methods were employed for gene expression data but are not reported on in this thesis due to constraints of space. The use of S curve did not yield positive results. This was expected as the random effects modelling showed no evidence of increase or decrease in gene expression with age.

A secondary analysis of the genetic data would involve studying the changes in gene amplitude using ultradian rhythms (4, 6, 8, and 12 hourly) rather than changes over 24 hours, to examine the strength of the rhythms. This would be followed by further work utilising the different amplitudes and R squared values in new random effects models.

Summary and Future work

This study highlights that the first four months for an infant are crucial and critical to development, maturation and adaptation to an independent life outside of the intrauterine environment and separate from the mother. The rapidity and extent of change may confer increased vulnerability to the infant if challenged by an external adverse factor such as an infection.

In summary this research again showed that infants demonstrate an 'abrupt' change in overnight core body temperature during night time sleep which may be used as a marker of physiological maturity, as shown previously in work by this group. There are obvious changes in infant behaviour and physiology which occur alongside the achievement of a diurnal temperature rhythm (patterns in melatonin secretion, sleep consolidation and a diurnal pattern of peripheral gene expression). Changes in cortisol secretion appear to be an early and important circadian rhythm that develops (pre temperature maturity) which may act as a trigger or act as a catalyst or key to the development of other circadian rhythms.

As suggested by previous work and further supported by this study, although the order of development in general stays the same, the timing of the maturation may be different for individuals (infants in this case). The same physiological events give rise to physiological maturity at different ages in different babies.

Developmental periods which have been studied more extensively such as prenatal growth and development and puberty have given clear examples of development being ordered, sequential and universal. This study opens the door to several lines of

enquiry with regards to development in the postnatal period. Are the physiological changes in the postnatal period also consistently ordered and irreversible? Why do they not occur simultaneously? What determines their order? Can the speed of change be altered or entrained by changes in routine, feeding, lighting etc? What are the implications for programming of adult disease, future morbidity and mortality? Who is more vulnerable and why? What is the relationship between immature physiological state and social deprivation? How is this relationship, if present, mediated? Is it connected with 'chaotic' lifestyles, nutrition or exposure to tobacco smoke?

As a priority it would be interesting to further explore the following -

- The physiological systems in infants known to be delayed in maturation e.g. IUGR, Twins, deprived groups, ethnic minority comparisons, premature infants, infants at risk of developmental disability/SIDS.

Future work might include:

- Refinement of definition of maturity of circadian rhythms in melatonin and cortisol secretion, with possible collection of data for leptin, ghrelin, growth hormone which also have robust circadian rhythms
- Development of potential markers of at risk infants for common infant morbidities and who are at risk of mortality (e.g. develop fetal HRV doppler flow studies inutero; buccal swab measurements of melatonin/cortisol/other hormones of growth factors/ peripheral gene cycles).

- Maximise technique for measuring cycling gene expression in adults and infants from buccal swabs – do experiments in lab controlled setting to dampen down 'noise' vs. home studies
- Study of the effects of different infant care routines on infant physiology (Gina Ford vs. demand feeding and infant led bed times, places of sleep) using anthropological methods
- More detailed sleep work to define the ontogeny of sleep in first postnatal months (possibly using behavioural observation and portable PSG)
- Examine further effect of illness and social circumstances on physiology
- Further work on infant separation from mother-infant dyad and the consequences of this on infant physiology

Directing our scientific enquiry into areas that simultaneously include topics of scientific value, with the search for answers to questions parents want to know will allow the continuation of researcher-carer partnerships which were invaluable in this project. Refining our knowledge of postnatal physiology and development will increase our understanding of the implications of physiological malfunction and delayed maturation. This may help contribute to the understanding of foetal origins of adult diseases such as those included in Syndrome X (Diabetes mellitus, Coronary Heart Disease, Hypertension- all associated with Obesity) and neurodevelopmental conditions which have a disruption of circadian rhythmicity as a prominent feature. Finally it may contribute the final pieces of the jigsaw puzzle known as the disease entity Sudden Infant Death Syndrome. The work is labour intensive and detailed but the privilege of working hand in hand with mothers and fathers of newborn infants

and helping to influence the choices they make regarding the care of their most precious possessions makes it worthwhile.

“There is a science of infant care but infant care is not a science”

Martin Ward Platt, 2010

And finally the old age adage still rings true

“Mum [and Dad] know best”

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Illustrations