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Impact of *Per1* and *Per2* clock genes on the reproductive outcome and physiological functions in female mice

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In memory of Helena Pilorz

“Der Widerstand gegen Darwin und Wallace geht zum Teil auf unser Unvermögen zurück, uns das Verstreichen von Jahrtausenden vorzustellen, ganz zu schweigen von Äonen. Was bedeuten 70 Millionen Jahre für Wesen, deren Lebenszeit höchstens ein Millionstel davon beträgt? Wir sind wie Schmetterlinge, die einen Tag und eine Nacht lang umherflattern und denken, es sei die Ewigkeit.” (Carl Sagan 1980).

Contents

List of abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
AVP	argininee vasopressin
Bmal1	brain and muscle ary1 hydrocarbon receptor nuclear translocator-like 1
Ck1ε	casein kinase 1 ε
Clock	circadian locomotor output cycles kaput
CO_2	carbon dioxide
CORT	corticosteroid metabolites
CRH	corticotrophin-releasing hormone
Cry	cryptochrome
CSN	central nervous system
CT	circadian time
Dec	differential embryo-chondrocyte expressed gene
DD	constant darkness

E-oscillator	evening oscillator
E	early stage of pregnancy
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
HPA axis	hypothalamic- pituitary-adrenal axis
kg/g	kilogram per gram
L	last stage of pregnancy
LD	light/dark conditions
LH	luteinising hormone
LL	constant light conditions
M-oscillator	morning oscillator
M	middle stage of pregnancy
MeOH	methanol
min	minutes
MJ/kg	megajoule per kilogram
mRNA	messenger ribonucleic acid

N	sample size
O_2	oxygen
p	error probability
PAG axis	pituitary-adrenal-gonadal axis
Per	*Period* gene
Per1$^{(-/-)}$	B6.129S7-Per1^{tm1Brd}
Per2$^{(-/-)}$	B6.129S7-Per2^{tm1Brd}
Rev erbα	nuclear receptor subfamily 1, group D, member 1
RHT	retinohypothalamic tract
RIA	radioimmunoassay
SE	standard error
SD	standard deviation
SCN	suprachiasmatic nuclei
τ	tau, free-running period length
VIP	vasoactive intestinal peptide
ZT	*zeitgeber*

1. Introduction

In the course of the evolution of life on earth, organisms adapted well to the daily rotation of our planet around itself and its yearly rotation around the sun. One of such adaptations is the acquisition of a regulating mechanism - the endogenous circadian clock - that can be synchronised to daily and seasonal changes in external time cues such as light, ambient temperature, and availability of food. Such an endogenous mechanism enables animals to anticipate environmental conditions, so as to be able to perform behavioural patterns at advantageous times during day or night and throughout the year.

In the past decade biologists have mainly focused on the molecular mechanisms of the biological clocks in vertebrates, insects, plants and even cyanobacteria. One of the most interesting discoveries made in the past is that the circadian rhythms are ubiquitous. They are present in each cell and each tissue in all organisms. In mammals the major component of the circadian system is in particular a light input pathway from the eyes to a self-sustained master circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus which generate the biological rhythms, and the output pathways that regulate numerous molecular physiological and behavioural functions. One of the characteristic features of the endogenous clock is a rhythm that is slightly deviant from 24 hours. The daily light signal corrects this deviation by resets or phase shifts so that the endogenous clock is then synchronised to the environmental 24 hours rhythm. This light information is transmitted via specialised photoreceptors to the retino-hypothalamic tract and synchronises the multitude of oscillations in cells and tissues. The transmission of the light information to the major pacemaker leads to the activation of proteins that reset the circadian pacemaker's core autoregulatory transcription-translation loop. The pineal gland is the end organ of the photoneuroendocrine axis and it transduces the circadian rhythms of neuronal activity from the SCN into an endocrine signal, the hormone melatonin (Axelrod J. 1974, Bartness and Goldman 1989). Thus, the photoperiodic message consists of alternating high melatonin levels during the night followed by low melatonin level during the day (Steinlechner 1998).

In photoperiodic species melatonin is the regulatory motor of seasonal reproduction transmitting the information about the day length to the neuroendocrine-gonadal axis (Bartness *et al.* 1993, Cassone 1990). Through this hormone system the organism modifies the secretion of the gonadotropin releasing hormone (GnRH). GnRH itself regulates the secretion of luteinising hormone (LH) and follicle stimulating hormone (FSH) that stimulate the secretion of sex steroid hormones and gametogenesis, respectively. The preovulatory GnRH surge is primarily controlled by two types of input to GnRH neurons and the surrounding interneurons: hormonal feedback for maturing ovarian follicles and circadian output from the SCN (van der Beek 1996, Levine 1997). The absence or dysregulation of both input pathways, particularly neuronal efferents from the SCN containing the neuropeptides argenine vasopressin (AVP) and vasoactive intestinal peptide (VIP) that trigger directly onto GnRH neurons, disrupt GnRH surge release and cause anovulation (Brown-Grant and Raisman 1977, van der Beek 1996). Non-photoperiodic organisms such as house mice (*Mus musculus*), some laboratory mouse strains and laboratory rats, however, do not make use of the melatonin signal to time their reproductive effort even though they show seasonal changes in the pattern of melatonin secretion similar to those in photoperiodic organisms (Bronson 1985, Ebihara *et al.* 1986, Stehle *et al.* 2002, Kennaway *et al.* 2004). Van der Beek (1996) suggested that reproduction in this species may be modulated by the pituitary-adrenal-gonadal axis (PAG axis). Nevertheless, the principle of temporal organisation and function of estrus related events that are connected with fertility is the same in non-photoperiodic as well as in photoperiodic mammals. There are substantial data from different rodent species demonstrating that estrus-related events such as timing of proestrus, surge of LH and FSH release, ovulation, increase in progesterone secretion, and onset of sexual receptivity are precisely timed by the internal circadian system and occur thus at specific times of day (Rusak and Zucker 1979, Eskes 1984, Turek 1985). This fact has been supported by showing that ovulation continues to occur near the expected time of day after an acute exposure to constant light or constant darkness (McCormack and Sridaran 1978, Campbell and Turek 1981). This well defined temporal program of all endocrine and neuronal events associated with gonadal development through all reproductive stages leads to reproductive success

during the life span of each organism (Turek and van Cauter 1994). Furthermore, the interval between successive recurrences of ovulation, behavioural receptivity and release of pituitary ovulation-associated hormones (LH and FSH) is relatively constant and species specific (Fitzgerald and Zucker 1976). For example rodents' estrus cycle is characterised by 4-5 days and the human menstrual cycle lasts 25-35 days.

It is well established that reproduction is a seasonal phenomenon in many species (Hoffmann 1981c, Bronson and Heidemann 1994, Steinlechner 1998). For example, animals of the temperate zone with short gestation time such as Djungarian hamsters (*Phodopus sungorus*) (Hoffmann 1979, Stetson *et al.* 1986, Davis *et al.* 1987), deer mice (*Peromyscus maniculatus*) (Whitsett and Miller 1982, Blom *et al.* 1994), and voles (*Micortus montanus*) (Horton 1984, Lee *et al.* 1989) show a sexual development and reproductive competence that are triggered by seasonal changes. Their mating and birth takes place usually in spring or summer. Accordingly, in larger mammals that show longer gestation time (e.g. deer and sheep) mating occurs in autumn or winter with birth the following spring. This kind of adaptation to the recurring environmental changes over the year enables many organisms to increase their fitness by reproducing at appropriate time of year with favorable environmental conditions (Morin *et al.* 1977, Boden and Kennaway 2006). Spring is the time of year that is most rich in essential nutrients and has advantageous climatic conditions that support the survival of each organism and especially that of neonates. Particularly lactation is commonly a critical period that is energetically most expensive (König and Markl 1988, Hammond and Diamond 1992, Degen *et* al. 2002). Hence, it is essential for a lactating female to give birth and to raise its offspring under environmental conditions that are optimal for survival. The synchronisation of the accurate breeding cycles with the appropriate season or time depends on the measurement of day length by the pineal gland. Thus, seasonal breeding animals show reaction to changes of the photoperiod switching from the period of reproductive activity to periods of reproductive quiescence. For example in many small rodents like hamsters and ground squirrels long days of spring and summer have a stimulatory influence on gonadal growth and reproductive activity while short days during autumn

and winter inhibit reproduction and delay sexual maturity until the following spring in animals born at the end of the breeding season (Steinlechner and Niklowitz 1992, Gorman and Zucker 1995, Park *et al.* 2006). In this case the seasonal change in day length is the primary environmental cue for stimulating gonadal activity and reproductive behaviour during the appropriate time of the year.

Certain species are facultative breeders. They live in relatively unpredictable environments where reproduction is opportunistically keyed to the presence of favorable conditions. Mice and rats are opportunistic breeders that can reproduce successfully throughout the year as long as the food supply is sufficient for successful breeding (Cassaing 1984). Reproduction is an energy-consuming process, particularly for females; therefore food availability and not the photoperiodic signal through melatonin is supposed to be essential for successful reproduction in these species (Bronson and Marsteller 1985).

1.1 Timing and basis of puberty and ageing

Puberty and adolescence represent the metamorphosis of the child or juvenile into the adulthood (Sisk and Foster 2004). Responsible for this developmental transformation are biological rhythms, particularly circadian rhythms that establish temporal order throughout life. This circadian rhythm undergoes dramatic changes in the course of life, especially in the early ontogenetic development and in old age (Weinert and Schuh 1988, Turek *et al.* 1995).

The developmental change during puberty is characterised by maturation and adaptation to the environment, resulting in reproducible and stable rhythms of biological systems such as secretion of gonadotropins and sex hormones that influence and regulate the timing of ovulation, mating, duration of pregnancy and lactation. These biological rhythms show a high amplitude and a characteristic phasing with respect to other biological processes and the external environment. Such a temporal structure lasts only for a certain length of time to provide optimal functioning of the biological system with a maximum of efficiency and welfare before the circadian system starts to break down.

The onset of puberty results from arousal of a complex neuroendocrine system that promotes the ultimate attainment of fertility reflecting morphological, physiological and behavioural development, causing an increase in frequency of pulsatile GnRH secretion. The very remarkable feature of the secretion of GnRH is that the main population of neurons, which mediate a connection between GnRH producing neurons and the SCN, are localised in different sub-regions within the SCN (van den Pol and Tsujimoto 1985) as well as in the medial septum, diagonal band of Broca, and in the preoptic area (Ebling 2005). Recent understanding of the interaction between GnRH neurons and the SCN provides the explanation for the pronounced circadian rhythm of the LH surge and associated sex-hormones that regulate the rhythmicity of the estrus cycle (Everett and Sawyer 1950, Legan *et* al. 1975). However, it is still unknown which pathways act directly upon GnRH neurons, or which neurons change and modify the adequate timing of the onset of puberty, inducing a high frequency of GnRH secretion at puberty (Sisk and Foster 2004, Ebling 2005).

Change in daily photoperiod is a good predictor for oncoming favourable periods and seasonal breeding species use this cue to time puberty and breeding accurately. The information about this daily photoperiodic change that is measured by SCN is transmitted by melatonin to the GnRH neurons (Turek and Van Cauter 1994). Thus, melatonin seems to function as an important component of the circadian system that is involved in regulating maturity and fertility in photoperiodic species.

It is well known that mothers have a huge influence on the development of their offspring already during the gestation phase providing their fetuses with essential substances such as nutrients and oxygen through the placenta and umbilical cord. In this stage they transmit also the information about the day lengths to the fetus via melatonin and thus programme the photoperiodic responses of the young in the postnatal phase (Weaver and Reppert 1986, Elliot *et al.* 1989). As for many breeders the photoperiod signals the optimal time of year for puberty onset (Ebling and Foster 1989, Foster and Ebling 1988) maternal melatonin in the pre- and postnatal phase (via placenta or maternal milk) may prepare the newborn for the optimal onset of puberty.

Moreover, there is substantial variation in the timing of puberty: some species reach puberty during their first year, others when they are one year old, and others after 2 or even more years (Follett 1991). Particularly in Djungarian hamsters (*Phodopus sungorus*) the onset of puberty is well established (Bronson 1989, Place *et al.* 2004, Park *et al.* 2006). Pups born into long or increasing day lengths reach sexual maturity much earlier than those born into short or decreasing day lengths; their reproductive maturation is then delayed (Yellon and Goldman 1984, Kauffman *et al.* 2003). In this context the advanced and the delayed maturity related to long and short day conditions, respectively, are adaptations to the environmental conditions where food availability is highest during the summer and lowest during winter. Hence, the optimal time of puberty during a year results in successful pregnancy and offspring care (Horton and Rowsewitt 1992).

In the case of laboratory rats and mice that were maintained for many generations under constant environmental conditions photoperiodic effects on puberty are less common (Clark and Price 1981). Nevertheless, there are some rat and mouse strains e.g. Fisher 344 rats, CBA and C3H mice that do retain photo-responsiveness and have functional melatonin that translates the photoperiodic information into a hormonal signal (Ebihara *et al.* 1986, Leadem 1988). However, findings on rats without melatonin expression suggest that vasoactive intestinal peptide (VIP) and arginine vasopressine (AVP), peptides synthesized also in the SCN are involved in the regulation of the timing of the secretion of GnRH and thus in the modulation of the preovulatory LH surge (Kriegsfeld *et al.* 2002). Taken together there are multiple signals that determine timing of puberty onset regulating the pubertal GnRH increase (Fig. 1) but the integrating mechanism regulating the balance is up to now unknown.

With increasing age, however, all biological functions such as reproductive capacity decrease progressively. This is exemplified in laboratory female rodents demonstrating that ageing females show higher embryonal postimplantation failures, aneuploidies, miscarriage and low litter size (Parkening *et al.* 1978, Liu and Keefe 2002). In addition, changes in all biological functions make the old organism less able to adapt well to changes in the temporal structure of the environment and this limits its life span. Transplantations of fetal SCN into old and young SCN-lesioned animals

which restore or improve the circadian behavioural rhythms, respectively, (Viswanathan and Davis 1995) support the assumption that the age-related changes in circadian behaviour and physiological functions are connected with age-related changes of electrical rhythms in SCN and dispersed SCN neurons (Yamazaki *et al.* 2002). This reduced electrical activity rhythm of SCN neurons in aged animals is an additional possible explanation for the decrease in amplitudes of all biological rhythms (Satinoff *et al.* 1993). The decreased amplitudes were described in many rodent species such as rats, mice, and hamsters (Ramaley 1974, Weinert and Schuh 1984a, Cai and Wise 1996, Paris and Scarbrough *et al.* 1997, Davis and Viswanathan 1998). The amount of the activity and the circadian amplitude decreased and became more fragmented in all animals. However, some mouse strains showed a very clear rhythm in their activity until their final days of life (Wax 1975, Teena and Wax 1975). Interestingly, the amplitude of various biological systems does not change simultaneously with the age in an individual. For example the corticosterone rhythm in ageing rats declines earlier than the body temperature rhythm (Paris and Ramaley 1974, Yunis *et al.* 1974). In adult animals the SCN entrains the peripheral oscillators. A decrease in neurotransmitter production of the SCN and low amplitude of electrical activity in ageing animals, however, might weaken the ability of the SCN to entrain peripheral oscillators e.g. adrenal glands and ovaries (Ruby *et al.* 1998).

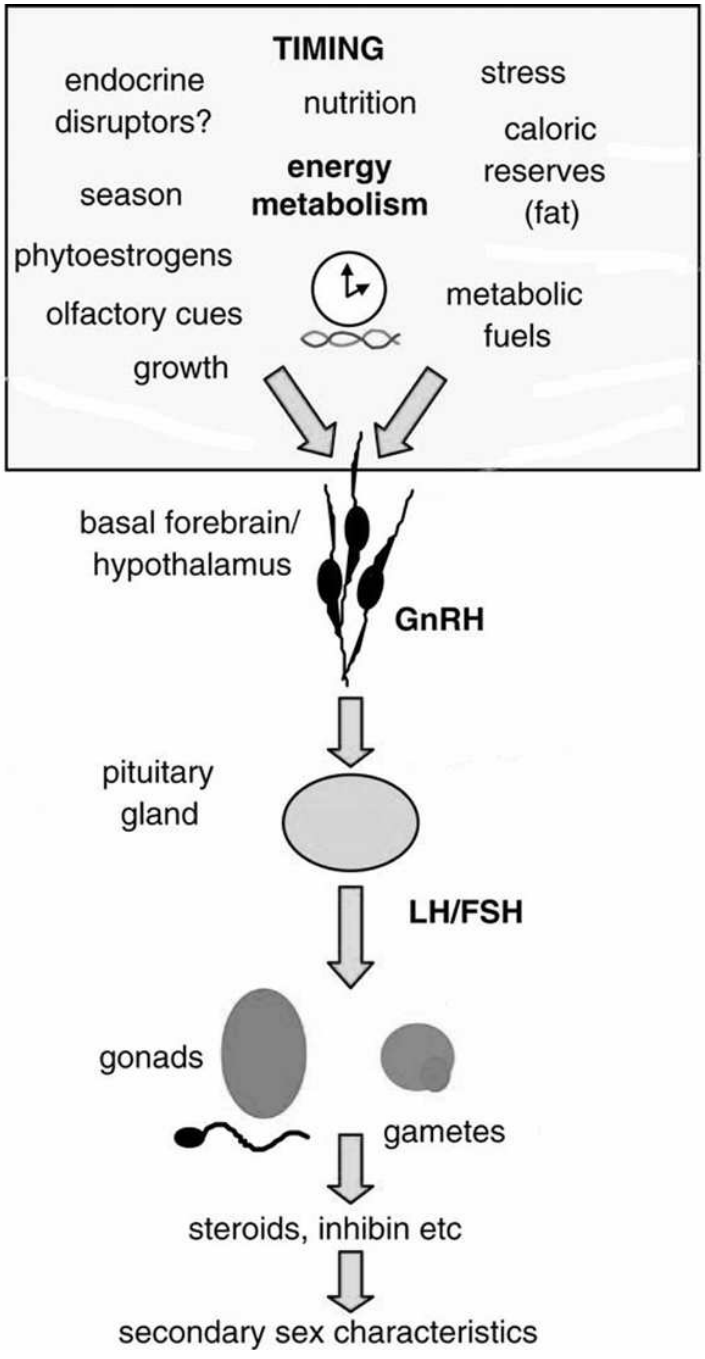

Fig. 1: The timing of regulation of puberty by the central control of GnRH secretion. It reflects the integration of multiple internal and external cues acting upon a genetically determined process. FSH: follicle-stimulating hormone, GnRH gonadotropin-releasing hormone (adapted from Ebling 2005).

1.2 The hypothalamic-pituitary-adrenal axis

Stress in its broadest sense has a substantial impact on a variety of physiological functions. One of the main mediators of the response to stress is the hypothalamic-pituitary-adrenal axis (HPA-axis) (Fig. 2) The HPA axis does not only respond to stressors but it also coordinates circadian events such as food intake, sleep/wake cycle, courtship and sexual behaviour. This diurnal activity of the HPA axis results in a peak of glucocorticosteroid hormone secretion at the onset of the active period and at the preovulatory stage in females. This peak is generally controlled by two mechanisms from the SCN: a stimulating and an inhibiting component (Buijs *et al.* 2003). The activation of the HPA axis at the circadian peak that occurs generally one hour before onset of activity or is caused by a stressor leads to the production of the corticotropin-releasing hormone that stimulates the release of adrenocorticotropic hormone (ACTH) into the bloodstream. In the adrenal cortex ACTH itself stimulates the production of glucocorticosteroids in the adrenal gland (cortisol in humans, corticosterone in rodents) (Nelson 2005).

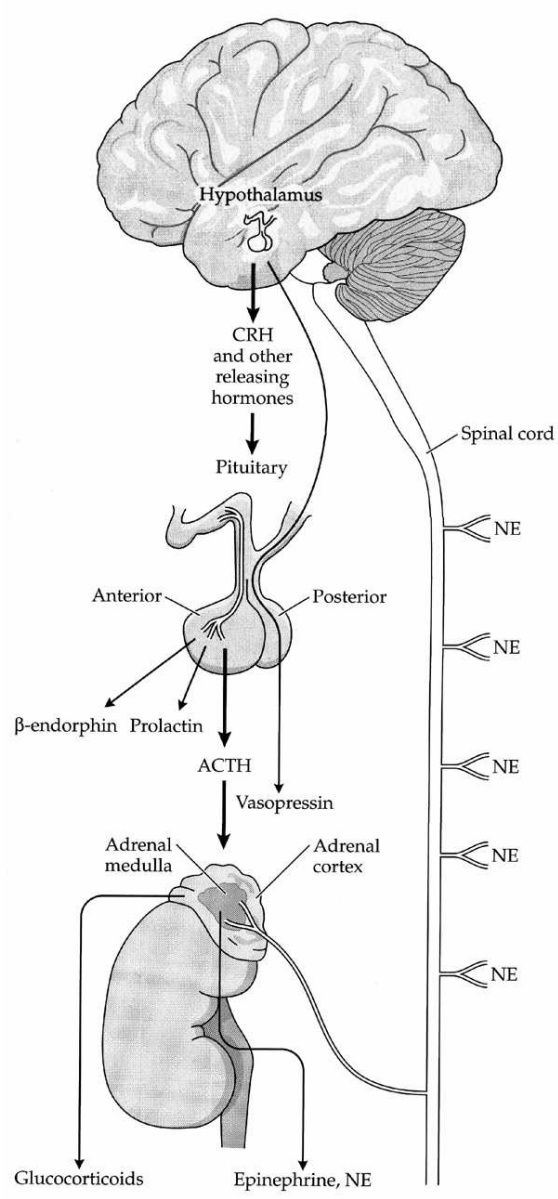

Fig. 2: Schematic diagram of the major components of the hypothalamic-pituitary-adrenal (HPA) axis. External stimuli as a stressor initiate a cascade of events which leads to stimulation of corticotropin-releasing hormone (CRH) and vasopressin (AVP) release from hypothalamic neurons. Activation of sympathetic pathway initiates release of catecholamins for the adrenal medulla that act on various target organs and tissues. CRH and AVP stimulate the release of adrenocorticotropic hormone (ACTH) from anterior pituitary that in turn stimulates glucocorticoid release for the adrenal cortex. Prolactin and AVP are often released during stress (adapted from Nelson 2005).

1.3 Relationship between glucocorticosteroids and reproduction in females

The response to stress mediated by the HPA axis can differ between males and females in many organisms. Studies on laboratory rats and mice have demonstrated a higher basal and stress-induced hormone secretion in females than in males (Brett *et al.* 1983, Touma *et al.* 2004, Cavigelli *et al.* 2005). Females also show alterations in basal and stress-induced HPA activity during the estrus cycle where the highest levels occur during the preovulatory period (Nichols and Chevins 1981, Cavigelli *et al.* 2005). The rise in corticosterone at proestrus may be physiologically important in relation to the mobilisation of fuel supplies at this stage of estrus and thus facilitates the greater energy expenditure associated with energetic requirements of mating behaviour (Weizenbaum *et al.* 1979). Usually at this time the females become more active with the objective to find a male for potential copulation. Simultaneously they decrease other essential activities such as food and water intake (Bell and Zucker 1971). At the same time, however, an excessive stress-induced increase of corticosterones acts on the reproductive function in a suppressive way (Welsh *et al.* 1999). These suppressive effects of stress on female reproductive physiology and behaviour influence three components: disruption of ovulation, impairment of the uterine maturation associated with impaired implantation, and inhibition of receptive behaviour (Wingfield and Sapolsky 2003). In this case corticosteroids exert direct inhibitory effects on gonadal steroid secretion and sensitivity of target tissues to sex steroids (Magiakou *et al.* 1997). In addition stress-induced secretion of hormones inhibits the release of GnRH, thus greatly reducing the amount of LH. Hence, corticosteroids appear to inhibit reproductive hormone production by acting at both the central nervous system (CSN) (Dubey and Plant 1985) and the pituitary (Ringstrom and Schwartz 1985).

1.4 Molecular organisation of the mammalian circadian clock

The molecular structure that describes the mammalian circadian clock is derived from the model of the *Drosophila* circadian clock. The cellular rhythmicity of the SCN is generated by a series of interlocking positive and negative feedback gene transcription and translation loops (Fig. 3). To date there are more than 10 genes known that shape the basis of cellular rhythmicity including *Per1*, *Per2*, *Per3*, *Clock*, *Bmal1*, *Cry1*, *Cry2*, *Dec1*, *Dec2* and *Rev erbα* (Table 1). The core components comprising the autoregulatory feedback loop of the mammalian circadian oscillator are *Bmal1* and *Clock* genes. These genes are components of the positive feedback transcription and translation loop. Their protein products form a heterodimer and bind to a specific gene sequence in the promoter region of *Per1*, *Per2*, *Cry1*, and *Cry2* genes and drive their transcription (Gekakis *et al.* 1998, Kume *et al.* 1999). Conversely, the translated CRY/PER heterodimers enter the nucleus and repress their own transcription through inhibition of the transcription activation by the BMAL1/CLOCK heterodimer. Thus, the negative loop is generated by the transcription of *Per1*, *Per2*, *Cry1*, and *Cry2* genes. Additionally, the degree of the phosphorylation of the PER and CRY by casein kinase 1ε may alter the protein stability and hence the speed of the feedback loop (Lowrey *et al.* 2000). The PER2 protein alone has a functional effect on the SCN oscillation amplifying the *Bmal1* expression (Shearman *et al.* 2000, Yu *et al.* 2002). *Rev-erbα* (Adelmant *et al.* 1996, Onishi *et al.* 2002, Sato *et al.* 2004) and *dec1* and *dec2* genes (Grechez-Cassiau *et al.* 2004) are additional clock components that are involved in the transcription of *Bmal1* by inhibiting its expression. All in all this entire complex mechanism is involved in displacing or inhibiting the transcription of CLOCK/BMAL1 heterodimer and thus inhibiting the transcription of *Per* and *Cry* genes. The period of this process is approximately 24 hours.

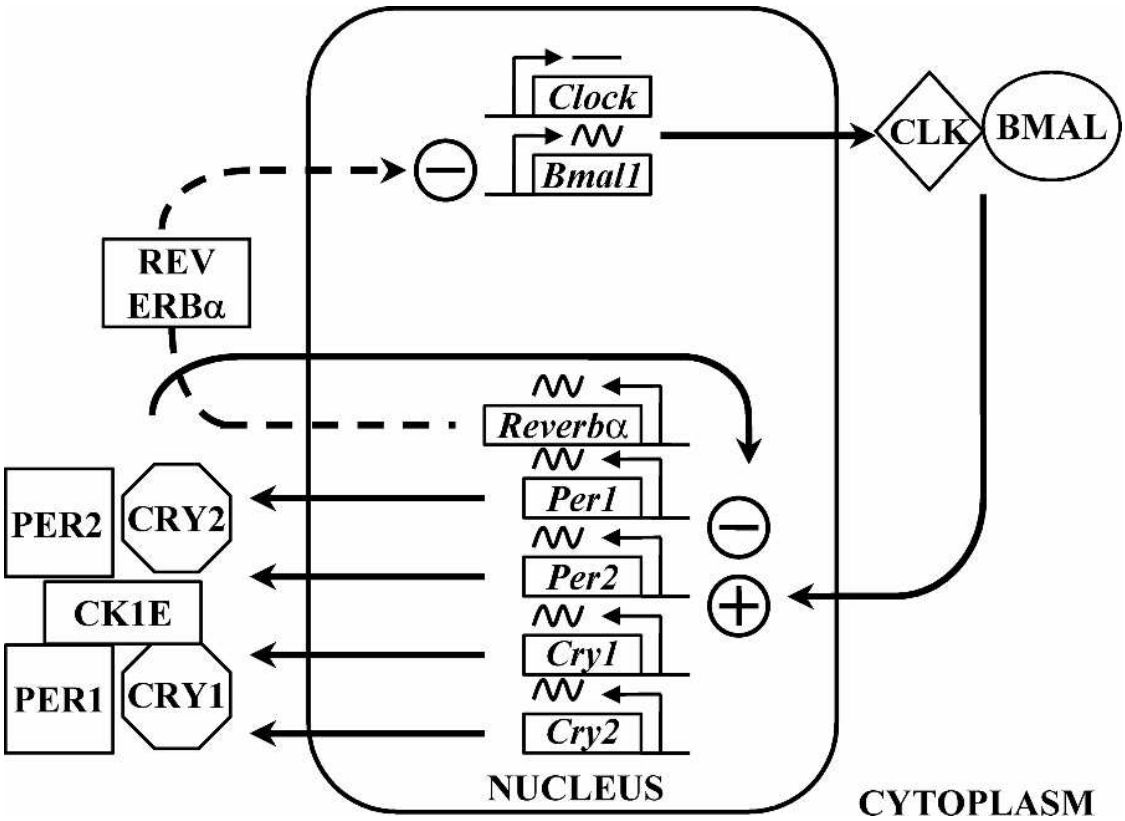

Fig. 3: Proposed molecular mechanism of the circadian oscillator
Positive drive is characterised by the CLK/BMAL heterodimer complex which initiates transcription of P*er1*, *Per2*, *Cry1* and *Cry2*. Proteins from these genes which form complex with casein kinase 1ε inhibit CLK/BMAL1 induction of *Per* and *Cry* genes. Meanwhile, REV ERBα protein inhibits *Bmal1* transcription. The negative loop consists of the PER1/CRY1 and PER2/CRY2 heterodimers which repress their own transcription through inhibition of the transcription activation by the BMAL1/CLOCK heterodimer. Clock expression is normally constitutive while *Bmal1* is rhythmic and in antiphase to the *Per* and *Cry* gene expression. A secondary loop not shown here involves *dec1* and *dec2* (adapted from Kennaway 2005).

Table 1: Clock-genes, which play a role in the generation of cellular rhythmicity in vertebrates (adapted from Kennaway 2005)

Gene name	Abbrevation	Alternative name	Homologe
Period 1	*Per1*		
Period 2	*Per2*		
Period 3	*Per3*		
Circadian Locomotor Output cycle kaput	*Clock*		*Npas2* (*MOP4*)
Brain muscle ARNT-like protein 1	*Bmal1*	*MOP3*	*Bmal2*
Cryptochrome 1	*Cry1*		
Cryprochrome 2	*Cry2*		
Differentiated Embryo Chondrocytes 1	*Dec1*	*Stra13, Sharp2, BHLHB2, Clast5*	
Differentiated Embryo Chondrocytes 1	*Dec2*	*Sharp1, BHLHB3*	
Nuclear receptor subfamily 1 group D member 1	*Rev erbα*		
Timeless	*Tim*		

1.5 The dual oscillator model

In diverse activity patterns of vertebrates Aschoff (1954), Pittenbrigh (1960) and Daan (1976) noted that the activity commonly includes two distinct components, namely onset and end of activity, that move in opposite directions with changes in photoperiod as well as with changes in the intensity of illumination. These activity patterns have been characterised by two peaks of activity, the first major peak that occurs in the early subjective night and a later component that precedes dawn. This kind of activity pattern provided an indicator for a two oscillator model that includes the phenomenon of "splitting" of circadian rhythms of activity and bimodality with one large peak of activity followed by a small peak. In 1976 Pittendrigh and Daan following earlier suggestions of Aschoff (1960) and Hoffman (1960) proposed the two-oscillator-model suggesting that the mammalian circadian pacemaker consists of a morning oscillator (M) following dawn and an evening oscillator (E) tracking dusk (Pittendrigh and Daan 1976b). This model provides specific predictions towards a pacemaker missing either E or M oscillators, or even both. The different phenotypes of mutant mice e.g. *mPer1*^{Brdm1}, *mPer2*^{Brdm1}, *mCry1*$^{(-/-)}$ and *mCry2*$^{(-/-)}$ (Albrecht *et al.* 2001) exhibited possible indications of missing either the M or E oscillator or even both in the double mutants (van der Horst *et al*.1999). Hence, Daan *et al.* (2001) modulated or supplemented the M - E oscillator model suggesting that the molecular mechanism of the circadian clock relates to the two oscillators. Further they predicted that M oscillator deficient animals are supposed to show suppressed advance shifts in the phase response curve, and animals lacking E oscillator should show suppressed delays. This prediction was confirmed by Albrecht *et al.* (2001) and Steinlechner *et al.* (2002). Both groups demonstrated in *Per* mutant mice that *Per2* gene expression is delayed in *Per1* mutants and vice versa in *Per2* mutants. Moreover, Steinlechner *et al.* (2002) have confirmed the prediction of Daan *et al.* (2001) that M deficient animals should show shorter period under constant condition and animals lacking E should reveal a shorter period length under increasing constant light conditions. In this case they used also *Per1* and *Per2* mutant mice as models which demonstrate the lack of M and E oscillators, respectively. Thus, the

data of *Per* mutants tally reasonably well with the predictions from the hypothesis of Daan et al. (2001). However, there are experiments with double knockouts (Oster *et al.* 2002) and with *Cry* knockouts under constant light conditions (Spoelstra *et al.* 2004) that contradict this theory. Thus, further investigations are required to confirm or refute this theory.

1.6 Aims and scope of the current study

The aim of this study was to investigate whether *Per1* and *Per2* clock genes have an impact on reproduction in adult and ageing females. For this study I used *Per1* and *Per2* mutant females with the genetic background B6.129S7. As this mouse strain does not produce detectable amounts of melatonin any influence of melatonin on gene expression as well as on the physiological functions could be ruled out.

The current thesis addresses three topics with regard to the fertility in female mice: reproductive outcome, glucocorticosteroid rhythm, and onset of puberty.
The second chapter concerns reproductive outcome in adult and ageing *Per* mutant female mice. I discuss the reproductive outcome in female mice focussing on pre- and postnatal phases. As I did not find any differences in the reproductive success between young adult $Per1^{(-/-)}$, $Per2^{(-/-)}$ and wild-types females I focused on the study of physiological and behavioural parameters such as estrus cycles, body condition, digestibility of food, food intake and protein preference, maternal behaviour and number of produced offspring, restricted to ageing female mice. Additionally, to find out whether the maternal condition of *Per* mutants has an impact on the development of their offspring I also examined the condition of their pups from birth until weaning. As ageing *Per* mutant females exhibited an impairment in fertility and fecundity as well as showing enormous changes in their estrus cycle compared to wild-type females their uteri were removed at the end of the experiment and checked for the number of embryonic implantations that were then compared with the number of successfully produced offspring.

The results concerning postimplantation failures during pregnancy gave us an indication of an altered stress response of the transgenic mice as compared to the wild-type that could negatively influence embryonic development as well as maternal care. Therefore, the levels of faecal corticosteroid metabolites were determined in order to establish basal unstressed values of the daily profile of corticosteroid rhythms in young adults and old females as well as the corticosteroid concentration at the four estrus stages (Chapter 3). To avoid any additional stress we used a non-invasive method for determining the glucocorticosteroids. Moreover, to investigate whether the stress hormones influence activity in female mice I compared patterns of locomotor activity, particularly during estrus cycle as well as the amount of activity with the corticosteroid level.

Furthermore, I assumed that the low reproductive success in transgenic mutants could also be associated with their advanced development as compared to the control group. Thus, the development of the females was investigated by recording the onset of vaginal opening and first regular estrus cycle in the absence of males under LD conditions (Chapter 4). In addition, in connection with the M - E oscillator model accounting for the fact that the females lacked either morning or evening oscillator we examined whether the free-running period (≠ 24 hours) may influence the onset of the puberty. Therefore, locomotor activity and estrus cycle were recorded in transgenic females under constant light conditions.

Chapter 2

Based on a manuscript submitted to: *Journal of Reproduction*; in revision

2

Low reproductive success in *Per1* and *Per2* mutant mouse females due to accelerated ageing?

Violetta Pilorz and Stephan Steinlechner

Abstract

The major mechanism regulating daily behavioural and physiological rhythms is based on feedback loops among several clock genes and their protein products and is located in the suprachiasmatic nuclei of the hypothalamus. Recent studies on mice with mutations in the *Clock* gene have shown that this mutation disrupts estrus cyclicity and interferes with successful pregnancy. In our study we investigated changes in the estrus cycles and reproductive outcome in correlation with the functionality of the endogenous clock in female mice. In order to determine whether the two molecular components of the main clock, *Per1* and *Per2* genes, have an effect on the length of the estrus cycle and the reproductive success we used *Per1*-deficient and *Per2*-deficient females. We show that although fecundity of young adult *Per* mutant females does not differ from that of wild-type females, middle-aged *Per* mutant mice are characterised by lower reproductive success than the control group. This may be a consequence of irregularity and acyclicity of the estrus cycle of the mutants that is similar to that of old wild-type females. Besides, we demonstrate that *Per* mutant females have significantly more embryonal implantations in the uterus than successfully delivered offspring. We discuss whether *Per1* and *Per2* mutations cause an advanced ageing resulting in acyclicity, and hence in poor reproductive success.

2.1 Introduction

The suprachiasmatic nuclei of the hypothalamus (SCN) play an important role in coordinating circadian rhythms of numerous physiological functions as well as of behaviour. In this way they allow the organism to anticipate the changing environment and thus increase its survival (Aschoff 1964, Moore and Greenwald 1974, Zucker *et al.* 1976). Reproduction is one of the most important factors enabling the animal to optimise its biological efficiency and hence its fitness. The secretion of sex hormones that regulate reproductive functions such as estrus cycle, pregnancy and lactation is characterised by optimal timing and rhythm (Turek and Van Cauter 1994, Johnson and Day 2000, Dolatshad *et al.* 2006). This rhythmic behaviour of pregnant and lactating females also forms the cyclic environment for foetuses and neonates before the development of their retinohypothalamic tract (Weaver and Reppert 1995). Thus, maternal condition, health and intact rhythmicity may affect reproductive success. Disruption of the circadian signal caused by SCN lesions interrupts cyclic ovulation (Gray *et al.* 1978). Specific changes in the molecular function in the SCN, e.g. mutation of C*lock* in mice have caused a disrupted estrous cyclicity and poor reproductive outcome associated with increased foetal absorption during pregnancy and high pregnancy failure (Kennaway 2004, Hoshino *et al.* 2006). *Clock* mutations also affect the growth of pups negatively (Miller *et al.* 2004, Dolatschad *et al.* 2006).

In different species of mammals it has been shown that the ability to produce offspring progressively declines in ageing females. This decline is reflected by smaller litters in polytocous species, increasing intervals between litters or individual births, increased resorptions, abortions and stillbirths (Talbert 1977). One of the measurable markers of reproductive decline is the vaginal estrus cycle. Its length and frequency reflect the hormonal milieu (Nelson *et al.* 1982). Changes in these parameters may give information about age-related changes in the hormonal control of reproduction. Ageing female rats exhibit an increase in irregularity in estrous cycles (Meites *et al.* 1976, Lu *et al.* 1979, Matt *et al.* 1987). Labhsetwar (1969) found that 21 month-old rats with irregular estrus cycles show an increase in follicle-

stimulating hormone (FSH) content and a lower luteinizing hormone (LH) concentration. The reduced LH content of older rats is explained by a continuous negative feedback of estrogen on the hypothalamus (Redding 1972). However, studies on rats have shown that disrupted connection to the SCN may also result in estrous acyclicity and infertility (Brown-Grant *et al.* 1977, Wiegand *et al.* 1980).
Per1 and *Per2* clock genes (Zheng *et al.* 1999 and 2001) belong to the important molecular components of the pacemaker and are associated with the feedback loops of transcription and translation of genes such as *Clock* (Vitaterna *et al.* 1994), *Bmal1* (Bunger *et al.* 2000), as well as *Cry1* and *Cry2* genes (Vitaterna *et al.* 1999). Steinlechner *et al.* (2002) have shown in male mice that the expression of *Per1* and *Per2* clock genes can be accelerated and decelerated by light, respectively. This phenomenon is consistent with the hypothesis that *Per1* is important for advancing and *Per2* for delaying the clock (Albrecht *et al.* 2001, Daan *et al.* 2001).

All these investigations regarding the effects of *Per* genes on physiological functions and behaviour were made on male mice but there are no reports about the impact of these genes on the physiological functions and the reproductive success in female mice. Thus, in the present study we investigated the impact of *Per1* and *Per2* clock genes on reproductive function associated with maternal behaviour and the food consumption during pre- and postnatal phases as well as the estrus cycle length in middle-aged females, using *Per1* and *Per2* mutant mice. In addition, the body weights of the offspring were recorded to monitor their development.

2.2 Material and methods

Animals
We used homozygous B6.129S7-*Per1*^{tm1Brd} (*Per1*$^{(-/-)}$) (Zheng *et al.* 2001) and B6.129S7-*Per2*^{tm1Brd} (*Per2*$^{(-/-)}$) (Zheng *et al.* 1999) as well as wild-type female mice B6X129.S7 as a control group for our experiments. 30 virgin females ranging in age from 7 to 9 months at the beginning of the experiment were housed individually in polycarbonate transparent cages type III containing wood shavings as bedding material. The animals were divided into three groups of 10 individuals: in a control

group represented by wild-type B6.129S7, in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ mutant females. They were maintained at 22 ± 1°C under a light-dark cycle of 12h light : 12h dark with lights on at 22.00 h and off at 10:00 h. The room was equipped with two red lamps which were permanently on and provided < 6 lux at cage level during the dark phase. Food and water were given *ad libitum*. 10 males of each strain were kept in the same room. For copulation each female in proestrus was placed in the cage of a male for one day and then put back in her own cage. During the non-reproductive and reproductive phases all females were weighed daily. Towards the end of the gestation period the cages of pregnant females were checked daily for the presence of offspring. The day on which young were found was recorded as the date of birth (day 1). To determine the reproductive success of the three strains we recorded litter sizes from the first and second parturition immediately post partum and on the day of weaning. After parturition weights of mothers and pups were recorded daily until the 10th day after weaning.

All experiments including animals were in accordance with the animal protection laws of the Federal Republic of Germany and the guidelines of the European Union. Moreover, the experiments were approved by the district government of Hannover.

Examination of estrus cycle

To distinguish the different phases and length of the estrus cycle vaginal smears were taken daily in the mice's activity phase between 11.00 - 13.00 h; i.e. 1 – 2 h after lights off. To facilitate our vision in darkness we used a red-light forehead-lamp of < 6 lux. The smears were obtained by inserting a fire-polished metal diluting loop into the vagina not further than 1mm so as to minimize the possibility of inducing pseudopregnancy (Sinha *et al.* 1978). The vaginal smear was transferred to a drop of saline solution on a microscopic slide. Dry smears were fixed in MeOH for 2 min, stained with methylene blue solution for 2 min and washed with deionised water. After staining they were evaluated microscopically at a magnification 10x60. The vaginal smears were taken daily for 6 weeks in order to identify the length of estrus cycle and to monitor the estrus status for successful copulation with a male at the end of this period. After 1.5 months each female in proestrus was placed for 24 hours in a cage with a male and was then checked for a vaginal plug to verify copulation.

The smears were classified into different estrus stages according to the description of Nelson *et al.* (1982). Prolonged diestrus and permanent estrus for at least 15 days were considered as anestrus. An estrus cycle of > 6 days was considered as prolonged and one of < 3 days as irregular.

Embryonic implantations in uterus

At the end of the experiment all remaining females of 10-13 months of age (wild-type females N = 7, *Per1*$^{(-/-)}$ N = 6 and *Per2*$^{(-/-)}$ N = 10) were killed by CO_2 and their uteri were removed. The uteri were stained using 10% ammonium sulphide solution in accordance with the description of Kopf *et al.* (1964) for counting the implantation scars.

Protein selection

Protein preference experiments were conducted during the non-reproductive and reproductive phases of females. Three isocaloric diets containing 14%, 20% and 30% protein (Altromin, Germany) were offered to the females of all three genotypes. Non-reproductive and pregnant females received 10.00 g (± 0.01 g) of each diet daily, while lactating females received 15.00 g (± 0.01 g) of each diet daily. To recognise the different diets, the food-powder was dyed with neutral test food colouring (Ceasar and Loretz GmbH Hilden, Germany) and shaped into little balls. The three colours red, yellow and green were rotated daily in order to avoid a colour preference. The food consumption was recorded daily by removing all food from the cage (including husks and pieces of food in the bedding) and separating according to diet (colour). Food was replaced after weighing, and at the end all bedding was replaced. The left-over isocaloric diets were dried to a constant weight and then weighed.

Average daily metabolic rate

We used metabolisable energy intake that is required by a caged animal to maintain its constant body energy content to represent average daily metabolic rate (ADMR) (Degen *et al.* 1998). Measurements were conducted on non-reproductive and twice on reproductive females (*Per1*$^{(-/-)}$, *Per2*$^{(-/-)}$ and wild-type) in the middle stage of

pregnancy (day 11-12) and on the 5th day of lactation (2nd parturition). Metabolisable energy intake was measured placing the non-reproductive and reproductive females with their offspring over 24 h in a cage with new nesting material and fresh bedding and providing them with weighed portions of food. After 24 h all food and all faeces were removed. The faeces were stored frozen at -20°C before measuring the caloric value. The faecal samples were dried at 65°C for 24 hours, weighed and homogenised, crushing the faeces with a mortar to powder. The gross energy content of the isocaloric dry diet was 12.67 MJ/kg. The gross energy content (kJ/g) of about 0.85 g homogenised faeces of each sample from the non-reproductive and reproductive females was determined by adiabatic bomb calorimetry (Automatic O_2 bomb calorimeter 6200, Parv Instrument Germany GmbH 442M) calibrated with the aid of an ascending mass of benzoic acid (26.454 MJ/kg). We calculated the ADMR for each female as follows:

ADMR = (gross energy intake kJ – faecal energy output kJ) / body mass (g)

Maternal behaviour

To analyse maternal behaviour the lactation that took 21 days was divided into three stages: 1) early stage of lactation: one day after parturition 2) middle stage of lactation: on the 11th day of lactation 3) last stage of lactation: one day before weaning. All three stages of lactation were videotaped for 24 h. However, maternal behaviour was observed only during the active phase, namely from 10.00 till 22.00 h under infrared light. The behaviour patterns of lactating primiparous (6 wild-type, 5 *Per1*$^{(-/-)}$) and multiparous (9 wild-type, 3 *Per1*$^{(-/-)}$ and 4 *Per2*$^{(-/-)}$) females were recorded continuously using the focal sampling method. The analysis of maternal behaviour was conducted using the Observer program (Noldus, Netherlands). The following behaviours were scored: Maternal care, i.e. behaviour directed at the young: 1) licking and grooming pups, 2) nursing: mother lying in nest either on her side or over the pups while nursing; self-directed activities: 3) feeding: mother going to the food containers and eating or drinking water from a water bottle, 4) resting: mother standing or lying outside the nest without any body contact to any offspring, 5) grooming: self-grooming and self-licking, 6) locomotor activity: walking, running and climbing on the cage lid.

Reproduction of young adult females

To compare the reproductive success of middle-aged animals with young adult females we used females of the same three strains as above, aged 2-6 months. The females were kept under the same light and temperature conditions as the middle-aged females, albeit in a separate room. The females gave birth twice. The pups were weaned on the 21st day post parturition. The litter sizes of the second pregnancy were recorded after parturition and on the day of weaning.

Statistics

For statistical analysis data were tested for normal distribution using Kolmogorov-Smirnov-test. Differences in body mass, maternal behaviour, food intake, and ADMR were compared during the non-reproductive and reproductive stages using repeated–measures ANOVA. Differences among the groups were tested with Turkey's Honest Significant Difference-Test. In order to test differences between specific periods or two groups we used the paired t-test. Non-normally distributed data were tested with non-parametric tests either with Mann-Whitney U-test for independent data or Wilcoxon-test for dependent data. Results are presented as means ± SE or as ± SD. The differences were considered significant at $p < 0.05$.

2.3 Results

Reproductive success

The results of reproductive outcome of all three stains of middle-aged (9-12 months of age) and young adult females (2-6 months old) are presented in Table 1. All primiparous (1st parturition) and multiparous (2nd parturition) females of all three strains were successful in becoming pregnant (Table1). In contrast to *Per1* and *Per2* mutant females, almost every pregnant wild-type female gave birth and was a successful breeder (Table1).

Primiparous wild-type and *Per1* mutant females produced the same litter size as multiparous females, i.e. they did not differ in their reproductive outcome (U-test: $Z = 0.54$, $p = 0.61$). However, only 33.3% of multiparous *Per1* mutant females bred successfully, whereas 90% of the wild-type multiparous females bred. In contrast, *Per2* mutant females as primiparous females did not raise a single pup successfully (Table 1). The pups were obviously eaten by their mothers. As multiparous females they produced significantly smaller litter sizes than wild-type females did (U-test: $Z = 2.26$, $p > 0.05$).

Young adult *Per1* and *Per2* mutants produced significantly larger litter sizes - on average 7.9 and 7.0, respectively than the middle-aged (9-12 months of age) *Per* mutants (U-test: *Per1*$^{(-/-)}$: $Z= 2.26$ $p > 0.05$; *Per2*$^{(-/-)}$: $Z = 2.83$, $p > 0.05$). Furthermore, the litter size of young adult *Per* mutants did not differ from the litter size of the adult wild-type (Kruskal-Wallis-ANOVA: $Ch^2 = 3.76$, $FG = 2$, $p = 0.15$). Hence, primiparous as well as multiparous middle-aged mutant female mice *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ are characterised by a low reproductive success in comparison with the ageing wild-type females.

Table 1: Reproductive outcome of middle-aged and multiparous (2nd parturition) young adult wild-type, *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ female mice (each group N = 10).

Middle-aged females

	Wild-type		*Per1*$^{(-/-)}$		*Per2*$^{(-/-)}$	
parturition	1st	2nd	1st	2nd	1st	2nd
pregnant (n)	10	10	10	9	10	8
gave birth (n)	6	10	5	5	7	5
successful breeder (n)	6	9	5	3	0	4
mean litter size (± SD)	6.3 ± 2.2	5.1 ± 1.8	4.6 ± 2.5	5.7 ± 2.9	?	2.8 ± 1.7

Young adult females

	Wild-type	*Per1*$^{(-/-)}$	*Per2*$^{(-/-)}$
pregnant (n)	10	10	10
gave birth (n)	10	10	10
successful breeder (n)	10	10	10
mean litter size (± SD)	8.0 ± 2.3	7.9 ±1.9	7.0 ± 1.9

All of the following observations concern the middle-aged female mice only:

Estrus cycle

All wild-type females exhibited a regular estrus cycle (100% over 6 weeks). A four day estrus cycle in wild-type females occurred significantly more often than in *Per* mutant females (Kruskal-Wallis-ANOVA: $Ch^2 = 10.05$, FG = 2, $p < 0.001$) (Fig. 1). However, *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females were acyclic in 37.07% and 44.85% of this time, respectively. The remaining time was characterised by cyclicity of four, five and longer than 6 days, while *Per1* mutants exhibited more often 4 day cycles than prolonged cyclicity (ANOVA: $F_{2, 27} = 4.01$, $p < 0.05$, Post hoc-test. $p = 0.04$).

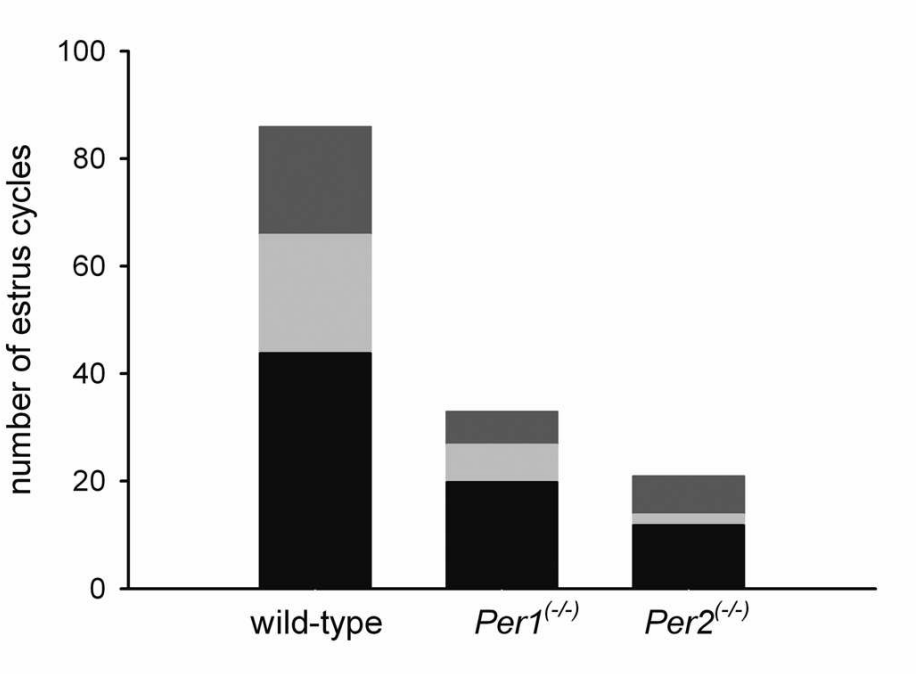

Fig. 1: Total number of estrus cycles over 1.5 months in all non-reproducing females of each strain, N = 10 each. Black bars: 4 day estrus cycle; light grey bars: 5 day estrus cycle; dark grey bars: > 6 day estrus cycle.

Implantations in uteri and live offspring

Per1$^{(-/-)}$ and *Per2*$^{(-/-)}$ females had a significantly higher number of embryonic scars in the uterus compared to the total number of their live offspring from the 1st and 2nd parturition (Fig. 2) (Wilcoxon-test: *Per2*$^{(-/-)}$: $Z = 2.93$, $p < 0.05$, $N = 10$; *Per1*$^{(-/-)}$: $Z = 2.02$, $p < 0.05$, $N = 6$). Wild-type females, however, did not differ significantly between the number of implantations and the number of live offspring (Wilcoxon-test: $Z = 1.82$, $p > 0.05$, $N = 7$). Moreover, *Per1*$^{(-/-)}$ females showed a significantly lower number of implantations than the control group (U-test: $Z = 2.45$, $p < 0.05$).

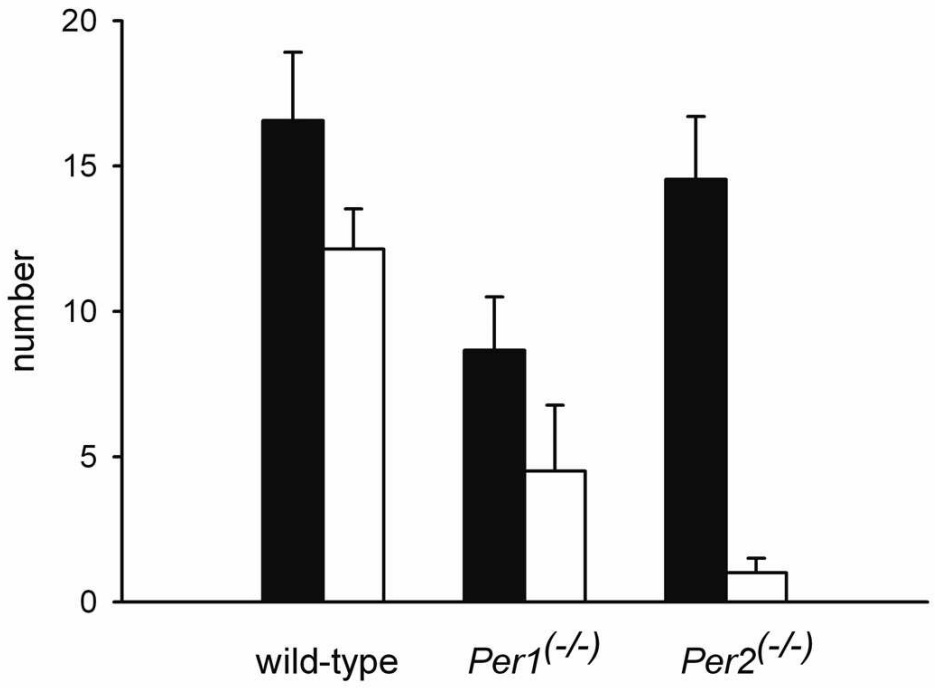

Fig. 2: Mean number of embryonic scars in uteri after two pregnancies and successful production of offspring in reproductive females. Wild-type: N = 7, *Per1*$^{(-/-)}$: N = 6, *Per2*$^{(-/-)}$: N = 10; Black bars: embryonic scars (± SE), white bars: number of live offspring = successfully reared until weaning (± SE).

Body mass of reproductive female mice

Both a*s* primiparous and as multiparous, the wild-type and *Per1*$^{(-/-)}$ females did not differ significantly in their body mass, neither during their non-reproductive nor during their reproductive phases (Fig. 3). Therefore, the body mass data of each strain were pooled. (rep. ANOVA: non-reproductive: wild-type: $F_{1, 13} = 3.79$, $p > 0.05$; *Per1*$^{(-/-)}$: $F_{1, 9} = 0.56$, $p > 0.05$; pregnancy: wild-type: $F_{2, 36} = 0.23$, $p > 0.05$; *Per1*$^{(-/-)}$: $F_{2, 21} = 1.28$, $p > 0.05$ and lactation: wild-type: $F_{1, 10} = 0.09$, $p > 0.05$; *Per1*$^{(-/-)}$: $F_{1, 9} = 0.34$, $p > 0.05$). The body mass increased from the non-reproductive to the lactation period in all three strains (Fig. 3). *Per2*$^{(-/-)}$ females were significantly heavier than wild-types and *Per1* mutants, both during the non-reproductive (rep. ANOVA: $F_{2, 15} = 13.41$, $p < 0.05$) and reproductive phase (rep. ANOVA: pregnancy: $F_{2, 17} = 8.70$, $p < 0.05$; lactation: $F_{2, 15} = 7.03$, $p < 0.05$). *Per1* mutant females had a lower body mass than the wild type females during the non-reproductive (rep. ANOVA: $F_{1, 12} = 18.97$, $p < 0.05$) and pregnant stages (rep. ANOVA: first stage: $F_{1, 13} = 7.32$, $p < 0.05$; middle stage: $F_{1, 13} = 7.58$, $p < 0.05$) but not when they were lactating (Fig. 3).

On the day before parturition all three genotypes had a significantly increased body mass (one-way ANOVA: wild-type: $F_{1,11} = 70.207$, $p < 0.05$; *Per1*$^{(-/-)}$: $F_{1,11} = 252.7$, $p > 0.05$; *Per2*$^{(-/-)}$: $F_{1,11} = 285.40$, $p > 0.05$) (Fig. 3). The loss of body mass after parturition was less pronounced in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females compared to the wild-type mice.

Lactating wild-type females showed significant changes in their body mass (ANOVA: $F_{20, 168} = 2.74$, $p > 0.05$) from the first day up to the last day of lactation. These changes were characterised by a decrease in body weight during the first three days, an increase till the 16th day of lactation (t-test: $t = 4.43$; $FG = 8$; $p > 0.05$) and an additional decrease till the last day of lactation (21st day). *Per1*$^{(-/-)}$ as well as *Per2*$^{(-/-)}$ lactating mutant females did not show significant changes in their body mass over the 21 days of lactation (ANOVA: *Per1*$^{(-/-)}$: $F_{20, 84} = 1.15$; $p = 0.32$; *Per2*$^{(-/-)}$: $F_{20, 63} = 0.29$; $p = 0.99$).

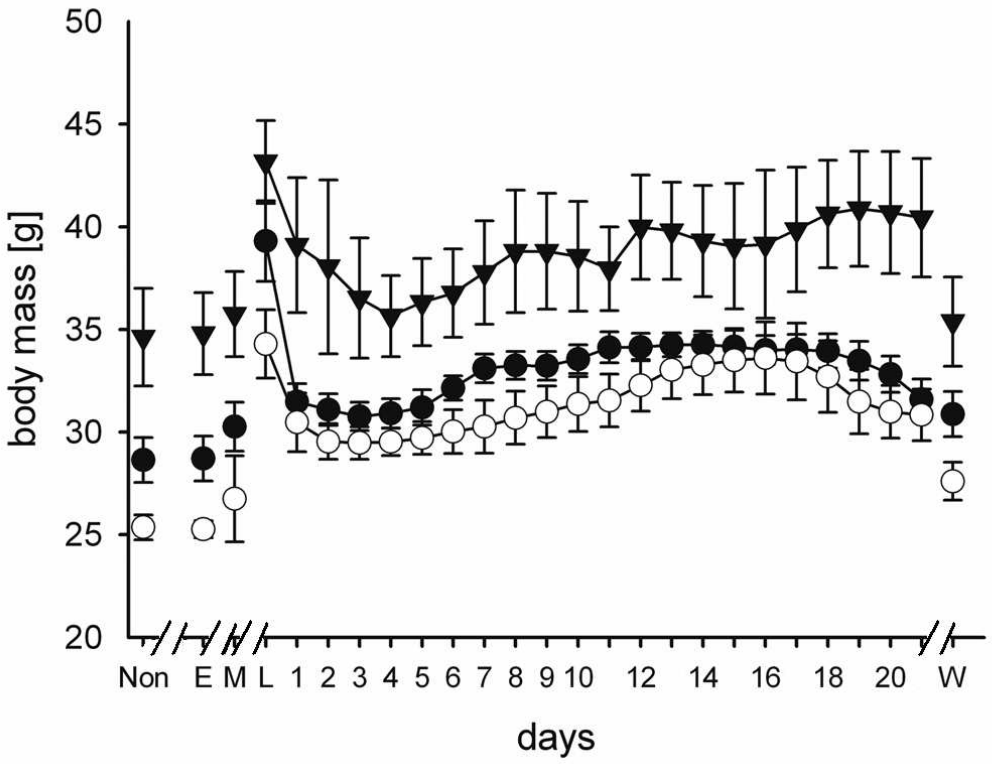

Fig. 3: Mean body mass (± SE) in non-reproductive and reproductive females. Black circles: wild-type females (control group); open circles: *Per1*$^{(-/-)}$ females; black triangles: *Per2*$^{(-/-)}$ females; Non: non-reproductive; E: early stage of pregnancy (first 3 days of pregnancy); M: middle stage of pregnancy (10-12 days of pregnancy); L: last stage of pregnancy (last 3 days before parturition); W: weaning (10 days after weaning).

Protein preference and food consumption

In contrast to non-reproductive wild-type females that preferred 20% protein content (rep. ANOVA: $F_{2, 24} = 1.28$, $p < 0.05$), *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females did not show any preference to a certain protein content (rep. ANOVA: *Per1*$^{(-/-)}$: $F_{2, 9} = 0.78$, $p = 0.79$; *Per2*$^{(-/-)}$: $F_{2, 9} = 0.78$, $p = 0.79$). During pregnancy (rep. ANOVA: *Per1*$^{(-/-)}$: $F_{4, 36} = 0.87$, $p = 0.49$; *Per2*$^{(-/-)}$: $F_{4, 27} = 0.28$, $p = 0.89$; wild-type: $F_{4, 72} = 0.43$, $p = 0.79$) and lactation period (rep. ANOVA: *Per1*$^{(-/-)}$: $F_{2, 12} = 0.31$, $p = 0.74$; *Per2*$^{(-/-)}$: $F_{2, 9} = 0.28$, $p = 0.11$; wild-type $F_{2, 24} = 1.28$, $p = 0.29$) all three strains did not prefer any particular protein content either. All three strains consumed the same amount of food per day during their non-reproductive phase (Fig. 4). The daily food consumption during pregnancy and lactation in all three strains was characterised by a significant

increase in food intake. Pregnant wild-type females increased their food consumption progressively (rep. ANOVA; $_{F3, 24}$ = 15.01, p < 0.001) correlating positively with the body mass (Spearman's: R = 0.26; t(N-2) = 3.61; p < 0.05). *Per1* mutant females increased their daily food consumption by 20% up to midterm pregnancy but without any relationship to body mass. *Per2* mutant females did not show any correlation either. Around day 16 the pups start to eat solid food and therefore we compared food consumption of the females only up to this day. During lactation all females of the three strains increased their food consumption (Fig. 4): Wild-type and *Per1* mutant females increased their food consumption by 67.6% and 63.3%, respectively up to the 16th day of lactation. *Per2* mutant females increased their food consumption till the 16th day of lactation by 40%. This increase, however, did not occur continuously as in wild-type females (Fig. 4).

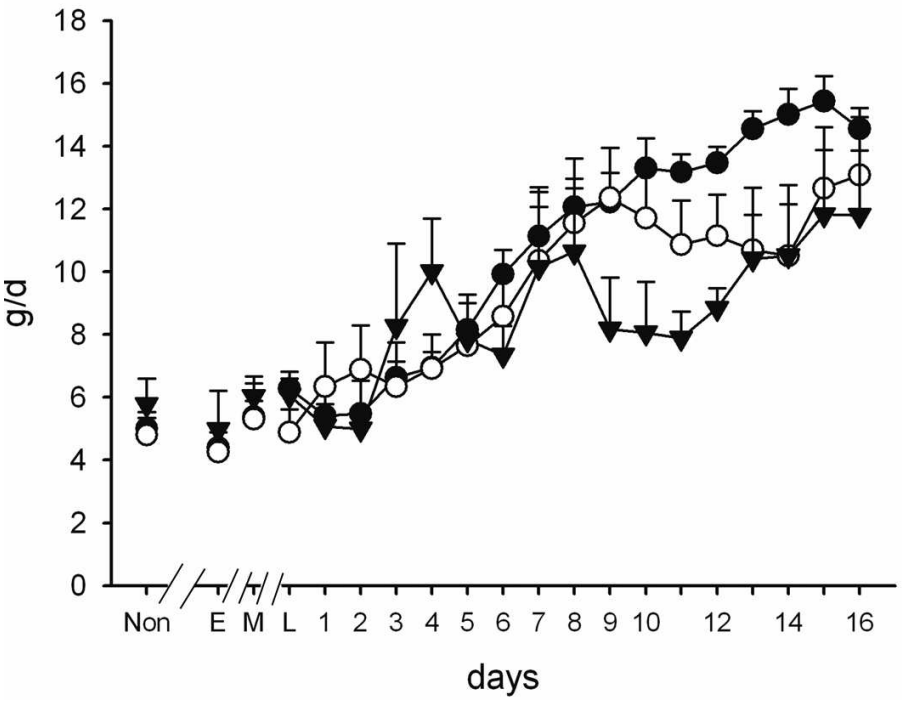

Fig. 4: Mean food consumption in non-reproductive and reproductive female mice (± SE). Black circles: wild-type females (control group); open circles: *Per1*$^{(-/-)}$ females; black triangles: *Per2*$^{(-/-)}$ females; non: non-reproductive, E: early stage of pregnancy (first 3 days of pregnancy); M: middle stage of pregnancy (10-12 days of pregnancy); L: last stage of pregnancy (last 3 days before parturition).

Average daily metabolic rate

The average daily metabolic rate of *Per1* mutant females increased from the non-reproductive period to the lactation period (ANOVA: $F_{3, 18} = 9.63$, $p < 0.001$) (Fig. 5). However, during pregnancy the ADMR of *Per1* mutant females was lower than that of the control group (U-test: $Z = 2.84$, $p < 0.001$) but similar to *Per2* mutant females (U-test: $Z = 0.87$, $p = 0.43$). *Per2* mutants did not show any significant variance in ADMR (ANOVA: $F_{3, 16} = 2.07$, $p = 0.14$). During non-reproductive and both reproductive periods their metabolisable energy intake was lower than that of the wild-type females (U-test: non-reproduction: $Z = 2.35$, $p < 0.05$, pregnancy: $Z = 0.87$, $p < 0.001$, lactation: $Z = 2.35$, $p < 0.05$).

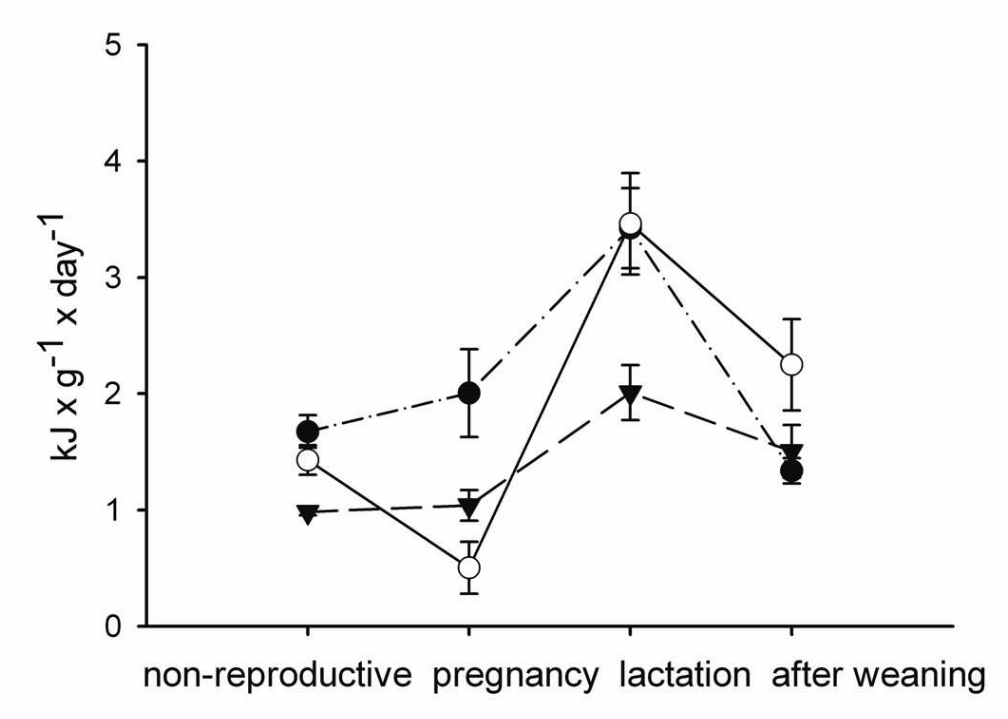

Fig. 5: Average daily metabolic rate in non-reproductive and reproductive females (± SE). Black circles: wild-type females (control group); open circles: *Per1*$^{(-/-)}$ females; black triangles: *Per2*$^{(-/-)}$.

Maternal behaviour

Since the behavioural parameters for primiparous as well as multiparous wild-type and *Per1* mutant females did not differ significantly, these data for the two

pregnancies were pooled (Wilcoxon; wild-type: first stage: Z = 0.73; p = 0.73; middle stage: Z = 0.31, p = 0.75; last stage: Z = 0.31, p = 0.75; ANOVA; *Per1*$^{(-/-)}$: $F_{5,18}$ = 0.25, p = 0.93). On the day after birth all three strains spent approximately 60% of their time in the nest, presumably nursing (Table 2). During the middle and last stage of lactation wild-type and *Per1*$^{(-/-)}$ females spent significantly less time in the nest (ANOVA: *Per1*$^{(-/-)}$: $F_{2, 12}$ = 7.58, p < 0.001; wild-type: $F_{2, 24}$ = 32.42, p < 0.001) (Table 2, and Fig. 6 A and B). This decrease in duration in the nest correlated negatively with an increase in feeding (Spearman's; *Per1*$^{(-/-)}$: R =.-0.71, t(N-2) = - 3.68, p < 0.001; wild-type: R = - 0.86, t(N-2) = - 8.33, p < 0.001) and resting outside the nest (Spearman's; *Per1*$^{(-/-)}$: R =.-0.57, t(N-2) = - 2.52, p < 0.001; wild-type: R = - 0.62, t(N-2) = - 4.03, p < 0.001). In contrast, *Per2*$^{(-/-)}$ females spent the same time in the nest during the three lactating stages (ANOVA: $F_{2, 9}$ = 0.27 , p = 0.77) (Table 2).

Table 2: Mean duration (h ± SEM) of being in nest of lactating female mice during the dark phase, i.e. active phase (total time 12h). First: first stage of lactation (day after parturition).

	Wild-type	*Per1*$^{(-/-)}$	*Per2*$^{(-/-)}$
First	7.09 ± 0,07	7.31 ± 0.09	5.72 ± 0.34
Middle	4.00 ± 0.15^{b}	4.97 ± 0.26^{b}	6.27 ± 0.16^{A}
Last	4.02 ± 0.09^{b,B}	5.09 ± 0.21^{b,B}	6.42 ± 0.25^{B}

b. significance within the groups vs. first stage of lactation
A: significance between *Per2*$^{(-/-)}$ and wild type
B: significance vs. the other genotypes
First: first stage of lactation (day after parturition); Middle: middle stage of lactation (11th day of lactation); Last: last stage of lactation (day before weaning)

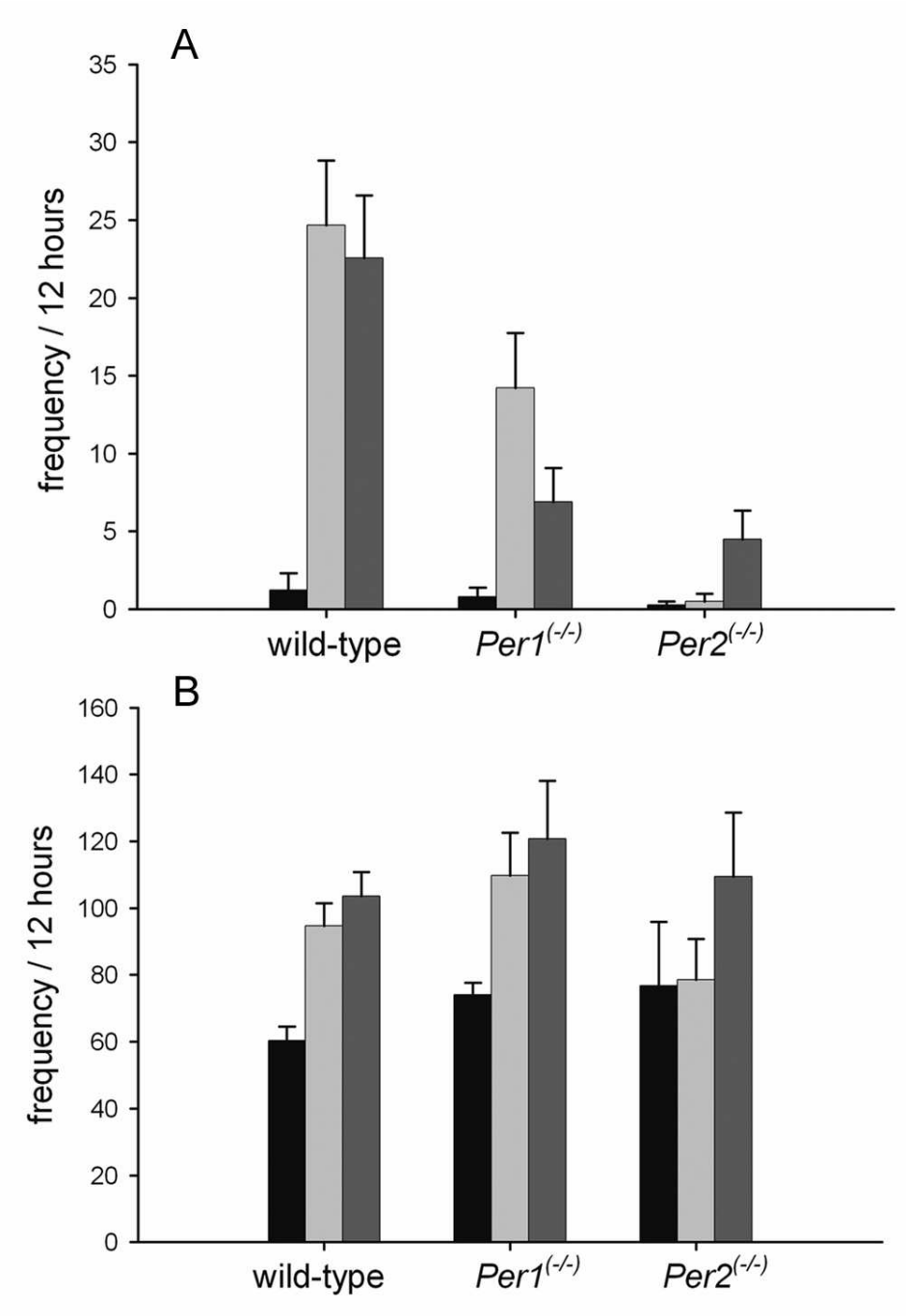

Fig. 6: Frequency of mean resting outside of nest (A) and mean food consumption (B) during the dark phase in lactating females (± SE). Black bars: day after parturition; light grey bars: 11th day of lactation; dark grey bars: day before weaning.

Body mass of offspring

The offspring of all three strains did not differ in their individual body mass development, neither during the lactation nor 10 days after weaning (Fig. 7), despite the fact that *Per2*$^{(-/-)}$ females produced significantly smaller litters than *Per1*$^{(-/-)}$ and wild-type females (see also Table 1).

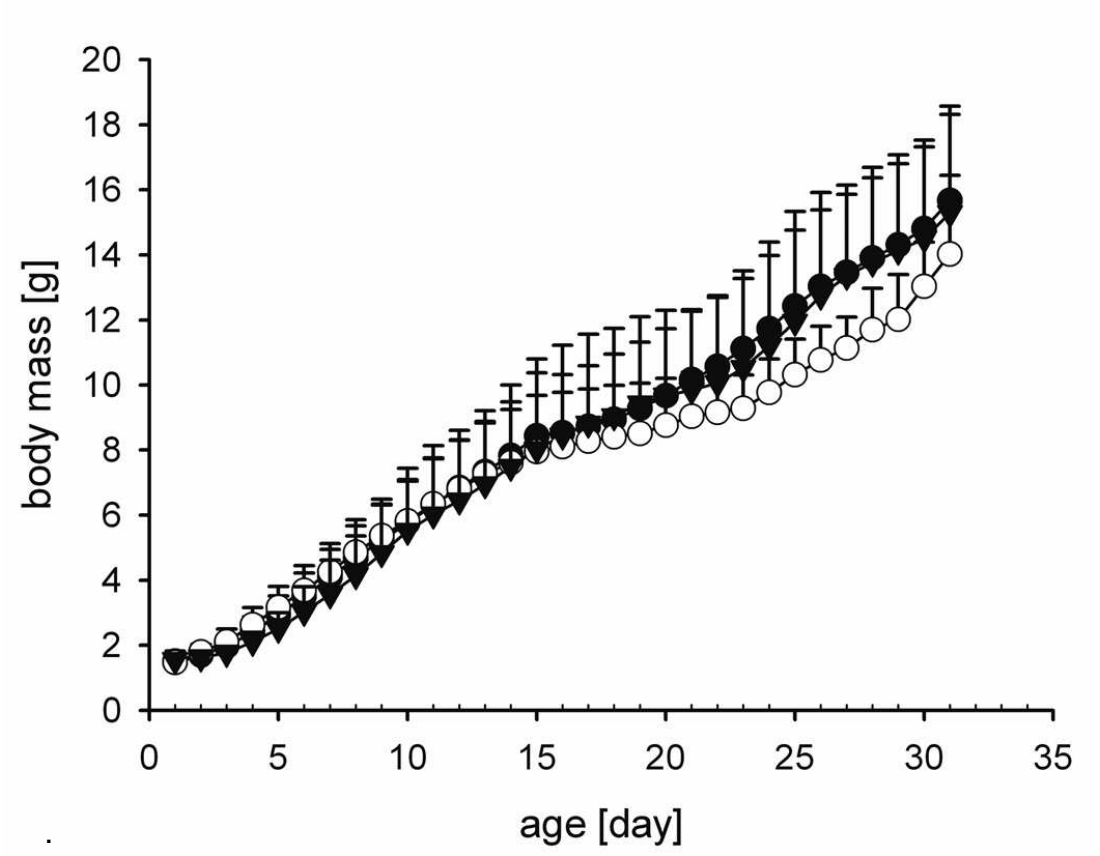

Fig. 7: Mean body mass (± SD) of offspring from the 1st day till 31st day of age. Black circles: wild-type offspring (control group); open circles: *Per1*$^{(-/-)}$ offspring; triangles: *Per2*$^{(-/-)}$ offspring. Day 1 represents day of the birth.

2.4 Discussion

Reproductive success

Young adult *Per1* and *Per2* mutant females have the same reproductive success as wild-type females, confirming experiences by other laboratories with breeding these strains (U. Albrecht and S. Daan, personal communication). Here we show, however, that the middle-aged *Per1* and *Per2* mutant females have a significantly lower reproductive success. They produce either less litters and/or smaller litter sizes than the middle-aged wild-type and young adult mice (Table 1). These results indicate an accelerated reproductive ageing of the *Per* mutants versus the wild-type strain. This conjecture is supported by the lower incidence of regular estrus cycles in *Per* mutants compared to wild-type females. *Per* mutants were acyclic for 41% of the observation period (1.5 months), while the wild-type females of the same age showed no acyclicity at all and the normal estrus cycle of four days occurred significantly more often in the control group. Acyclic phenomena, e.g. persistent vaginal cornification or leukocytosis, characterise the decline of cyclicity in rats and mice (Nelson *et al.* 1982, Matt *et al.* 1987, Markowska 1999). The initial phases of declining of cyclicity from the 13th month of age onwards are prolonged and irregular cycles and cause a delay in the preovulatory rise of estradiol (Nelson *et al.* 1981). These prolonged estrogen secretions in mice and rats have been explained as being a result of incomplete or delayed luteinization (Nelson *et al.* 1982, Matt *et al.*1987). Ageing female rats displaying irregular cyclicity and acyclicity (permanent estrus or permanent diestrus) are unable to perform successful gestation (Matt *et al.* 1986). This decline in fecundity is usually accompanied by a decrease in the number of live pups. Thus, the lower reproductive success of middle-aged *Per* mutants may be due, at least in part, to changes in the length of the estrus cycle and in its frequency. In addition, even mutant females who have a regular estrus cycle also often failed to reproduce successfully. Since only 33% (3 out of 9) of multiparous pregnant *Per1* and 50% (4 out of 8) of *Per2* mutants were successful in breeding they seem to be characterised by less fecundity compared to the control group where almost all pregnant females were successful breeders. In the present study none of

the middle-aged primiparous pregnant *Per2* mutant females were successful in rearing offspring. The number of successfully breeding *Per1* mutants is significantly lower than that of the control group, most obviously during the 2nd parturition.

Comparisons of implantation scars with the successfully bred offspring in the present study confirm that the decreased fecundity in middle-aged *Per* mutants is related to reproductive failures during gestation. Studies on rats have demonstrated that an induced or spontaneous delay of ovulation results in increases in abnormal development and subsequent death of embryos (Fugo and Butcher 1966, Fugo and Butcher 1971, Fossum *et al.* 1989). The delay of ovulation is affected by an early rise in the preovulatory levels of estrogen in relation to time of ovulation and hence it results in decreased implantation rate, increased pre- and post-implantation death of embryos, retarded development and embryonic abnormalities (Butcher and Pope 1979). Moreover, the study of Talbert (1971) has emphasised that the degenerating corpora lutea in older mice could be a result of early embryonic death rather than the cause of embryonic death. Thus, the uterus of aged animals appears to be responsible for post-implantation loss in those females with implantation sites (Parkening *et al.* 1978). Results from the present study reveal that the number of foetal implantation scars of *Per2* mutants does not differ from that of the control group. However, the total number of surviving pups in *Per2* and *Per1* mutants is significantly lower than the number of implantation scars. Thus, *Per* mutants seem to suffer from post-implantation loss. Previous studies on rabbits (Larson and Foote 1972) and hamsters (Parkening and Soderwall 1974) have suggested that a reduction in blood flow and vascular impairment of an aged uterus may cause reduced litter sizes that are related to post-implantation loss. The functional alterations causing decreases in both fertility and fecundity in ageing *Per1* and *Per2* mutant female mice are currently unknown. We can conclude, however, that age-related changes in the cyclicity of 9-12 month old *Per1* and *Per2* mutant females are qualitatively similar to those of 13-16 month old C57BL/6J female mice that were investigated by Nelson and co-workers (1982).

Energetic investment of females and maternal care

The period of pregnancy is characterised by an increase in metabolic rate (Trojan and Wojciechowska 1968) that is related to a rapid growth of the embryos, causing an enhancement of energy intake by the female (Migula 1969). Thus, increased food consumption during pregnancy is essential. However, *Per2* mutant females did not increase their food intake at all during pregnancy, while *Per1* mutant females exhibited an increase in food consumption during midterm pregnancy. The lack of increased food intake during pregnancy in *Per2* mutant females may be explained firstly by their high rate of post-implantation embryonic death and secondly by the low number of lactating females. The control group showed a continuous increase in food consumption in all three stages, equivalent to the rise of consumed food as reported for pregnant common voles (Migula 1969). The fact that *Per1* and *Per2* mutant females are lighter and heavier than the control group, respectively, is a further indicator that *Per1* and *Per2* clock genes regulate the metabolic rate differently and, hence, the *Per* mutants obviously utilise consumed energy in a different way. The average daily metabolic rate representing metabolisable energy intake required by an animal to maintain constant body energy content (Degen *et al.* 1998) is significantly lower in pregnant *Per* mutants compared to the wild-type females. This fact might be a further explanation for their failures during pregnancy that were obviously associated with post-implantation death of embryos or abortions due to insufficient energy levels for the development of their embryos.

Maternal behaviour such as nursing, licking and warming the naked offspring is important for the development and, thus, for the survival of their pups (König and Markl 1987). However, the maternal care can vary with the size of the litter (Priestnall 1972, König and Markl 1987). Females caring for large litters are known to produce more milk than females caring for small litters (Kumaresan and Turner 1967). Nevertheless, the time budget for nursing behaviour usually decreases gradually with the growth of the pups during the first 12 days, irrespective of the litter size (Chiang *et al.* 2002). From day 17 onwards the house mouse females spend more time away from the litter and the young start to eat solid food (König and Markl 1987). In the present study we demonstrate that immediately after parturition all three strains spent

60% of their time with the pups. This maternal behaviour corresponds to the maternal care of house mice right after delivery (König and Markl 1987). During the midterm of lactation young mice develop hearing, grow fur and they start to use their eyes (Fuller and Wimer 1966, König and Markl 1987). Generally, litter size and its energy costs are characterised by a positive correlation (Millar 1977, Sikes 1998). Additionally, in rats and mice it has been shown that net production and its energy cost are higher during lactation than during gestation (Kenagy *et al.* 1989). *Per1* mutant and wild-type females reach a plateau of their body mass around day 13 of lactation. Obviously this body weight gain by day 13 is associated with the demands of the quickly developing young. Thus, from day 13 onwards the lactating female mice exhibit the highest energy requirement. In the lactating house mouse it has been shown that the maximum body mass correlates with the maximal food consumption (König and Markl 1987). In the current study we have demonstrated that lactation period is characterised particularly in *Per1* mutants and wild-type females by the highest daily metabolic rate. *Per2* mutants, however, do not increase their metabolisable energy intake during lactation and it is significantly lower than that of the control group. Such a low energy requirement in lactating *Per2* mutant females is likely due to their small litter size.

Per2 mutant females show very unusual behaviour, spending during the whole lactation period the same time in the nest as on the day after parturition. This means that *Per2* mutants spend less time feeding and drinking for their own good plus for their milk production than the control group and *Per1* mutants. Equal growth in young from small and large litters requires that mothers of large litters ingest more nutrients or produce a higher milk quantity than those with small litters (Jameson 1998). Previous studies on rodents have shown that the offspring in small litters grow significantly more than the young in large litters. This is not the case for pups of *Per2* mutant females. Even though *Per2* mutants produced smaller litter sizes and spent more time in the nest during the whole lactation period than wild-type and *Per1* mutants the body mass of the offspring of the three genotypes during lactation did not differ from each other. Thus, the total energetic investment of *Per2* mutant females in only 2.8 pups must certainly be lower than that of wild type females with

larger litter sizes. *Per1* mutant females also spend significantly more time in the nest, while having a similar number of offspring as the control group. Nevertheless the offspring of *Per1* mutant females has the same body mass as those of wild-type females. The time that lactating *Per* mutants spend with their litter cannot necessarily be equated to nursing. The cause of the similar growth of *Per1* and wild-type pups in large litters might be milk transfer per pup with the same energy rate in *Per1* and wild-type females (Rogowitz 1996). Since the offspring of *Per1* mutant females does not gain more weight despite their mothers' intense time spent in the nest, the mothers do not seem to invest too much of their additional energy in their offspring but rather consume it themselves.

All in all, *Per1* and *Per2* clock genes do not seem to have any influence on the reproductive outcome in young adult females. However, between 9-12 months of age the *Per* mutant females are clearly less fertile than wild-type females. Furthermore, their estrus cycles, characterised by prolongation and acyclicity, were comparable to the estrus cyclicity of ageing 13-16 month old C57BL/6J female mice. Thus, these results suggest that *Per* clock genes cause an accelerated ageing resulting in poor reproductive fitness. Further studies are needed in order to clarify the impact of *Per* genes on the ageing process and on the hormonal level in association with SCN functionality.

Chapter 3

Manuscript in preparation

3

Age-related changes in corticosteroid secretion and in wheel-running activity during the estrus cycle in *Per* mutant female mice

Violetta Pilorz, Stephan Steinlechner, Henrik Oster

Abstract

The endogenous clock, located in the suprachiasmatic nuclei (SCN), controls the timing of numerous physiological functions and behaviour. Clock genes such as *Per1* and *Per2* are part of the molecular system of this main pacemaker and are involved in the regulation of the circadian rhythmicity of many processes including reproductive behaviour, estrus cycle and hormones. In the present study we have investigated the impact of *Per1* and *Per2* clock genes on the corticosteroid secretion in relation to the estrus cycle and locomotor activity in adult and old female mice. We show that a lack of *Per2* clock gene in adult female mice causes an impairment of daily faecal corticosteroid secretion both during dark and light phase. In addition, the corticosteroid level of *Per2* mutant females is lower during the preovulatory stages than that of wild-type females. Furthermore, the locomotor activtity in *Per2* mutant females does not show any variability during the estrus stages. *Per1* mutant females, however, reveal the same timing of corticosteroid secretion and increased locomotor activity during estrus as do wild-type females. Additionally, we demonstrate that 20 month-old acyclic *Per1* and wild-type females show reduced corticosteroid level during the active phase compared to the adult state. Old acyclic *Per2* mutants, however, exhibit rhythmicity in corticosteroid secretion. Moreover, old females have similar onset of the locomotor activity as do adult females.

3.1 Introduction

In mammals numerous physiological and behavioural processes are characterised by rhythmicity related to environmental changes such as alternation between light and darkness. Also physiological mechanisms underlying female sexual behaviour show cyclic nature (Nelson 2005). The hormones, estrogens and corticosteroids influencing each other in their secretion coordinate temporal reproductive behaviour and ovulation. This hormonal interaction represents an adaptive mechanism that has evolved in rodents to optimise the chances of fertilisation and ultimately to ensure the survival of the species (Horvath 1998, Levine 2002, Cavigelli *et al.* 2005). Several studies on rats and mice have identified changes of corticosterone concentration with a daily peak around the beginning of the daily activity cycle (Carey et al. 1995, Atkinson *et al.* 1997). Moreover, the glucocorticosteroid concentration associated with estrus cycle stage shows peak corticosterone levels highest at proestrus, accompanied by a maximum of estrogen secretion, and lowest at estrus stage (Carey *et al.* 1995, Atkinson *et al.* 1997, Touma *et al.* 2003, Cavigelli *et al.* 2005). Thus, this functional relationship between corticosteroids and estrogens seems to be characterised by the subsequent up and down regulation of corsticosterone and of estrogene secretions that may contribute to the probability of successful reproduction: energetic readiness for sexual behaviour, sexual motivation, sexual reflexes, and ovulation (Levine 2002).

In addition, there are findings indicating a role for glucocorticosteroids as temporal signals that may synchronise the oscillators (Sage *et al.* 2002) as well as entrainment factors for physiological and behavioural events at specific circadian times (Moore-Ede *et al.* 1977). This temporal function of glucocorticosteroid on reproductive behaviour is emphasised by a study on female hamsters showing that glucocorticosteroids affect the onset and amount of daily locomotor activity (Zucker *et al.* 1980). The earlier onset of activity and higher amount of activity during ovulation increases the female's odds of locating males and thus of copulation. Such patterns of advanced activity onset have been termed scalloping (Albers 1981).

The rhythmicity of these interacting hormones, estrogens and glucocorticosteroids, which frame the reproductive behaviour, is regulated similar to many other essential physiological processes by the main pacemaker, the suprachiasmatic nuclei (SCN). The clock genes *Per1* and *Per2* clock genes are part of the molecular system of the master clock (Sun *et al.* 1997). There is markedly growing interest regarding the effects of *Period* genes in different processes and pathways e.g. corticosteroid secretion (Dallmann *et al.* 2006), oxygen consumption (Dernbach 2002), and sleep (Kopp *et al.* 2002). Dallmann *et al.* (2006) demonstrated in *Per1* mutant male mice that a deficit of the *Per1* gene is associated with severe consequences for the regulation of glucose metabolism and glucocorticosteroid secretion reflected by impaired daily glucocorticoid metabolite (CORT) rhythms in faeces.

Previous studies indicate also that the circadian rhythm of glucocorticosteroid changes dramatically in age-related diseases such as Alzheimer's disease (Hatfield *et al.* 2004). Normal ageing, however, may also induce changes in the circadian rhythmicy of corticosteroid secretion (van Cauter *et al.* 1996) that is characterised by progressive loss of control of the hypothalamic-pituitary-adrenal axis (HPA-axis) in rats and humans, resulting in hypersecretion of glucocorticoids during times of stress.

Although numerous mouse models for a wide range of human stress-related disorders have been developed surprisingly little is known about the impact of the endogenous clock. In the present study we focussed particularly on the effect of *Per* genes on basal activity regulation over the HPA-axis during the estrus cycle in relation to age. For the male mouse it is known that old mice are characterised by an irregular secretion pattern of corticosterone explained by reduced hippocampal mineralocorticoid and glucocorticoid receptor mRNA (Dalm *et al.* 2005). Accordingly, we expect to detect also in female mice alterations in HPA axis activity that relate to the estrus cycle. Furthermore, we measured the locomotor activity that was expected to show an activity pattern during estrus cycle reflecting estrogene and corticosterone levels (Cavigelli *et al.* 2005).

3.2 Material and methods

Animals

We used homozygous B6.129S7-Per1^{tm1Brd} (*Per1*$^{(-/-)}$) (Zheng *et al.* 2001) and B6.129S7-Per2^{tm1Brd} (*Per2*$^{(-/-)}$) (Zheng *et al.* 1999) as well as wild-type female mice (B6X129.S7) as a control for our experiments. In order to compare adult and old females of each group N = 10 we used adult females 4-7 months old as well as old females aged 18-20 months. The females were housed individually in polycarbonate transparent cages type II containing wood shavings as bedding. They were kept under light-dark cycle (12h light : 12h dark) with lights on at 22.00 hr and the room temperature was maintained at 22±1°C. Food and water were given ad libitum.

Examination of estrus cycle

To distinguish the different phases and length of the estrus cycle vaginal smears were taken daily in the mice' activity phase between 11.00 – 13.00 hr. To facilitate our vision in darkness we used a red-light forehead-lamp < 6 lux. The smears were obtained by inserting a fire-polished metal diluting loop into the vagina, not more than 1mm to minimize the possibility of inducing the pseudopregnancy (Sinha *et al.* 1978). The daily vaginal smear was transferred to a drop of saline solution on a microscopic slide. Dry smears were fixed in MeOH for 2 min stained with methylene blue solution for 2 min and washed with deionised water. After staining they were evaluated microscopically at a magnification 10x60. The vaginal smears were taken for 1.5 months in order to identify the length and variability of estrus cycles. The smears were classified into different estrus stages according to the description of Nelson *et al.* 1982. Prolonged diestrus and permanent estrus for at least 15 days were considered as acyclic estrus. An increased estrus cycle (< 6 days) was considered as prolonged and reduced one (> 3 days) as irregular.

Running wheel activity

The running wheel activity was monitored in adult and old wild-type, *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ mutant females. The running wheel (diameter 15 cm) was fixed to the cage lid. A magnet was attached to the outside of the wheel and each passing of this magnet by a reed relay corresponds to a single wheel revolution. The signal was recorded by a computer using an I/O card and stored together with time of day as a number of wheel revolutions every 6 min.

CORT-concentration during the estrus cycle

Corticosteroid metabolites (CORT) were measured from faecal samples in order to avoid any additional stress caused by handling and collecting blood samples. In this experiment faecal samples were collected only once a day, namely three hours after the light was switched off. This time point reflects the peak of CORT secretion in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ mutant male mice as shown by Dallmann *et al.* (2006). For the collection of faeces each female was placed in a cage with new wood shavings as bedding. As we measured metabolised corticosteroids which represent a 4 hours time lag of the plasma corticosteroid level (Touma *et al.* 2004, Cavigelli *et al.* 2005) we can be sure that placing the females in a new cage does not influence measurable CORT concentration. After one hour all faeces were removed and stored at -20°C for the CORT analysis. Moreover, before collecting faecal samples the estrus cycle was recorded using vaginal smears (see above).

Daily profile of CORT

In order to determine the daily CORT secretion of adult and old *Per* mutant and wild-type females we kept 6 animals of each group individually in metabolic cages for collecting urine and faeces separately. Before starting with the sampling the animals where habituated in these metabolic cages for 3 days. Afterwards the faeces were collected for 7 days at 3 h intervals and stored at -20°C in a plastic tube for the CORT analysis by RIA.

Faecal corticosteroid extraction

Before extraction the frozen faecal samples were thawed, dried in a vacuum - exsiccator over night, weighed and crushed with a mortar into a powder. Afterwards each sample was transferred into a 15 ml centrifuge tube to which 10 ml Ethanol (100%) was added. Each sample was then boiled in a water bath for 20 min. Upon boiling the samples were centrifuged for 15 min at 900 xg for 15 min and the supernatant was drained off into a glass dish. 5 ml Ethanol was then added to the sample tubs, vortexed for 1 min, re-centrifuged for 15 min and the supernatant added to the glass dish with the previous supernatant. Supernatants were then dried on a hot plate at 30°C, re-constituted with 1 ml methanol and then stored at -80°C for further processing.

For determining of CORT concentration of each faecal sample we used the commercially available RIA KIT (125I RIA Kit, for rats and mice MP Biomedicals, LLC Diagnostics Division, Orangeburg, NY). The assay protocol was adapted to the mouse model as described by Cavigelli *et al.* (2005).

Statistical analysis

All data are given as means (± SEM). In order to test for statistical differences between the genotypes we used repeated measurement ANOVA and Turkey Post hoc test for the differences between and within the variables. In order to compare the CORT level between the different time points in a daily profile we used one-way ANOVA. All statistical tests were carried out using Statistica. For the calculation of the amount of wheel running activity of each female we analysed their activity from four successive estrus cycles lasting 4-5 days in adult females (Nelson *et al.* 1982). In order to analyse the activity of females with acyclic estrus we chose 10 progressive days of acylicity: consistent diestrus or estrus (Felicio *et al.* 1984). All three genotypes showed a high individual variability of wheel-running activity in the successive estrus cycles. This high variability may conceal the potential variability within one estrus cycle. To eliminate this high variability between the estrus cycles we set the highest number of wheel revolutions within one estrus cycle as 100%. The period length of the locomotor activity was analysed using Chi-periodogram analysis written by T. Ruf. Differences were considered as significant at $p < 0.05$.

3.3 Results

Daily CORT secretion profile and its concentration during an estrus cycle

Adult *Per1* mutant females showed similar daily CORT secretion as did wild-type females (ANOVA: *Per1*$^{(-/-)}$: $F_{7, 48} = 7.34$, $p < 0.001$; wild-type $F_{7, 48} = 4.98$, $p < 0.001$). The daily profile is characterised by a low CORT level during the light phase and a high CORT level during the dark phase while the peak of CORT occurs between ZT 15 and ZT 18 (Fig. 1A and 1B). In contrast, adult *Per2* mutant females do not show a pronounced daily rhythm (ANOVA: $F_{7, 48} = 1.79$, $p = 0.11$) (Fig. 1C). Moreover, the CORT concentration in *Per2*$^{(-/-)}$ during the dark phase is significantly lower than that of *Per1* mutant females and the control group (rep. ANOVA: $F_{14, 144} = 3.51$, $p < 0.001$; Post hoc Turkey: *Per1* and *Per2* mutants: $p < 0.001$; *Per2* and wild-type females: $p < 0.05$). To investigate effects of age on the excretion pattern of corticosteroid metabolites we compared the daily CORT concentration of adult females with that of old females. Concerning the daily profile of steroid metabolite secretion we found that old *Per1* and *Per2* mutant females display a daily rhythm of CORT secretion similar to that of the control group (ANOVA: *Per1*$^{(-/-)}$: $F_{7, 48} = 2.39$, $p = 0.04$; *Per2*$^{(-/-)}$: $F_{7, 46} = 5.51$, $p < 0.05$, wild-type: $F_{7, 48} = 3.96$, $p < 0.05$). In contrast to the CORT concentration of the adult females, however, old *Per1* and wild-type females produce lower CORT concentration during the dark phase (ANOVA: *Per1*$^{(-/-)}$: $F_{1, 96} = 17.59$, $p < 0.001$; wild-type: $F_{1, 103} = 18.33$, $p < 0.001$). Surprisingly, old *Per2* mutant females exhibit significantly higher CORT level in the faeces at ZT 18 than the adult females (Turkey test: $p < 0.001$). However, old *Per2* mutant females produce per day in total the same CORT concentration as do adult *Per2* mutant females (ANOVA: $F_{1, 93} = 0.15$, $p = 0.70$) (Fig. 1C). Contrary to this, adult *Per1* mutant females exhibit in total more CORT in their faeces than old females (t-test unpaired: *Per1*$^{(-/-)}$: $t = 3.26$, $df = 110$, $p < 0.001$).

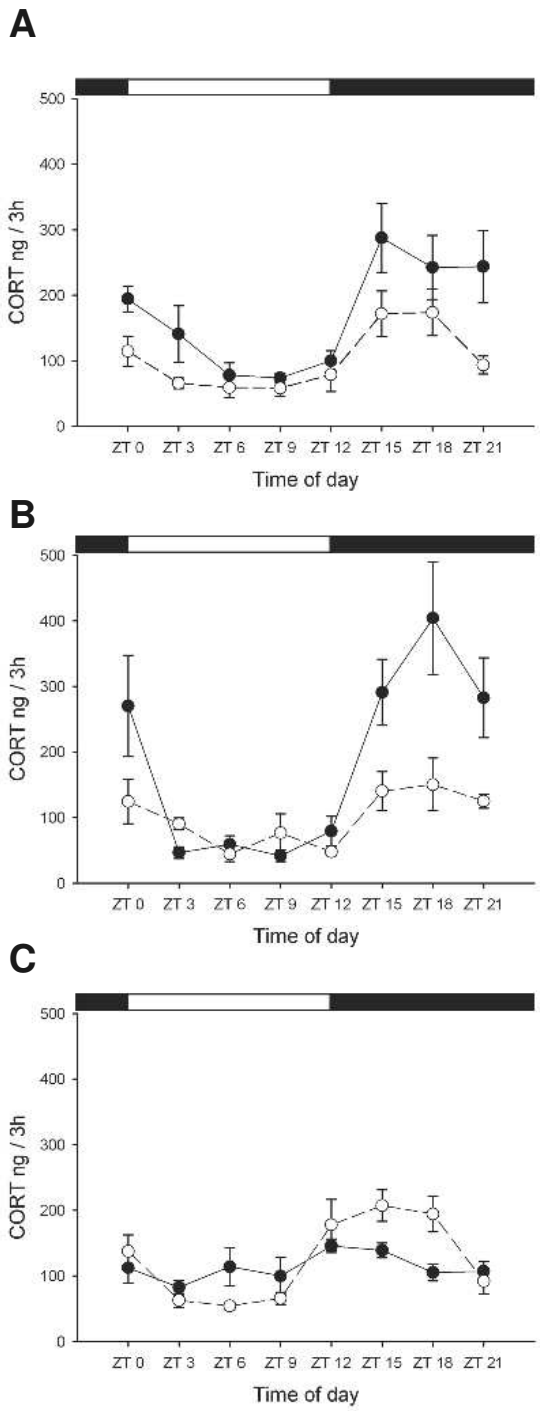

Fig. 1: Changes in mean CORT concentration during 24 hours in adult and old females. The samples were collected at 3-h intervals throughout 7 days. Figure A: wild-type; B: *Per1*$^{(-/-)}$; C: *Per2*$^{(-/-)}$. Black bars above the panels indicate dark phases of the lighting schedule. Each sample represents mean ± SEM; N = 6 in each group. Open circles: adult females; black circles: old females.

To unravel whether the CORT concentration of *Per* mutant mice reflects the estrus cycle we compared the CORT concentration during the four estrus cycle stages. Similar to the wild-type females, *Per1* mutant females display a high CORT concentration during proestrus (456.52 ng / 3h, ± 69.56 SEM) and a low CORT concentration when they are in estrus (122.81 ng / 3h, ± 24.43 SEM) and metestrus (129.95 ng / ± 3h, 31.45 SEM) (ANOVA: $F_{2, 26} = 14.45$, $p < 0.001$). *Per2* mutant females do not display any variation of CORT concentration throughout the estrus cycle (ANOVA: $F_{3, 29} = 1.19$, $p = 0.33$) (Fig. 2). They excrete the same CORT concentration in all four estrus stages. This CORT concentration corresponds to the CORT concentration at estrus and metestrus in *Per1* mutants and wild-type (Fig. 2).

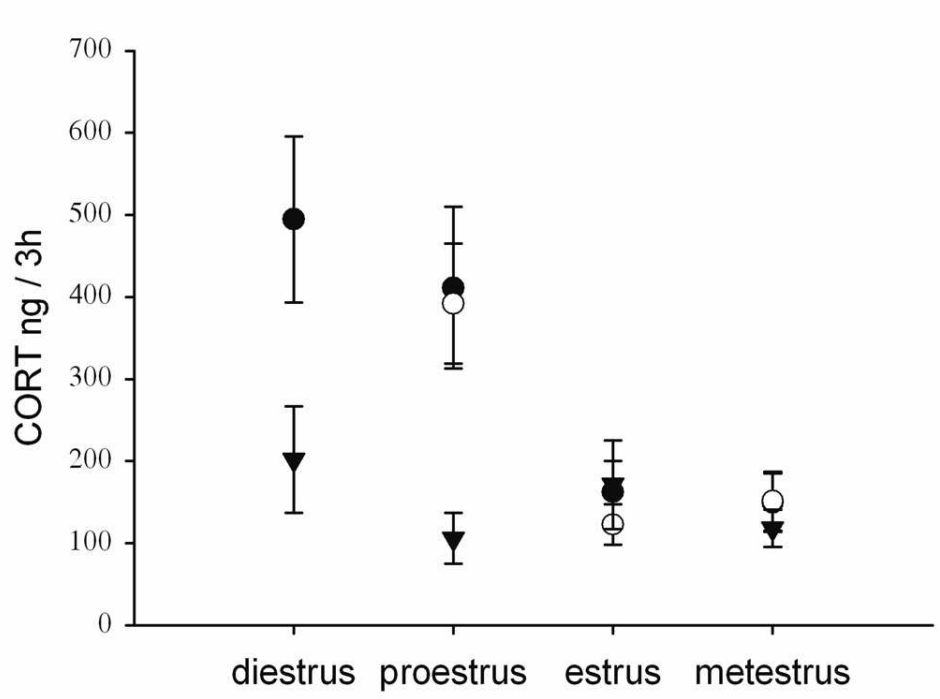

Fig. 2: Changes of CORT concentration in adult female mice during the four estrus cycle stages in the active phase (dark phase) (mean ± SEM). The samples were collected three hours after the light was switched off. Black circles: wild-type (N = 10); white circles: *Per1*$^{(-/-)}$ (N = 10 at proestrus, estrus and metestrus, N = 0 at diestrus); black triangles: *Per2*$^{(-/-)}$ (N = 10).

Locomotor activity during estrus cycle

To determine whether the daily CORT concentration of adult and old females, correlates with the amount of activity during an estrus cycle we analysed running wheel activity for four successive estrus cycles of 4 days each in adult and during acyclicity in old females.

Adult *Per1* mutant and wild-type females show significantly higher running wheel activity at estrus than during the other estrus stages (rep. ANOVA: *Per1*$^{(-/-)}$: $F_{3, 9} = 8.66$, $p = 0.003$; wild-type: $F_{3, 7} = 16.56$, $p < 0.001$) (Table 1). In contrast, adult *Per2*$^{(-/-)}$ females do not show any variation in activity at the particular estrus stages (rep. ANOVA: $F_{3, 7} = 1.16$, $p = 0.39$) (Table 1).

Table 1: Proportion of amount of wheel-running activity (± SEM) during an estrus cycle in adult females.

	Wild-type	*Per1*$^{(-/-)}$	*Per2*$^{(-/-)}$
Proestrus	67.58% ± 8.17	71.17% ± 8.91	67.40% ± 8.66
Estrus	93.72% ± 4.13 [b]	92.81% ± 4.40 [b]	87.82% ± 6.91
Metestrus	64.01% ± 7.89	65.58% ± 8.47	67.92% ± 20.96
Metestrus II	65.93% ± 8.17	74.23% ± 6.23	65.70% ± 7.23

b: significance within the groups vs. estrus

However, the total daily wheel activity of both adult *Per* mutant females does not differ from that of the wild-type females (Kruskal–Wallis-test: $H (2, N = 30) = 2.66$, $p = 0.26$). In contrast to adult *Per2* mutant and wild-type females old acyclic females (permanent estrus or diestrus) display significantly lower wheel activity (U-test:

Per2$^{(-/-)}$: U = 14, Z = 1.71, p < 0.001; wild-type: U = 8, Z = - 3.03, p < 0.05). Surprisingly, adult *Per1*$^{(-/-)}$ females exhibit the same wheel activity as do the old females (U-test: *Per1*$^{(-/-)}$: U = 33, Z = -0.62, p = 0.53) (Fig. 3). Furthermore, old *Per1*$^{(-/-)}$ females reveal significantly more activity than the old *Per2*$^{(-/-)}$ females (U-test: U = 19, Z = 2.06, p < 0.05). Although there seemed to be tendency towards higher activity in old *Per1*$^{(-/-)}$ mice no statistical significant differences between old *Per1*$^{(-/-)}$ females and old wild-type females could be detected (U-test: U = 18, Z = 1.73, p = 0.08). Furthermore, the onset of locomotor activity does not differ in all three young adult and old genotype females (Fig. 4).

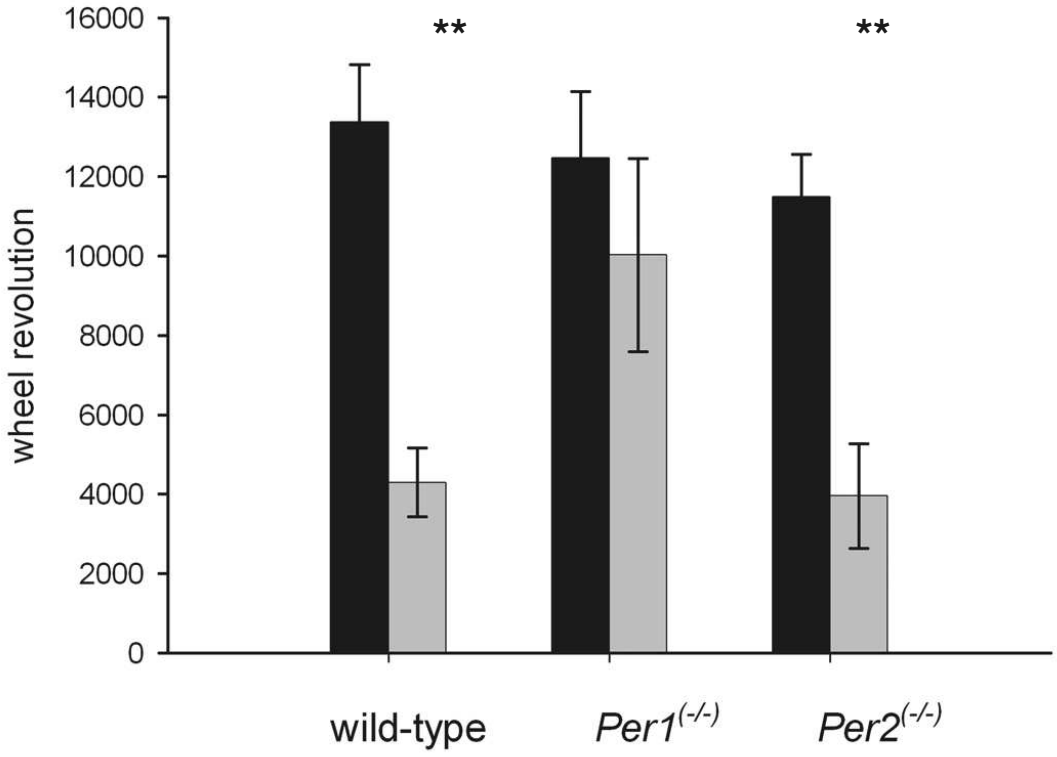

Fig. 3: Mean running wheel activity of adult and old females during one estrus cycle of 4 days in adult females and in acyclic old females (± SEM). Black bar: adult females; grey bar: old females. 2 asterisks represent highly significant.

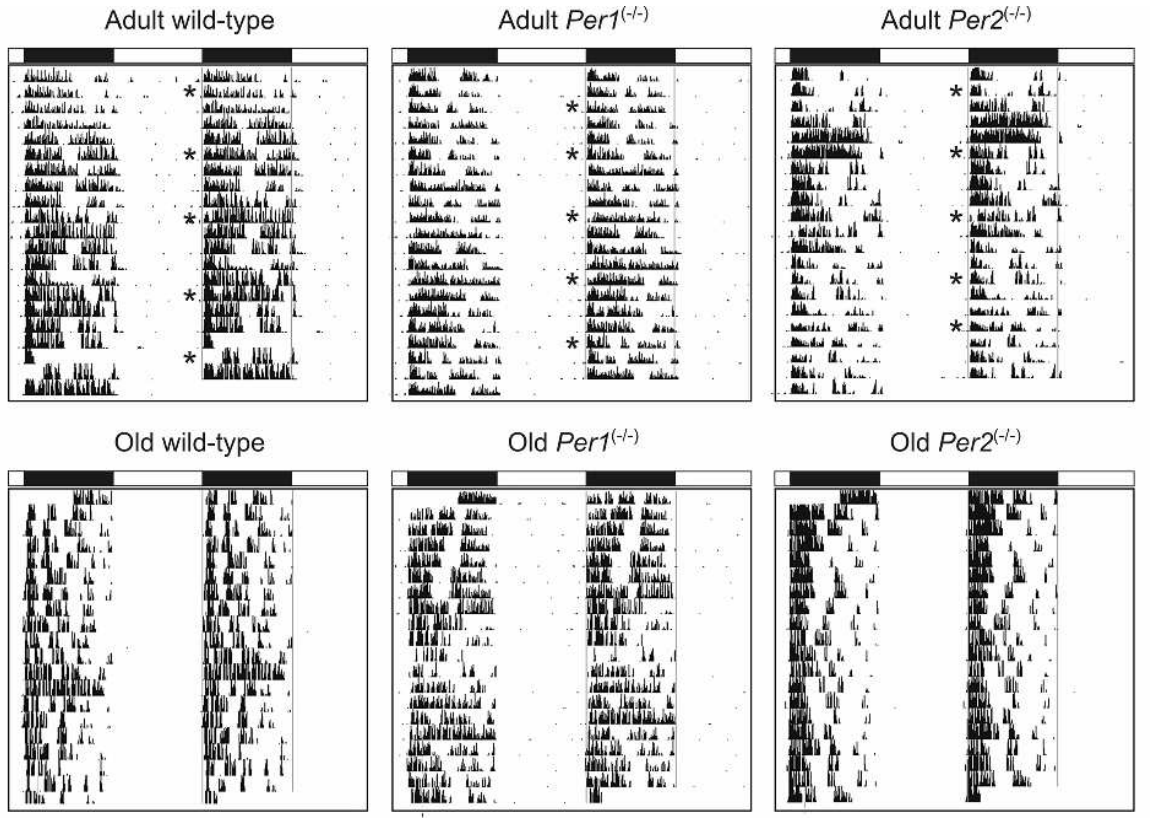

Fig. 4: Double plots of locomotor activity of adult and old females over 20 days. Asterisks in adult females represent the proestrus stage. All three old genotypes are acyclic during the 20 days. Black bars above the abscissa indicate dark phases of the lighting schedule.

3.4 Discussion

In contrast to the adult *Per2*$^{(-/-)}$ male mice (Dallmann *et al*. 2006), *Per2*$^{(-/-)}$ female mice do not show a daily rhythm in CORT secretion. Moreover, their CORT concentration is even lower during the activity phase than that of the control group and *Per1*$^{(-/-)}$ females which are characterised by daily CORT rhythmicity with a peak of CORT in the dark phase. Many mammals, including nocturnal animals like rats and mice exhibit a circadian rhythm in circulating glucocorticoid concentration occurring just prior to the onset of the activity period (Good *et al.* 2003, Touma *et al.* 2003, Millspaugh and Washburn 2004, Cavigelli *et al.* 2005). This increase of corticosteroid concentration before the onset of activity and awakening is associated

with activation of physiological processes such as metabolic rate and body temperature (Levine 2002). The activity of the HPA axis is normally analysed taking blood samples. To avoid killing a large number of animals due to repeated measurements and inducing additional stress by handling we used the non-invasive method of analysing faecal corticosteroid concentration. This method allows the determining of temporal dynamics of corticosterone production of a single individual over days and months (Kishibayashi *et al.* 1995, Touma *et al.* 2004; Cavigelli *et al.* 2005). The daily dynamic of faecal corticosterone metabolites with a peak in the activity phase in adult wild-type and *Per1* mutant females is consistent with the result of CORT peak in mice (Touma *et al.* 2003, Dallmann *et al.* 2006) and rats (Cavigelli *et al.* 2005). Additionally, the faecal corticosteroid concentrations reflect plasma corticosteroid concentrations (Good *et al.* 2003, Touma *et al.* 2003, Cavigelli *et al.* 2005) with a time lag of approximately 4 hours that is related to gut passage in a mouse (Touma *et al.* 2003). Thus, the peak of faecal CORT between ZT 15 and ZT 18 in wild-type and *Per1* mutant female mice matches the peak corticosteroid concentration in plasma that occurs at the end of the resting phase (Touma *et al.* 2003, Cavigelli *et al.* 2005).

In numerous studies on rats and mice it has been shown that there are also sex differences which appear most pronounced at the peak of the circadian rhythm (Critchlow *et al.* 1963, Hiroshige *et al.* 1973, Touma *et al.* 2003, Cavigelli *et al.* 2005). The female pattern of the HPA activation has particular functional significance with respect to the cyclic manifestation of behavioural estrus. This pattern of glucocorticosteroid levels in females, with peak corticosterone levels highest at proestrus and maximal estrogen secretion, may contribute to the probability of successful survival by regulating the timing of reproduction (Atkinson and Waddell 1995). Also in our study we demonstrate that there are sex differences regarding daily CORT secretion between the *Per* mutants. The study on *Per* male mice (Dallmann *et al.* 2006) showed that the lack of *Per1* but not *Per2* gene has severe consequences for the regulation of glucose metabolism and the HPA axis with impaired daily CORT rhythm and markedly elevated levels during the day. Contrary to this, the lack of *Per1* gene in female mice does not seem to have any negative effect on the timing of the HPA axis. However, the lack of *Per2* clock gene appears to

have a similar effect as does the *Per1* clock gene deficiency in male mice. Moreover, *Per2*$^{(-/-)}$ females have a constantly lower daily CORT level compared to the wild-type females and *Per1* mutant males. Consequently the intact *Per2* clock genes seem to have a stimulatory effect on the glucocorticosteroid secretion shortly before the onset of activity as well as during the active phase. According to the M - E oscillator model (Pittendrigh and Daan 1976, Daan *et al.* 2001) *Per2* clock genes represent the evening oscillator tracking the dusk and are characterised by increased expression in the second half of the day (Steinlechner *et al.* 2002). Therefore, it is likely that there is a potential link between *Per2* clock gene and timing of normal corticosteroid secretion with increased level during the early dark phase (active phase).

During the estrus cycle the CORT concentration of *Per2*$^{(-/-)}$ females is lower at proestrus and diestrus compared to wild-type and *Per1*$^{(-/-)}$ females, and thus does not differ from the remaining estrus stages: estrus and metestrus. The high peak of CORT during proestrus in wild-type and *Per1*$^{(-/-)}$ females corresponds to the findings of studies in female rats (Atkinson and Waddell 1997, Cavigelli *et al.* 2005). Female rodents exhibit alternations in basal and stress-induced HPA activity during the ovulatory cycle, with highest levels observed during the preovulatory period, diestrus and proestrus (Atkinson and Waddell 1995, Levine 2002, Cavigelli *et al.* 2005). In rat females the peak of CORT can even be two or three times higher during proestrus than peaks during estrus or metestrus (Cavigelli *et al.* 2005). Numerous studies have shown that the timing of such corticosteroid secretion affects the timing of sex steroid secretion and the onset of reproductive behaviour in females such as searching for potential male for copulation (Atkinson and Waddell 1995; Levine 2002). Thus, the low CORT level at proestrus in *Per2*$^{(-/-)}$ females might affect negatively the reproductive behaviour impairing or lacking the motivation components of sexual behaviour (Levine 2002). However, *Per2*$^{(-/-)}$ female mice of this age living under laboratory conditions do show a 4-5 days estrus cycle like the control group and reproduce as successfully as do wild-type females (Chapter 2). As the mating under laboratory condition is induced by human hand transferring a female into a male's cage the probability of copulation taking place is increased. Under natural conditions, in the field, however, the *Per2*$^{(-/-)}$ females would presumably not show such an alert sexual behaviour searching actively for a male. This assumption is confirmed by the

lack of significant difference in the wheel-running activity during the four estrus stages. Considering the averages of the locomotor activity, however, the *Per2* mutant females seem to show a tendency for a higher activity during estrus. Wild-type and *Per1* mutant females show significantly higher activity during estrus than during the other estrus stages. The variations of the activity in all four stages within and between the individuals emphasise the missing significant differences between the estrus stages. The fact that four out of 10 *Per2* mutant females show significantly higher activity during estrus compared to the remaining stages indicates a variability regarding activity during an estrus cycle within the strain. Therefore, to clarify the variability within *Per2* mutant females further investigations with larger sample size are required. Wheel-running in rodents might be a primary reinforcer that like sexual behaviour is linked to the kind of motor activation associated with behavioural estrus (Wang 1923, Slonaker 1925, Baranczuk and Greenwald 1973, Takahashi and Menaker 1980, Carmichael *et al.* 1981, Turek and Gwinner 1982). In female rats (Wollnik and Turek 1988) and hamsters (Fitzgerald and Zucker 1976) it has been shown that the rate of running in adult females follows a 4-5 day period, corresponding to the stage of their estrus cycle. Proestrus is characterised by increased estrogen and glucocorticosteroid levels. This hormonal change is associated with behavioural changes such as increased sexual proceptivity and increased wheel running (Markowski *et al.* 2001). These changes reflected in behaviour are often referred to as behavioural estrus. Surprisingly, in our study we have shown that *Per1* mutant and wild-type females exhibit significantly higher running wheel during estrus and not during proestrus. Hence, the estrus stage did influence locomotor patterns in both these strains, although to a much lesser extent than in rats (Wollnik and Turek 1988). Investigations on the influence of specific estrus cycle days on locomotor activity showed in the rat consistently higher locomotor activity in the proestrus compared to other estrus stages (Colvin and Sawyer 1968, Wollnik and Turek 1988). Also in out-bred female mice daily variations of locomotor activity have been shown that may reflect the ovarian cycle (Weinert 1996). In contrast, in the recent studies in BALB/c, C57BL/6 and C3H/He inbred mice no change in locomotor activity during an estrus cycle has been found (Koehl *et al.* 2003, Kopp *et al.* 2006). Moreover, Kopp *et al.* (2006) have shown that the

correlation between locomotor activity and estrus cycle in different mouse strains is expressed differently. Although the influence of the estrus cycle on the pattern of locomotor activity is subtler in mice than in rats and hamsters, the present results suggest that the strains B6.129S7 and B6.129S7 Per1^{tm3} show higher activity during estrus compared to the other stages, proestrus, metestrus and diestrus in spite of low CORT levels during estrus. In contrast to the studies on rats and hamsters, wild-type and *Per1* mutant females exhibit a low CORT level during proestrus that is commonly related to an increased locomotor activity. Thus, the question crops up as to what extent the CORT level has an effect on the activity during estrus cycle in our strains. To clarify this interaction between CORT concentration and estrogen level in relation to the activity there are further investigations necessary particularly a determination of estrogen level and its correlation with the CORT level during the estrus cycle.

Further support for the influence of the estrus cycle on locomotor activity patterns in adult females was provided by the reduction of the daily variation of activity levels in 20 month-old mice characterised by acyclicity compared to the young mice. Surprisingly, adult *Per1*$^{(-/-)}$ females do not differ significantly in overall running activity from the old females. However, considering the total daily CORT secretion in *Per1*$^{(-/-)}$ and wild-type females the CORT concentration decreases significantly compared to the adult females. This decrease in glucocorticosteroids in old animals corresponds to the results in 16 month-old male mice (Dalm *et al.* 2005). In contrast to the young *Per2* mutant females old *Per2*$^{(-/-)}$ females exhibit a rhythmicity in daily CORT secretion with a peak in the dark period at ZT 18. It might be argued that the metabolic cage could be a stressor particularly for old *Per2* mutant females as it has been shown in rats (Erikson *et al.* 2004). To avoid such an effect we habituated all females for three days. Indeed, the wild-type and *Per1* mutant females showed a diurnal rhythmicity in the excretion of CORT in faeces as do adult mice (Touma *et al.* 2004, Dallmann *et al.* 2006) and rats (Cavigelli *et al.* 2005) from other studies. Moreover, the result regarding increased CORT secretion during the dark phase in 20 moth-old *Per2*$^{(-/-)}$ females shows similarities to the increased CORT level in ageing male mice of 9 months of age (Dalm *et al.* 2005) and ageing rats (Sapolsky *et al.* 1986a, Issa *et al.* 1990). However, this increase in CORT in old *Per2*$^{(-/-)}$ females occurs during the dark phase and not during the light phase as in the

case for ageing males. The elevated secretion pattern of corticosterone in the 9 month-old mice has been explained as a consequence of reduced hippocampal mineralocorticoid and glucocorticoid receptor mRNA expression (Dalm *et al.* 2005). This explanation for the elevated CORT level might also be applicable to old *Per2*$^{(-/-)}$ female mice. However, this aspect needs to be proven by further examination.

Ageing or being old is characterised by a deterioration in many biological processes such as metabolic rate, body temperature, locomotor activity, and the rhythm in hypothalamic-corticotrophin-releasing hormone mRNA (Weinert and Timiras 2003). These rhythm disturbances in old organisms are thought to be due to an ageing SCN that relies on changes in the number of neurons influencing the period length of activity (Valentinuzzi *et al.* 1997, Yamazaki *et al.* 2002). Thus, there are many studies on ageing male rats (Van Gool *et al.* 1987) and hamsters which have revealed a shortening of the period length (Pittendrigh and Daan 1974, Morin 1988, Pohl 1993, Davis *et al.* 1998). For laboratory mice, however, no uniform age-dependent changes of period length were found (Teena and Wax 1975, Witting *et al.* 1994, Kopp *et al.* 2006). Therefore, it is difficult to demonstrate the ageing process using activity in mice. Although we are also unable to show in our study age-dependency in the period length and in the onset of activity we do demonstrate that the total activity in old female mice differs from that of adult females with the exception of *Per1* mutants.

In the current study we have shown that the lack of *Per2* gene has an impairing effect on the daily secretion of CORT: there is a low CORT level in the dark phase (activity phase) as well as in the light phase (resting phase). Moreover, *Per2* mutant females have lower CORT levels during the preovulatory stage than the control group and *Per1* mutant females. This preovulatory stage is characterised by increased locomotor activity that is generally associated with sexual motivation. Hence, the low CORT level during estrus in wild-type and *Per1* mutant females is not in accordance with the high locomotor activity at this stage. Moreover, their CORT level is significantly higher during proestrus but the locomotor activity is as low as during die- and metestrus. *Per2* mutant females do not show such a locomotor variability during the estrus cycle as do wild-type and *Per1* mutant females. Thus, this lower variability in locomotor activity in *Per2* mutants may reflect an absence of

sexual motivation associated with the lack of variability in the CORT. Additionally, we have shown that there are indeed age-dependent influences on CORT secretion and the estrus cycle pattern in all three strains. In contrast to the lowered CORT level in wild-type and *Per1* mutant females *Per2* mutants show an atypical pattern of CORT secretion demonstrating rhythmicity in 20-months-old *Per2*$^{(-/-)}$ females. This phenomenon might be explained by a changing in the HPA-axis sensibility. Nevertheless, this aspect is particularly relevant for further studies investigating the interactions of the circadian system and the hormonal changes contributing to reproductive senescence in transgenic mouse models.

Chapter 4

Manuscript in preparation

4

Per1 and *Per2* clock gene mutations in female mice alter the timing of sexual maturity and estrus cyclicity

Violetta Pilorz and Stephan Steinlechner

Abstract

The recently discovered rhythmic expression of clock genes in ovaries supports the growing evidence that the circadian rhythmicity may play an important role in optimizing fertility. To investigate whether clock genes have an influence on the puberty onset we used *Per1* and *Per2* mutant female mice and determined the age of the vaginal opening and the occurrence of the first regular estrus cycle under LD (12 light : 12 dark) and LL (constant light) conditions. We observed under LD condition that *Per1* and *Per2* mutant females display a significantly earlier onset of puberty compared to the LD condition. Furthermore, under constant light conditions *Per1* and *Per2* mutant females show longer and shorter period length than 24 hours, respectively. This period length of the *Per* mutants corresponds with the onset of puberty, *Per2* mutants display an advanced and *Per1* mutants a delayed onset of puberty compared to LD conditions. Under constant conditions wild-type, *Per1* and *Per2* mutant females have regular estrus cycle patterns that are similar to those in LD condition. Taken together these results suggest that the endogenous clock genes *Per1* and *Per2* are involved in the timing of the sexual maturity in female mice.

4.1 Introduction

The key to successful reproduction in many organisms is the optimal timing of ovulation. The control of ovulatory cyclicity involves complex interactions between the hypothalamus, pituitary and ovary (Freeman 1988). Since puberty reflects the maturation of all systems required for optimal reproductive performance it might be expected that circadian rhythmicity may play a role in puberty onset. For many seasonal breeders such as Djungarian hamsters (*Phodopus sungorus*) (Hoffmann 1973, Adam *et al.* 2000; Park *et al.* 2006), deer mice (*Peromyscus maniculaturs*) (Millar and Wille 1979, Whitsett and Miller 1982) and voles (*Microtus pennsylvanicus*) (Lee 1993, Meek and Lee 1993, 1994) the photoperiod signals the optimal time of year for puberty onset. Thus, the puberty onset of these long-day breeders changes with day length in a way that long durations of darkness inhibit, and long day lengths accelerate sexual maturity (Reiter 1980; Kaufmann *et al.* 2003; Yellon *and* Goldman, 1984). Furthermore, the photoperiods of increasing or longer day lengths are associated with higher rates of reproduction and with larger number of animals with prime reproductive conditions, whereas shorter day lengths are associated with lower rates of reproduction (Baker and Ranson 1932, Steinlechner and Niklowitz 1992). It is known that under constant environmental conditions e.g. constant light, the endogenous period of a circadian oscillator in the organism is expressed in overt rhythms. These rhythms deviate from 24 h and are called circadian rhythms (Aschoff 1981). Thus, constant conditions may be a proper approach to revealing the timing of inherent developmental events such as onset of puberty. Fiske (1941) used this approach and showed in a study in rats that constant light accelerates vaginal opening by 6 days compared to those under LD conditions.

Normally the seasonal optimal timing of the onset of puberty is a result of adaptation to beneficial environmental conditions such as the availability of adequate food and water and moderate climatic conditions (Steinlechner and Niklowitz 1992). However, puberty is not only influenced by these external factors but also by internal components representing a complex sequence of maturational events which lead to the secretion of gonadal steroids driven by an increased frequency of the pulsatile

secretion of gonadotropin-releasing hormone (GnRH) (Fritzgerald and Zucker 1976, Bourguignon *et al.* 1990, Kriegsfeld *et al.* 2002, Sisk and Foster 2004; Ebling 2005, de la Iglesia and Schwartz 2006). Even though increased neuronal production and high frequency of GnRH secretion are the neuroendocrine trigger of pubertal development, the determinants which decide the precise timing of puberty onset are not well defined, and thus the integrating mechanism is still unknown (Nelson *et al.* 1990, Kriegsfeld *et al.* 2002, Sisk and Foster 2004, Ebling 2005).

The environmental signal for changes in reproductive competence is the change in the photoperiod which together with an endogenous timing system located in the suprachiasamtic nuclei (SCN) controls the daily circadian rhythmicity of many physiological and behavioural functions (Reiter 1980). Thus, the circadian output from the SCN plays a major role in the regulation of female reproductive rhythms (de la Iglesia and Schwartz 2006). Moreover, it has been shown that the onset of estrus in female rodents such as rats (*Rattus norvegicus*) (Wang 1923, Richter 1927), deer mice (*Peromyscus maniculaturs*) (Cushing 1985) and Syrian hamsters (*Microcetus auratus*) (Alleva *et al.* 1971; Fritzgerald and Zucker 1976; Morin 1980) is associated with increased activity and an advance in the circadian rhythm of activity. Studies on *Clock* mutant mice (Miller *et al.* 2004) and *tau*-mutant hamsters (Lucas *et al.* 1999) have demonstrated that circadian luteinizing hormone (LH) regulation can be negatively affected by *Clock* and *tau* mutations leading to irregular estrus cycles. Neuronal projections from the SCN to GnRH producing neurons provide a substrate for direct modulation of circadian rhythms by reproductive state (de la Iglesia *et al.* 1999; Kalsbeek and Buijs 2002). Thus, neuronal pathways located in the SCN may serve as substrate for the circacian control of ovarian steroids that lead to the generation of the gonadotropin-releasing-hormone (GnRH) and LH surge (de la Iglesia *et al.* 1999, Kriegsfeld *et al.* 2002). Generally the clock genes are important components of the transcription/translation feedback loop in the SCN responsible for regulating the timing of physiological and behavioural processes exhibiting circadian rhythmicity. Particularly the *Per* genes play an important role in timekeeping of numerous biological processes (Hall and Rosbach 1988, Daan *et al.* 2001). Mutations at this locus in *Drosophila* can either, speed up, slow down, or obliterate various developmental processes of the flies (Kyriacou *et al.* 1990). Thus, the *Per*

proteins seem to be required not only in the adult stage to induce circadian rhythms but they may also have a function in the earlier developmental stages.

In the present study we want to investigate this particular aspect in female mice that are deficient for either the *Per1* or the *Per2* gene. We investigated the onset of puberty by determining the age at the onset of vaginal opening and the age of the first regular estrus cycle under two different light conditions, namely under LD 12 : 12 and LL, i.e. constant light. Previous studies on female hamsters and rats have shown that in these animals the locomotor activity is influenced by the estrous cycle (Morin *et al.* 1977, Campbell and Schwartz 1978, Albers *et al.* 1981, Wollnik and Turek 1987). The average number of wheel revolutions is highest on the day of ovulation both in LD and LL. Moreover, the activity onset in Syrian hamsters (Carmichael *et al.* 1981, Swann and Turek 1982) and rats (Wang 1923, Wollnik and Turek 1981) occurs earlier in proestrus and estrus than in the other estrus cycle stages. Since we do not know whether *Per* genes have an impact on the length of the estrus cycle, we also recorded locomotor activity while monitoring the estrus cycle. In addition, to investigating the influence of light conditions on the development of the young females we measured their daily body mass.

4.2 Material and methods

Animals

We used homozygous B6.129S7-*Per1*^{tm1Brd} (*Per1*$^{(-/-)}$) (Zheng *et al.* 2001) and B6.129S7-*Per2*^{tm1Brd} (*Per2*$^{(-/-)}$) (Zheng *et al.* 1999) as well as wild-type female mice (B6X129.S7) as a control for our experiments. To determine whether the light conditions such as LD and LL have an influence on the onset of puberty in all three strains we divided the females into two different groups: one group (N = 10 of each strain) contained females which were born and maintained under light-dark cycle (12h light : 12h dark) with lights on at 22.00 h and the second group was born and kept under constant light condition LL N = 10 of each strain. The room with LD regime was provided with two red lamps of < 6 lux which were kept permanently on. Steinlechner *et al.* (2002) have shown in the study on male mice that more than 300

lux causes arythmicity in the locomotor activity of the wild-type mice. To ensure rhythmicity in all three strains under LL condition we therefore chose a light intensity between 50 and 150 lux at the cage level.

All pregnant females had been kept from the day 15 of pregnancy under LD or LL light conditions. The cages of pregnant females were checked daily for the presence of offspring towards the end of the gestation period (21 days). The day on which young were found was recorded as the date of birth (day 1). On the 21st day postpartum all juvenile females were weaned and housed individually in polycarbonate transparent cages type II containing wood shavings as bedding. The laboratory temperature was maintained at 22 ± 1°C. Food and water were given ad libitum. The body mass of each individual in LD was recorded daily in the dark phase 2 hours after the light was turned off. The body mass of females kept under LL conditions was recorded also daily two hours after onset of activity (i.e at CT14).

All experiments including animals were in accordance with the animal law of the Federal Republic of Germany and the guidelines of the European Union. Moreover, the experiments were approved by the district government of Hannover.

Assessment of onset of puberty

Puberty onset was determined by daily examination for vaginal opening. To avoid exposure to the odor of males, and thus impact of males on the onset of puberty, all females in LD and LL were maintained in an experimental room without any male. The daily examination began on postnatal day 21 and continued until an opening of the vagina was observed. From this day on we started taking daily vaginal smears for eight successive weeks to examine the onset of regular estrus cycles of 4-5 days.

Examination of estrus cycle

To distinguish the different phases and length of the estrus cycle, vaginal smears from adult wild-type and *Per* mutant females of each group (N = 10) kept under LD and LL condition were taken daily in their activity phase, i.e. two hours after lights off in LD and two hours after the onset of activity in LL. To facilitate vision in darkness we used a red-light forehead-lamp < 6 lux. The smears were obtained by inserting a fire-polished metal diluting loop into the vagina, not more than 1 mm to minimise the

possibility of inducing pseudopregnancy (Sinha *et al.* 1978). The vaginal smear was transferred to a drop of saline solution on a microscopic slide. Dry smears were fixed in MeOH for 2 min, stained with methylene blue solution for 2 min and washed with deionised water. After staining they were evaluated microscopically at a magnification 10x60. The vaginal smears were taken for 1.5 months in order to identify the length of estrus cycle. The smears were classified into different estrus stages, according to the description of Nelson *et al.* (1982). Prolonged diestrus and permanent estrus for at least 15 days were considered as acyclic estrus. An increased estrus cycle (< 6 days) was considered as prolonged and reduced one (> 3 days) as irregular.

Running wheel activity

The running wheel activity was monitored in wild-type *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ mutant females under LD and LL conditions. Running wheel activity was recorded by using a running wheel with a diameter of 15 cm. The running wheel was fixed to the cage lid. A magnet was attached to the outside of the wheel and each passing of this magnet by a reed relay corresponds to a single wheel revolution. The signal was counted by a computer using an I/O card and recorded as number of wheel revolutions every 6 min.

Statistics

For statistical analysis data were tested for normal distribution using Kolmogorov-Smirnov-test. Differences in the onset of vaginal opening and of estrus cycle, as well as in locomotor activity and body mass among the groups wild-type *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ were compared among the females under LD and LL conditions using a repeated-measures ANOVA. Moreover, differences among the three groups were tested with Turkey's Honest Significant Difference-Test. To test differences between specific periods or two groups we used paired t-test. Non-normally distributed data were tested with non-parametric tests either with Mann-Whitney U-test for independent data or Wilcoxon-test for dependent data as appropriate. For the investigation of the amount of wheel-running activity of each female we analysed their activity from four successive estrus cycles lasting 4-5 days each in adult

females (Nelson *et al.* 1982). All three genotypes showed a high inter- and intra-individual variability of wheel-running activity in the successive estrus cycles. To eliminate this high variability between the estrus cycles we decided to set the highest number of wheel revolutions within one estrus cycle as 100%. The period length of the locomotor activity was analysed using Chi-periodogram analysis written by T. Ruf. Differences were considered as significant at $p < 0.05$.

4.3 Results

Onset of puberty and the first regular estrus cycle

Under LD conditions *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females display significantly advanced vaginal opening (U-test *Per1*$^{(-/-)}$: $p < 0.001$; *Per2*$^{(-/-)}$: $p < 0.001$) (Fig. 1A) as well as an earlier appearance of the first regular estrus cycle (U-test: *Per1*$^{(-/-)}$: $p = 0.002$; *Per2*$^{(-/-)}$: $p < 0.001$) (Fig. 1B) compared to the wild-type females. The mean of the onset of vaginal opening in wild-type females is 36.5 days ± 1.39 SEM whereas in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ it is 26 days ± 1.05 and 28.9 days ± 0.66 SEM, respectively.

Under constant light conditions *Per1*$^{(-/-)}$ as well as wild-type females have significantly delayed vaginal opening (U-test: wild-type: $p < 0.0001$; *Per1*$^{(-/-)}$: $p < 0.0001$) and first regular estrus cycle (U-test: wild-type: $p = 0.001$; *Per1*$^{(-/-)}$: $p = 0.005$) compared to *Per2*$^{(-/-)}$ females. Furthermore, *Per2*$^{(-/-)}$ females in LL show an earlier vaginal opening (U-test: $p < 0.001$) and regular estrus cycle (U-test: $p = 0.02$) than under LD conditions. Contrary to this, *Per1*$^{(-/-)}$ females exhibit significantly later vaginal opening (U-test: $p < 0.001$) under LL condition than under LD. Wild-type females, however, display the same vaginal opening and the first regular estrus cycle in LD as in LL condition (Fig. 1A and 1B).

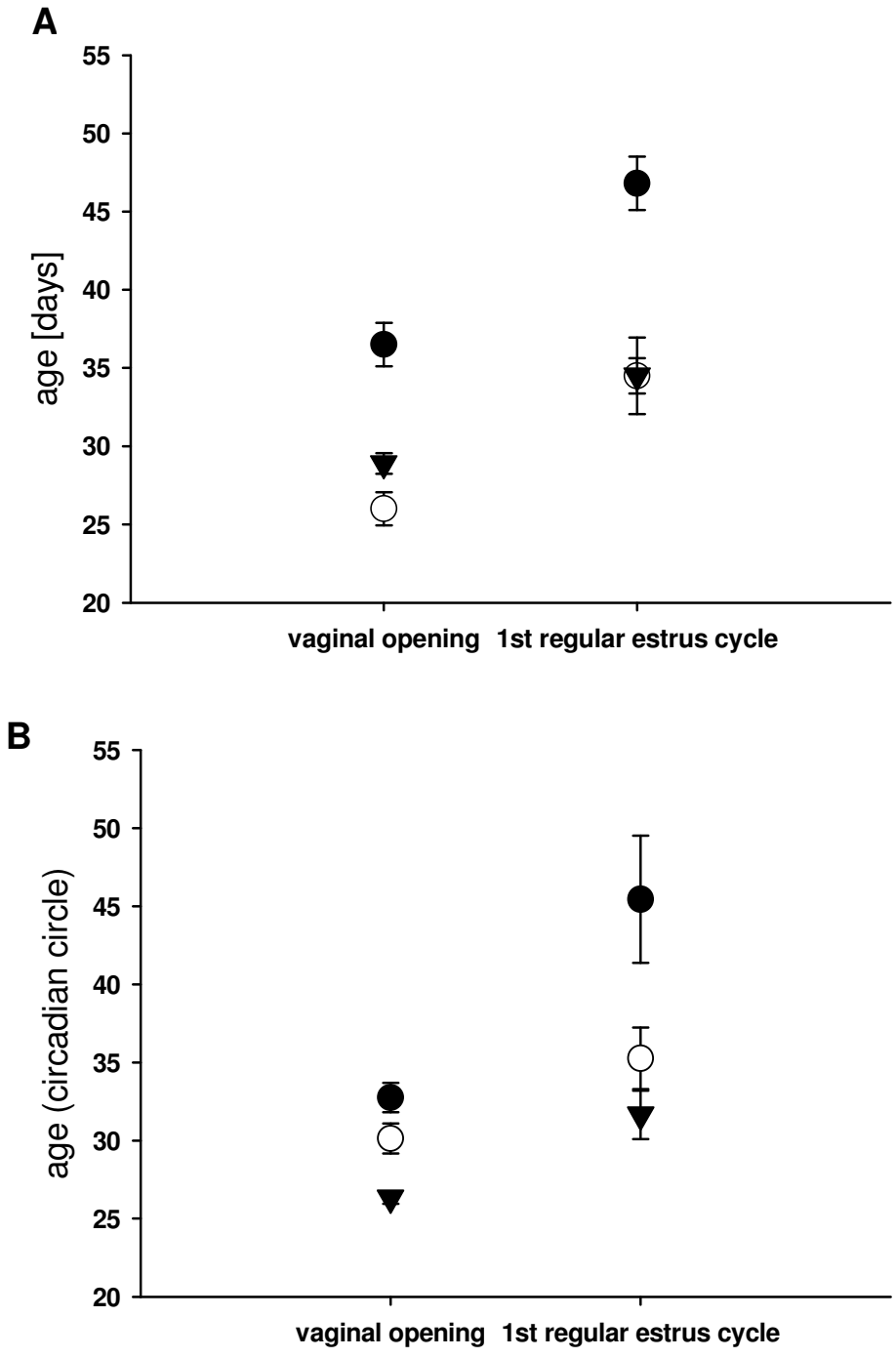

Fig.1: A: represents the mean ± SE age at the onset of vaginal opening and first regular estrus cycle in all three genotypes under LD condition. Fig B represents the mean ± SE age at the onset of vaginal opening and first regular estrus cycle of all three genotypes under LL condition. Black circles: wild-type; open circles: *Per1*$^{(-/-)}$; black triangles: *Per2*$^{(-/-)}$.

Wheel-running activity during estrus cycle under LD and LL conditions

All mice kept under LD 12 : 12 conditions were synchronised and thus show an activity rhythm with a period length (τ) of 24 hours (Fig. 2). Under LL conditions, wild-type and *Per1* mutants displayed circadian locomotor activity rhythms with a period length longer than 24 h, with *Per1* mutant mice showing a significantly larger τ than wild-type females (U-test: $p < 0.001$) (Fig. 3). Although both strains *Per1*$^{(-/-)}$ and wild-type females have a longer period length under LL their total activity over one 4-days estrus cycle is significantly lower (U-test: *Per1*$^{(-/-)}$: $p = 0.003$; wild-type: $p = 0.008$) than under LD condition. *Per2* mutant females in constant light show a period length shorter than 24 hours again with less activity than under LD condition (U-test: $p = 0.005$).

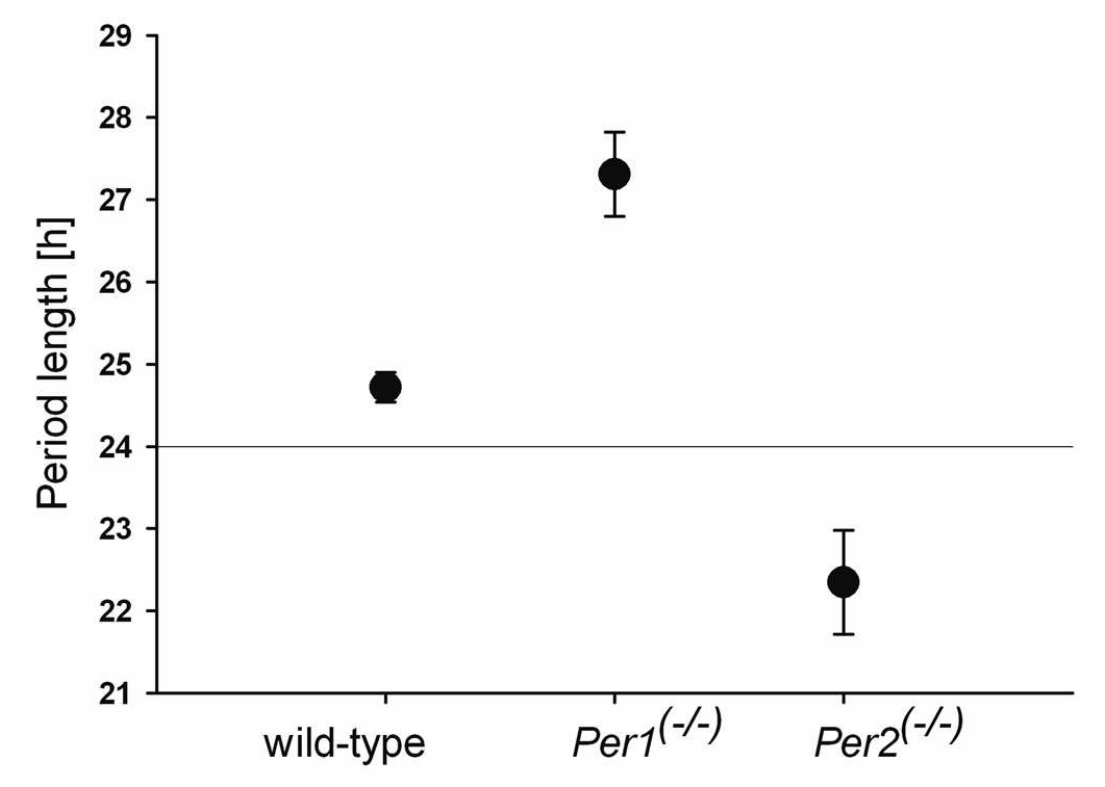

Fig. 2: Mean ± SE period length in all three genotypes under LL conditions (black circles). Horizontal line represents τ of 24 hours in all three genotypes under LD.

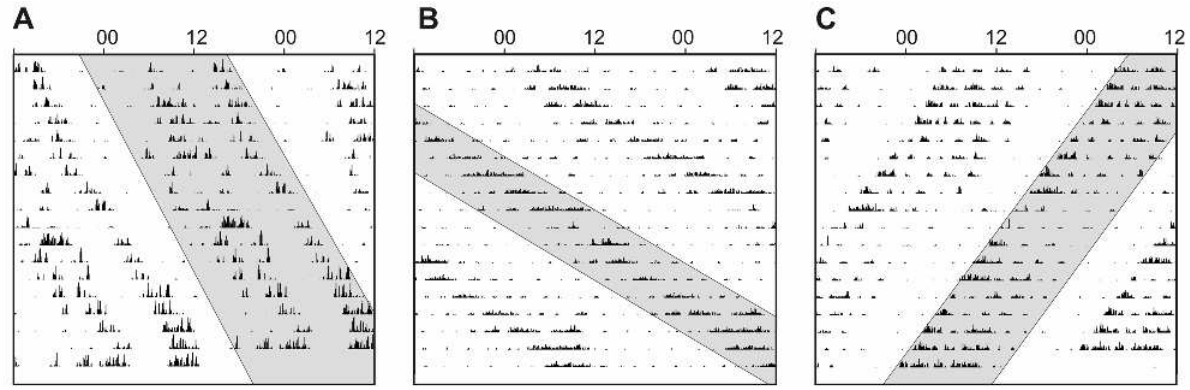

Fig. 3: Double plots of locomotor activity of will-type (A), *Per1*$^{(-/-)}$ (B), and *Per2*$^{(-/-)}$ (C) females under constant light condition (LL). Grey background to highlight the change of τ as well as onset and offset of activity under LL conditions.

To investigate whether the period length under LD and LL has an impact on the length and frequency of estrus cycle, we analysed the number of estrus cycles and their length under these two different light conditions over 8 weeks. Mice kept under LD displayed from the beginning of the regular estrus cycles three estrus cycle patterns such as 4, 5 day, > 6 day which did not differ between the three strains (ANOVA: $F_{(4,\ 72)} = 2.11$, $p = 0.09$) (Fig. 4A). However, the five days estrus cycle pattern occurred significantly more often in wild-type females than 4 day (Turkey's: $p = 0.03$) and > 6 day cycles (Turkey's: $p = 0.009$). Contrary to this, *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females do not exhibit any difference in the number of certain estrus cycle patterns of 4 days, 5 days and more than 6 days (ANOVA: *Per1*$^{(-/-)}$: $F_{(2,\ 24)} = 0.33$, $p = 0.72$; *Per2*$^{(-/-)}$: $F_{(2,\ 24)} = 0.19$, $p = 0.83$). Under LL condition all three strains do not differ significantly in the number of estrus cycle pattern (ANOVA: $F_{(6,\ 108)} = 1.31$, $p = 0.26$). They show more often 5 day and > 6 day estrus cycle pattern than 3 day estrus cycle (Fig. 4B). Additionally, all three strains do not exhibit any daily variability in the amount of locomotor activity during the estrus cycle (Fig. 5).

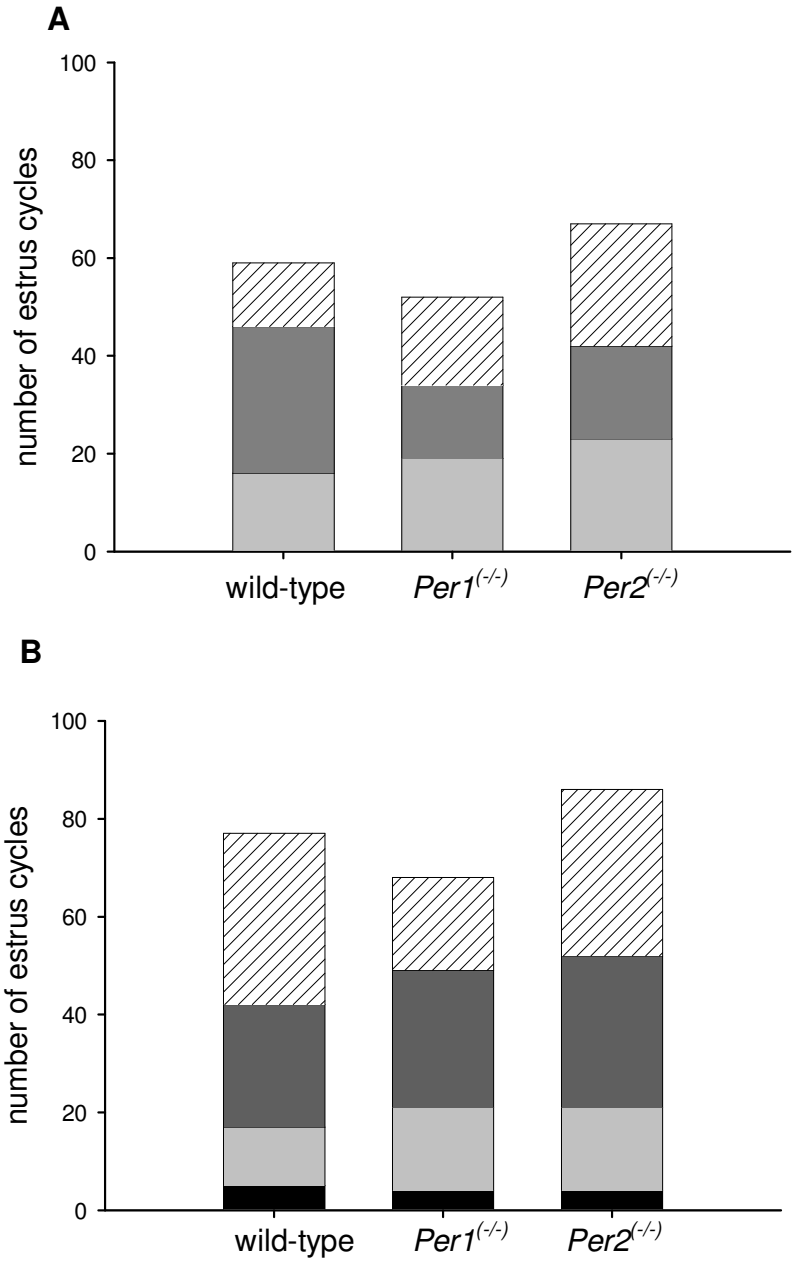

Fig. 4: A: represents number of estrus cycle patterns in all three genotypes over 8 weeks under LD regime; B: represents number of estrus cycle patterns in all three genotypes over 8 weeks under LL regime; dashed bars: > 6 day estrus cycle; dark grey bars: 5 day estrus cycle; light grey bars: 4 day estrus cycle; black bars: 3 day estrus cycle.

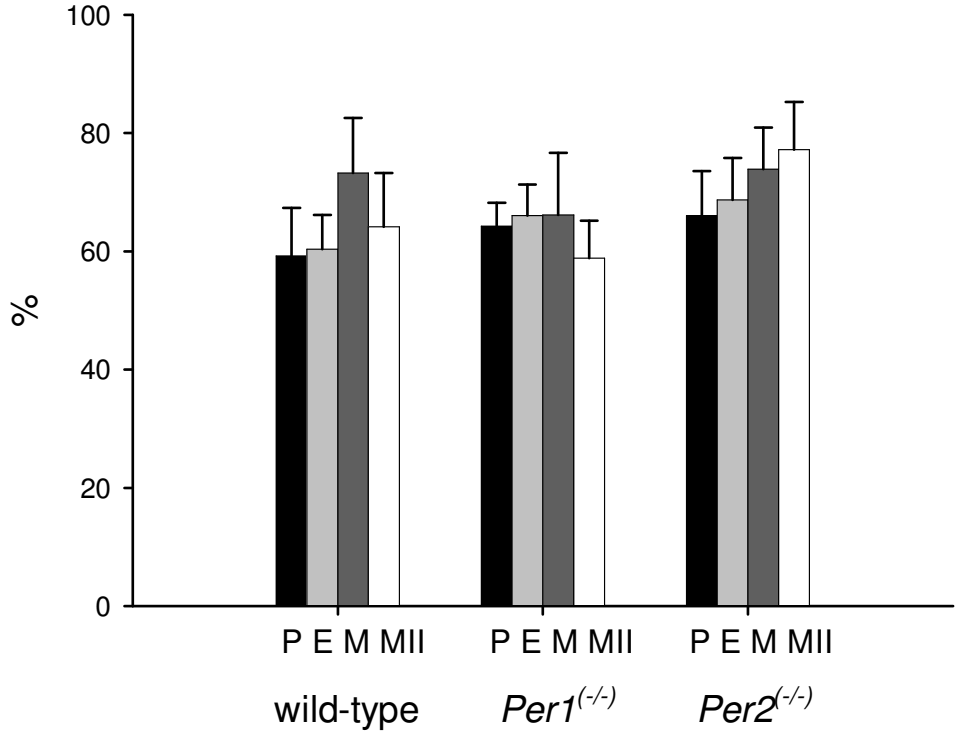

Fig: 5: Proportion of amount of running-wheel activity (± SEM) during an estrus cycle of adult females under constant light conditions (LL); P: proestrus; E: estrus; M: metestrus; MII: metestrus II.

Body mass under LD and LL conditions

The development of the body mass under LD conditions does not differ between the three strains (rep. ANOVA: $F_{(2, 27)} = 0.72$, $p = 0.48$) (Fig. 6A). However, under LL conditions wild-type (ANOVA: $F_{(1, 18)} = 9.35$, $p = 0.007$) and *Per2*$^{(-/-)}$ females (ANOVA: $F_{(1, 18)} = 18.18$, $p < 0.0001$) are significantly heavier than under LD conditions. *Per1*$^{(-/-)}$ females do not show any differences in body mass between development in LD and in LL conditions (Fig. 6A and Fig 6B). Consequently, under LL condition *Per1*$^{(-/-)}$ females are significantly lighter than wild-type (Turkey's: $p = 0.03$) and *Per2*$^{(-/-)}$ (Turkey's: $p = 0.003$) females.

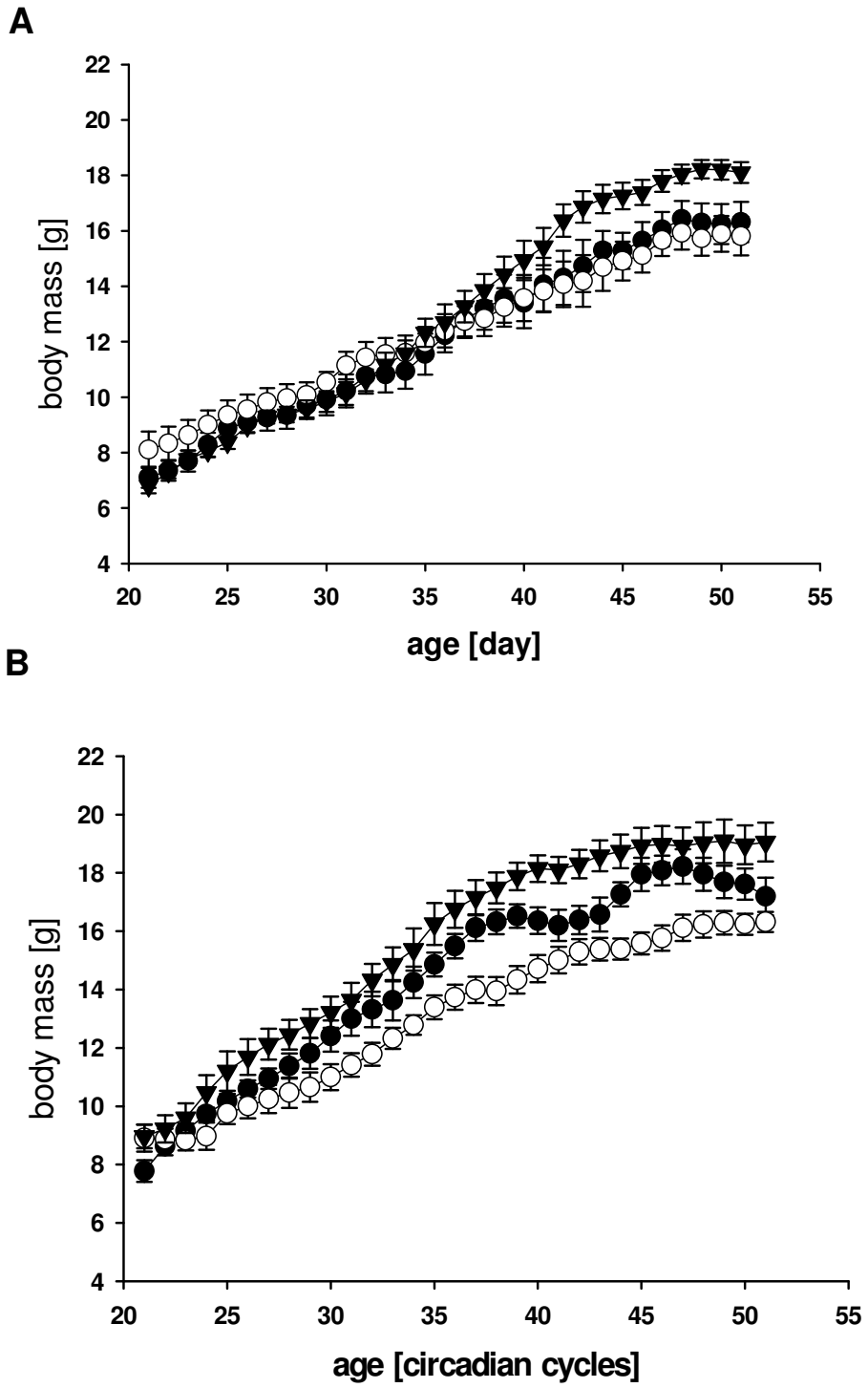

Fig. 6: Body mass (mean ± SE) of all three genotypes under LD condition (A) and under LL condition (B). Day 21 represents the day of weaning; open circles: wild-type: open square: *Per1*$^{(-/-)}$, black triangles: *Per2*$^{(-/-)}$.

4.4 Discussion

In the present study we report on the onset of puberty in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ female mice under different light conditions, namely under LD 12 : 12 and LL regime. In the absence of a mature male we observed under LD conditions an advanced onset of puberty in *Per1*$^{(-/-)}$ and *Per2*$^{(-/-)}$ females compared to wild-type females. This is characterised by accelerated vaginal opening and accelerated appearance of the first regular estrus cycle. Normally female laboratory mice housed with an adult male display the first ovulation at about 37 days of age whereas females in the absence of a mature male are sexually mature at about 57 days of age (Vandenbergh 1967). Even though in the present study the wild-type females with the genetic background B6.129S7 were housed in the absence of a male they displayed much earlier sexual maturity at an age of 36.5 ± 1.39 SEM days than the mice investigated by Vandenbergh (1967). Recent studies on different mouse strains have shown that genetic factors modulate the timing of puberty by 50 - 80% (Nelson *et al.* 1990, Keri *et al.* 2000, Herman-Giddens *et al.* 2001, Sun *et al.* 2002, Eaves *et al.* 2004, Nathan *et al.* 2006). Thus, this difference in the timing of puberty onset in female mice can be a consequence of the genetic differences among the various inbred strains.

Numerous previous studies on laboratory rodents have already shown that circadian rhythms play a critical role in the estrus cycle indicating a relationship between the hypothalamic-pituitary-gonadal axis (HPG-axis) and SCN (de la Iglesia *et al.* 1999, Kriegsfeld *et al.* 2002, Sisk and Foster 2004, de la Iglesia and Schwartz 2006). This relationship between both anatomical structures has been confirmed particularly by disrupting the communication between the SCN and GnRH producing neurons, resulting in estrus acyclicity and infertility (Brown-Grant *et al.* 1977, Nunez *et al.* 1977, Gray *et al.* 1978, Weigand *et al.* 1980). Also lesions of the SCN in hamsters cause an abolishment of circadian rhythmicity and entrainment as well as inhibition of ovulation (Setson and Watson-Whitmyre 1976). Furthermore, specific mutations in circadian clock genes such as *a* mutation of *Clock* may result in irregular estrus cycles and anomalous proestrus LH release which leads to impaired fertility and fecundity (Miller *et al.* 2004, Kennaway *et al.* 2005, Dolatshad *et al.* 2006).

Since the estrus cycle in rodents has been intensively studied in association with the circadian clock, showing a relationship between the SCN and the HPG-axis during the estrus cycle and reproduction, it is apparent that the circadian clock could be also the initiating or regulating mechanism for the onset of puberty. Moreover, it has been shown that the SCN is involved in the secretion of GnRH that regulates not only the estrus cycle but initiates also the onset of puberty (Fitzgerald and Zucker 1979, Silverman *et al.* 1994, Jennes *et al.* 2002). Interestingly, although many recent studies about circadian clock genes focus on reproduction, none of them have investigated whether a single clock gene could have an impact on the regulation of sexual maturity.

The effect of *Per1* and *Per2* mutations has been investigated up to now only in male mice showing that these genes shorten or lengthen, respectively, the free-running circadian period under constant light condition (Albrecht *et al.* 2001, Steinlechner *et al.* 2002). Steinlechner *et al.* (2002) elucidated this phenomenon by the different expression of the *Per* genes by light and thus supported the morning–evening oscillator model of Aschoff (1954), Pittenbrigh and Daan (1976) and Daan *et al.* (2001). In their model Daan *et al.* (2001) suggested that the *Per1* gene is part of the morning oscillator being accelerated by the light and *Per2* gene is part of the evening oscillator being decelerated by the light. In *Per* mutant females we have also shown the same impact of constant light on the period length in locomotor activity as it has been shown in male mice (Steinlechner *et al.* 2002). Under constant light condition both strains *Per1* and *Per2* mutants exhibit a period length longer and shorter than 24 h, respectively. According to the period length in locomotor activity *Per2* mutant females have advanced and *Per1* mutant females delayed timing of puberty onset when compared with the puberty onset under LD.

Under LD condition wild-type females show the same onset of puberty in LD as well as in LL regime. Our results regarding onset of puberty in wild-type females under LD and LL conditions do not correspond with the study of Drickamer (1975) who showed that mice under constant light become sexual mature later than under LD condition. Female rats, however, kept in LL exhibit vaginal opening 6 days earlier than rats on a LD regime (Fiske 1941). It seems that there are not only differences between the rodent species regarding the onset of puberty but also between different

mouse strains. Moreover, the impact of *Per* genes on the puberty onset under constant condition in mice corresponds to the impact of *Per* genes on the larval und pupal development in *Drosophila* where mutation at the *per* locus either speeds up (*Per*^{s} mutation) or slows down (*Per*^{L}) the development of the flies (Kyriacou *et al.* 1990).

Interestingly, contrary to wild-type females both mutant genotypes under LD condition show the same advanced onset of puberty. The advanced sexual maturity in *Per* mutant females may be an explanation for our previous results regarding reproduction in ageing females where *Per1* and *Per2* mutant females showed lower reproductive success than the wild-type females of the same age (Chapter 2). However, to corroborate these findings further investigations, in particular the relationship between GnRH that regulates the onset of sexual maturity and *Per1* and *Per2* clock genes at the puberty onset, are required.

Under LD conditions all three strains show after vaginal opening an estrus cycle characterised by a length of 4-5 days and > 6 days. Under LL conditions all three strains display similar estrus cycle patterns as in LD conditions. However, the different period lengths under this constant light condition in all three strains such as *Per1*$^{(-/-)}$ (τ = 27.31), *Per2*$^{(-/-)}$ (τ = 22.35) and wild-type (τ = 24.72) females must be taken into consideration. Hence, the estrus cycle lengths differ between the strains within LL and between LL and LD. In general, under LD condition vaginal smears are taken in the morning without considering the activity onset of the animals. This approach to determining the estrus cycle under constant conditions would provide a non-photic 24 h time signal. To avoid such *zeitgeber* signal we determined the estrus cycle always in the active phase of the females after checking the onset of activity. Therefore, we can ensure that the estrus cycle length measured under constant conditions corresponds to the period length under such condition.

Numerous lines of evidence indicate that the circadian system is essential for estrus cyclicity, particularly for the generation of the preovulatory LH surge (Everett and Sawyer 1950, Legan *et al.* 1975, Fitzgerald and Zucker 1976, Kriegsfeld *et al.* 2002). The estrus cycle of rats and hamsters is usually characterised by 4-5 days during which the females at proestrus and/or estrus show a higher amount of activity and an advanced activity onset, so-called scalloping (Morin *et al.* 1977, Albers *et*

*al.*1981, Zucker 1980, Wollnik and Turek 1987). This increased activity corresponds with the behaviour of rodent females under natural conditions searching for potential mating males around the time of ovulation (Lisk *et al.* 1983).

Per1$^{(-/-)}$, *Per2*$^{(-/-)}$ and wild-type females showed as a percentage the highest activity at estrus under LD 12 : 12 condition (Chapter 3). Under constant light condition all three genotypes exhibit the same amount of activity during all estrus stages. Moreover, we do not find any evidence for scalloping. This absence of advanced activity at ovulation is consistent with the study on C57BL/6 mice of Dolatshad *et al.* (2006) and Kopp *et al.* (2006). As the duration of individual estrus cycles in *Per* mutant and wild-type females under LL does not differ from the duration under LD condition we can assume that either *Per* genes do not have an effect on the estrus cycle length at all or there are differences in the timing of the LH peak during estrus between the three strains. These could not be determined from measuring the estrus cycle by vaginal smears only once per circadian cycle. Thus, to find out whether there are direct effects of *Per* genes on the secretion of LH further investigations are necessary.

All females show the same amount of locomotor activity in constant light condition and are less active than under LD condition. Night-active rodents generally have a lower activity under LL condition due to masking effects. Lower activity requires less energy expenditure and, therefore, the higher body mass of wild-type and *Per2* mutants is to be expected. This does not apply to *Per1*$^{(-/-)}$ females. In fact, the results of *Per1* mutant females revealed that when kept under constant light condition, they do not change their body weight compared to *Per2* mutants and wild-type females despite lower locomotor activity in LL condition. Apparently the constant illumination changes the physiological mechanisms related to body mass and also food consumption. Our results regarding body mass in all three strains do not show any effect on the timing of puberty. However, to follow up the unchanged body mass development under LD and LL conditions in *Per1* mutant female mice further investigations, such as measurements of metabolic rate and energy consumption are needed.

In the present study we have shown for the first time that the clock components *Per1* and *Per2* can affect the timing of puberty in female mice under LD

and LL condition. Furthermore, the period length of locomotor activity in *Per1* and *Per2* mutant females under constant light corresponds with the onset of sexual maturity showing a delayed and an advanced onset of puberty, respectively. In contrast to rats and hamsters kept in LL condition wild-type *Per1* and *Per2* mutant female mice are characterised by a regular estrus cycle that does not differ from that in LD condition. Nevertheless, the exact relationship between the *Per* genes of the circadian clock and the timing of sexual maturity requires further studies in particular on the molecular basis.

Chapter 5

5. Discussion

In recent years the knowledge about function and importance of biological rhythms has increased enormously. In particular the role of the SCN for the development and reproduction of various species has aroused great interest. It has been shown that successful development and reproduction requires a precise temporal coordination among numerous endocrine and behavioural events and in this context the circadian system has proved to be the key player. Previous studies on rats and mice have demonstrated that a disruption of the neuronal communication between the SCN and GnRH producing neurons, which is responsible for regulating reproductive functions, has severe impact on the estrus cycle and fertility (Brown-Grant and Raisman 1977, Wiegand *et al.* 1980, Wiegand and Terasawa 1982). Moreover, a disruption of a core gene component of the SCN i.e. the *Clock* gene, leads to changes in the length of the estrus cycle and to lower fertility and fecundity (Miller *et al.* 2004, Kennaway *et al.* 2005). These current findings with *Clock* gene mutant mice provide an involvement of the endogenous clock in regulating reproduction. In future, further investigations must be carried out to detect to what extent each of the other clock genes play a role in reproduction.

Recent studies concerning the disruption of *mPer1* and *mPer2* clock genes in male mice from our laboratory (Steinlechner *et al.* 2002, Dallmann 2004, Dallmann *et al.* 2006) have demonstrated that these genes support the two-oscillator model that was proposed by Pittendrigh and Daan in 1976 and which was later modified by Daan *et al.* (2001) who proposed a molecular basis for the two oscillators that track dusk and dawn, respectively. In this context, *Per1* has been suggested to represent a component of the morning oscillator being accelerated by light and *Per2* being part of the evening oscillator which is decelerated by light (Albrecht *et al.* 2001, Daan *et al.* 2001, Steinlechner *et al.* 2002). Accordingly, in the present study we have shown that under LL conditions the period length of the endogenous clock and the onset of puberty in *Per1* and *Per2* mutant females also tally with the M - E-oscillator model (Chapter 2). *Per2* deficient females exhibit earlier onset of puberty. Hence, according to the model these females would have an intact morning oscillator and a defect

evening oscillator. The reverse observation was made for young *Per1* mutant females (Chapter 3). In contrast, even though *Per1* as well as *Per2* deficient females exhibit under LD conditions a period length of 24 hours equal to wild-type females their onset of puberty occurs earlier than in wild-type females. Thus, it is likely that the deficiency of either *Per1* or *Per2* clock gene accelerates the onset of sexual maturity. In intact, coupled oscillators *Per1* and *Per2* clock genes seem to decelerate the onset of puberty in the wild-type females.

The onset of puberty is generally driven by a neuronal control of hierarchically organised hormonal processes; thereby an increased frequency of pulsatile secretion of GnRH triggers the onset of sexual maturity (Ebling 2005, Sisk and Foster 2004). The pulsatile secretion of GnRH is for the most part regulated by the neuropeptides VIP and AVP that are also expressed in the SCN. Recent studies have demonstrated that the *Per1* and *Per2* clock genes belong to the most important components of endogenous clock playing a role in light-induced phase shifting of the biological clock (Moriya *et al.* 2000, Nielsen *et al.* 2001). These two genes are also expressed in AVP and VIP synthesising neurons and are synchronised by core components of the clockwork, the SCN (Dardente *et al.* 2002). This fact justifies the assumption that *Per* genes could affect the expression of GnRH, and thus the onset of puberty as well as the PAG axis. For this reason, with regard to the onset of puberty it is necessary to deal with the question as to with which intensity the expression of *Per1* and *Per2* clock genes appears shortly before the onset of sexual maturity. Presumably both *Per* genes may affect the expression of VIP and AVP at the pre-pubertal stage and regulate the timing of the high frequency of pulsatile GnRH secretion in this way. Consequently, due to the accelerated sexual maturity in *Per* mutant females the onset of the ageing process may occur also advanced (Chapter 2).

An aged SCN is generally characterised by a dampening of circadian amplitude and pulsatile pattern which then express an altered circadian rhythm of molecular, physiological functions and behaviour such as physical activity (Wise *et al.* 1988, Monk *et al.* 1995, Van Cauter *et al.* 1996, Li and Satinoff 1998, Weinert 2000). Some (Davis and Menaker 1981, Pittendrigh and Daan 1974, 1976a, Posside *et al.* 1995, Valentinuzzi *et al.* 1997), but not all studies (Wax 1975, Ralph *et al.* 1990, Weinert and Weinert 1997) demonstrate that the period of activity rhythms in rats,

hamsters and mice shortens or lengthens with age. Particularly mice of different strains show high variability regarding changes of period length with age. Teena and Wax (1975) did not observe a significant age-related change in the period of the C57BL/6 strain. In the present study I show no variability in period length as well as in the onset of activity between adult and old females, and neither in both *Per* mutant females. However, the stability in the activity rhythm of old female mice could be damped by the absence of external cues (Weinert and Weiss 1997, Valentinuzzi *et al.* 1997, Weinert and Kompauerova 1998). Such condition is achievable under constant conditions, for instance under constant light where adult *Per2* mutant males are still rhythmic in contrast to DD (Steinlechner *et al.* 2002).

A further feature of ageing and being old is increased irregularity in the periodicity of hormone release such as GnRH, LH, FSH, and sex hormones causing changes in estrus cycle such as irregular estrus cycle (ageing phase) and acyclicity (old age), respectively. This implies a decline of embryonic pre- and postimplantation in uteri leading to a decrease in the number of live offspring (Nelson *et al*. 1982, Matt *et al.* 1986, Matt *et al.* 1987, Markowska *et al*. 1999). Accordingly, middle-aged *Per* mutant females exhibited earlier decline of estrus cyclicity that was characterised by irregularity and acyclicity, whereas middle-aged wild-type females still revealed a regular estrus cycle (Chapter 2). This irregularity in the estrus cycle in *Per1* and *Per2* mutant females resulted in lower reproductive success that could be confirmed by the high number of embryonal implantation scars in uteri compared to the low number of their successfully produced offspring. In addition, the number of successfully breeding *Per* mutants was lower compared to the wild-type females. Similar declines in estrus cyclicity as well as in fertility and fecundity have been shown in adult *Clock* mutant females (Millar *et al.* 2004, Kennaway *et al.* 2005, Dolatschad *et al.* 2006). Miller *et al.* (2004) explained the abnormalities in fetal reabsorption in *Clock* mutants by a lack of coordinated daily timing signal of GnRH release that leads to reduced levels of estrogen and progesterone. Normally, these two ovarian hormones are vital for maintaining uterine receptivity to developing fetuses during pregnancy and promoting parturition (Itskowitz and Hodgen 1988). Thus, the *Clock* mutation experiments on female mice provide an indication that the endogenous clock has an

impact on regulation of PAG axis and therefore reproduction during the whole reproductive age.

Per genes are also components of the endogenous clock and might be also involved in the reproductive process. Current results demonstrate *Per* genes influencing the onset of puberty by accelerating it but not affecting the fertility during the young adult phase. Adult *Per* mutant females show the same estrus cycle pattern as do wild-type females. This regularity facilitates their fertility and fecundity. Middle-aged *Per* mutant females, however, show lower fertility and fecundity compared to wild-type females (Chapter 2). Hence, it seems that *Per* genes do not affect the reproductive process in the same way as does the *Clock* gene in adult females changing estrus cycle and fertility. They regulate rather the developmental process: beginning of puberty and duration of reproductive capability in females and thus affect indirectly the reproductive process.

The low metabolisable energy intake in *Per* mutant females during gestation that may be associated with low fertility can be further indication for the progressive ageing in *Per* mutants compared to wild-type females. Energy expenditure is important factor that plays a crucial role during reproductive stages and decreases in ageing females (Luz and Griggio 1998). Generally, the periods of pregnancy and lactation are characterised by an increased metabolic rate (Trojan and Wojciechowska 1968) that is related to formation of new tissues such as placentae and fetuses, fat storage for lactation period as well as nourishment and development of the offspring in the postnatal phase. The increased energy expenditure stimulates food intake. Ageing, however, induces a decrease in the energy expenditure causing lower energy requirements. Such a decrease results in the reduction of basal metabolic rate (Shock *et al.* 1963). In the current study *Per1* and *Per2* mutant females did not increase their food intake during pregnancy. Therefore, low metabolisable energy intake in both *Per* mutant females during pregnancy is not surprising. This fact may be an explanation for the high embryonic post-implantation death rate or abortions (Chapter 2). A further explanation for the failures during gestation in ageing females may be age-related morphological changes in the corpora lutea of the ovaries (Harman and Talbert 1970) as well as a decay of spindle

components during the meiotic phase, a result of delayed ovulation (Alberman *et al.* 1972, Sugawara and Mikamo 1980).

During lactation both *Per* mutants showed a behaviour that appears to be less advantageous for the development of their offspring. Even though they spent an intense time in the nest during the whole lactation period - *Per2* mutants even 60% of their time - their offspring did not gain more weight compared to the wild-type females with larger litter sizes and less amount of time spent in the nest. This maternal behaviour in *Per* mutant females leads to less time for feeding and drinking for their own good and for milk production. Hence, there seems to be a strong trade off between the energy requirement for their pups and for their own needs. This might be associated with the age-related lower energy utilisation. Therefore, the middle–aged *Per* mutant females seem to mobilise an allocation of their metabolic energy and nutrients to support their own energetic needs and that of their developing offspring. The recent study on adult *Clock* mutant females has demonstrated that the *Clock* mutation leads to loss of daily rhythm associated with altered behaviour toward the pups showing shortened duration of nursing (Hoshino *et al.* 2006). This disrupted nursing rhythm has been explained by a disrupted daily rhythm of prolactin. Prolactin is the hormone that modulates the growth and development of the mammary gland, milk synthesis, the maintenance of milk secretion and also the maternal behaviour (Kacsoh and Nagy 1983, Aray *et al.* 1991, Freeman *et al.* 2000). In ageing rodents the prolactin level is reduced causing also lower milk production (Smith *et al.* 2005). In contrast to the *Clock* mutants, I have shown that the middle-aged lactating *Per* mutant females exhibit maternal behaviour (intense time in nest), even more intense than wild-type females. Since the pups of *Per* mutant females do not increase their weight more than those of the control group we can assume that the lack of *Per1* or *Per2* clock genes in middle aged females may affect the prolactin secretion levels and thereby reduce the amount of milk production. This assumption may be an explanation for the age-related reduction in milk production (Smith *et al.* 2005) but it is still unclear why *Per* mutant females spent such an intense time in the nest during the whole lactation period. The natural variations in maternal care e.g. pup-licking and arched-back nursing associated with differences in the interaction between oxytocin receptors and estrogen, have profound consequences for the development

of offspring (Newman *et al.* 2000a, Champagne *et al.* 2002). In rats it has been shown that enhanced interactions between estrogen and oxytocin receptors implicate increased maternal responsiveness to pups (Champagne *et al.* 2002). The notably intense maternal behaviour in middle-aged *Per* mutant females may lead to the assumption that the *Per* mutant dams have higher oxytocin sensitivity compared to the wild-type females. On this basis, one can speculate that maternal behaviour in *Per* mutant females and the development of their pups may be connected with advanced ageing causing functional deficits or change in hormone secretion during lactation. To elucidate the advanced ageing in *Per* mutant females additional experiments regarding alterations in hormone release affecting milk production and maternal behaviour are necessary.

Corticosteroid concentration is a further important factor that influences the hypothalamic-pituitary-gonadal axis, and can thus affect the estrus cyclicity (Xiao *et al.* 1999). Accordingly, several studies on female rodents have identified alterations in the basal as well as stress-induced corticosteroid level across the 4-day estrus cycle, with highest levels observed during the preovulatory period and maximal estrogen secretion (Buckingham *et al.* 1978, Nichols and Chevins 1981). Such a pattern of glucocorticosteroid levels in females may contribute to the probability of successful survival and for regulating the timing of reproduction (Atkinson *et al.* 2005). The upward modulation of corticosteroid at proestrus occurs in rodents, as a result of estrogen's ability to stimulate the corticotropin releasing hormone (CRH) gene transcription (Vamvakopulos and Chrousos 1993) in the afternoon in anticipation of mating behaviour. It may serve to mobilise a greater allotment of metabolic energy to support the energetic requirements of mating behaviour (Levine 2002). This change of corticosteroid level during the estrus cycle is consistent with our results in *Per1* mutant and wild-type females. Moreover, the daily dynamic of faecal corticosterone metabolites in both adult wild-type and *Per1* mutant females does not differ from that in wild-type male mice (Atkinson and Waddell 1997, Touma *et al.* 2003, Dallmann *et al.* 2006). In contrast to *Per1* mutant male mice *Per1* mutation in female mice does not have an effect on the corticosteroid secretion (Chapter 3). Hence, the function of *Per* gene regarding the timing of the secretion of

corticosteroid may be different between the genders. Controversially, the lack of *Per2* clock gene in adult females has apparently an impact on the timing of CORT secretion. These females do not show a daily rhythm of CORT secretion (Chapter 3). Experiments with SCN-lesioned rats have demonstrated that the SCN has a regulating effect on corticosteroid secretion (Buijs *et al.* 1993, Sage *et al*. 2002). The corticosteroid level in these SCN-lesioned animals was higher than that in the control rats. Consequently, the SCN-lesion provided an indication for an inhibiting effect of the intact SCN. In the current study the absence of an elevation of CORT concentration during the active phase in *Per2* mutant females indicates that the *Per2* clock gene might have a stimulatory effect on the secretion of corticosteroids or on the expression of AVP and VIP neuropeptides that stimulate corticosteroid secretion (Rivier and Vale 1983). However, the observed daily rhythm of CORT secretion in old *Per2* mutant females (18-20 months of age) with a peak in the dark phase (active phase), is inconsistent with the assumed stimulatory effect of *Per2* clock gene on the CORT secretion in adult females. However, the slight increase of CORT in old *Per2* mutant females may correspond to elevated CORT in ageing rats (Issa *et al.* 1990) and mice (Sapolsky *et al.* 1986a, Dalm *et al.* 2005). This increase in circulating CORT levels under basal conditions in aged animals is associated with loss of corticosterone sensitive neurons and receptors in the hippocampus leading to decreased sensitivity to circulating corticosteroids and hence dampening of the negative feedback efficacy (Sapolsky *et al.* 1986a, Dalm *et al.* 2005). However, it is still unclear why the old *Per2* mutant females show a peak of CORT similar to adult wild-type females.

As we assume that the impaired fertility and fecundity in *Per1* and *Per2* mutant females may be a consequence of advanced ageing it is likely that the age-related increase of CORT level could be an additional reason for the low fertility and change in essential physiological processes such as metabolic rate (Atkinson and Waddell 1995, Magiakou *et al.* 1997, Wingfield and Sapolsky 2003). However, to prove this hypothesis determination of basal corticosteroid concentration as well as corticosteroid concentration during reproduction in middle aged *Per1* and *Per2* mutant females is required.

In female laboratory rats (Sridaran and McCormack 1977, Wollnik and Turek 1988) and Syrian hamsters (*Mesocricetus auratus*) (Fitzgerald and Zucker 1976) it has been demonstrated that the animal's reproductive state can influence locomotor activity. The animals display an increase in running activity and an advanced activity onset when they are in estrus, also termed scalloping (Wang 1923, Slonaker 1925, Baranczuk and Greenwald 1973, Morin *et al.* 1977, Turek and Gwinner 1982). There are various studies on different rat strains and hamster species that report the fluctuation of the time of activity onset during the estrus cycle. However, due to the various rat strains and hamster species that were used in the studies there are controversial data regarding the occurrence of scalloping during estrus or proestrus or even during both estrus cycle stages. Therefore, it is difficult to generalise the occurrence of scalloping at a particular estrus cycle stage for a specific genus or only for single rat or for hamster species. Furthermore, inbred mouse strains such as BALB/c, C57BL/6 and C3H/He do not exhibit any change in locomotor activity during an estrus cycle (Koehl *et al.* 2003, Kopp *et al.* 2006). Also in the present study the mouse strains B6.129S7 and B6.129S7 *Per1*^{tm3} show a high individual variability of wheel-running activity in successive estrus cycles. However, the representation of the wheel revolutions as a proportion over the estrus cycle enabled us to show higher activity during estrus compared to the other estrus cycle stages in wild-type and *Per1* mutant females. *Per2* mutant females showed only a tendency for higher activity during estrus. Thus, the low CORT level at proestrus and low variability in the amount between the estrus stages might negatively affect the reproductive behaviour, for example lack in sexual drive (Levine 2002). Nonetheless, this high amount of locomotor activity at estrus in wild-type and *Per1* mutant females is not consistent with the low CORT level at this stage. As these two female groups exhibit a high CORT level at proestrus we should also expect a high activity. In order to clarify the interaction between CORT concentration and estrogen level in relation to activity additional investigations are necessary such as determination of estrogen and glucocorticosteroid concentrations during the estrus cycle.

All in all, contrary to the *Per2* clock gene that seems to stimulate the timing of CORT secretion shortly before the activity onset (darkness), *Per1* clock gene does not have an effect on the CORT secretion in female mice. The CORT level in *Per1*

mutant females differs neither in adult nor in old females from that in wild-type females. However, with regard to advanced ageing one could expect an advanced increase in basal CORT concentrations in both *Per* mutant genotypes that consequently might have a negative effect on their fertility. Moreover, in the present study there is no obvious time point at which the *Per* mutant females begin with their ageing process. In order to investigate this, continuous measurements of the onset of irregularity in estrus cycle are required.

The present study reveals many new aspects regarding the function of *Per1* and *Per2* clock genes during development, from birth until old age. I have shown that both clock genes affect the timing of puberty and subsequently may cause an advanced onset of ageing in female mice. This fact is associated with alterations in various molecular, physiological as well as behavioural functions of which only a few aspects could be investigated here. Thus, numerous further questions arose which might be essential to substantiate the function of the clock genes particularly in females. Here I have made hypotheses about potential effects of the *Per* genes with regard to the reproductive outcome in middle aged as well as timing of HPA axis activity in adult and old females. Moreover, regarding the timing of corticosteroid secretion I have demonstrated that *Per1* and *Per2* clock genes exhibit different functions between males and females. Therefore, it is necessary to investigate whether both *Per* genes reveal gender-related functional differences concerning various essential physiological processes. Furthermore, all molecular and physiological events connected with the SCN regulation constitute a highly organised complexity. Hence, it is likely that the lack of *Per* clock genes may influence other regulating components. In this context it is difficult to argue that one deficient component of the endogenous clock e.g. *Per1* or *Per2* clock gene may have a particular impact on the biological events. In addition, even though present results of middle-aged *Per* mutant females provide hints for accelerating ageing e.g. advanced onset of puberty it is also likely that the lack of *Per1* or *Per2* clock genes may have a negative effect on physiological functions and reproduction in progressive age. To elucidate whether *Per* genes accelerate the ageing process or their lack has an impairing effect on the physiological functions, continuous investigations are required

from young age until old age. Nevertheless, the results from the present study provide groundwork for further and more intense investigations of clock genes with regard to development and reproduction.

6. Abstract

The life-span and the life style of each organism is the product of the evolution that facilitates the organism and the species to survive successfully. One of the obvious adaptive features of all living organisms is the ability to change their behaviour on a daily (24 hour) basis in response to the daily rotation of the earth on its axis. One of such adaptations in mammals is the acquisition of a regulating mechanism, a circadian pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The core mechanism of the clock is based on two interlocking transcriptional/translational feedback loops involving different sets of clock genes including *Per1* and *Per2*. Both these clock genes play a major role as light responsive elements promoting phase shifts and thus permitting adjustments of physiological and behavioural rhythms to the external environment.

Successful reproduction also requires precise temporal coordination among various endocrine and behavioural events. Recent studies on mice with mutations in the *Clock* gene have demonstrated that this mutation disrupts estrus cyclicity and interferes with successful pregnancy. In present study I focused on the reproductive success with respect to the functionality of the endogenous clock, particularly *Per1* and *Per2* clock genes. Therefore I investigated the onset of sexual maturity, changes in the estrus cycles and reproductive outcome in young adult and middle-aged *Per1*-deficient and *Per2*-deficient female mice. I demonstrated that *Per1* and *Per2* clock genes have a delayed (*Per1*) or advanced (*Per2*) impact on the onset of puberty under constant light conditions. Besides, under LD conditions (12 light : 12 dark) both genes accelerate the sexual maturity. This fact may explain the advanced ageing in the *Per* mutant females resulting in lower fertility and fecundity. Furthermore I demonstrated that *Per2* clock genes have apparently a stimulatory impact on the timing of corticosteroid secretion. In this context, young adult *Per2* mutant females showed neither a daily rhythm in corticosteroid secretion nor variability in the locomotor activity during estrus cycle.

6. Zusammenfassung

Die Lebensdauer sowie die Lebensweise jedes Lebewesens sind das Produkt der Evolution, das die Überlebenschance eines Organismus und der Spezies erhöht. Eines der wichtigsten Charakteristika der Anpassung eines Lebewesens an die Umwelt ist die Fähigkeit, das Verhalten auf den täglichen Rhythmus (24 h) abzustimmen. Dieses begründet sich aus regulären Umweltveränderungen, die auf dem Phänomen der Erdumdrehung um die eigene Achse beruhen. Der Erwerb eines Regulationsmechanismus, einer circadianen Uhr, der in den suprachiasmatischen Nuklei (SCN) im Hypothalamus lokalisiert ist, stellt eine solche Anpassung dar. Der Grundmechanismus dieser Uhr beruht auf zwei ineinander greifenden Transkriptions- und Translationsschlaufen, die eine Reihe von Uhrengenen einschließt, unter anderem die *Per1* und *Per2* Uhrengene. Diese beiden Gene spielen als Antwortelemente auf Licht eine wichtige Rolle, indem sie Phasenverschiebungen bewirken. Dieses wiederum führt zu einer Anpassung der physiologischen und Verhaltensrhythmen an die externen Umweltverhältnisse.

Erfolgreiche Reproduktion benötigt ebenfalls präzise zeitliche Koordination in den zahlreichen endokrinologischen Funktionen und Verhaltensprozessen. Kürzlich erschienene Studien an Mäusen mit einer *Clock*-Gen Mutation haben gezeigt, dass diese Art von Mutation den Östruszyklus stört sowie eine erfolgreiche Tragzeit behindert. In der vorliegenden Studie konzentrierte ich mich auf den Einfluss der inneren Uhr, insbesondere der Gene *Per1* und *Per2*, auf den Reproduktionserfolg. Dafür untersuchte ich den Beginn der Geschlechtsreife, die Veränderung des Östruszykluses und den Reproduktionserfolg in jung-adulten sowie altwerdenden *Per1* und *Per2* mutierten weiblichen Mäusen. Ich habe in dieser Studie erstmals zeigen können, dass *Per1* und *Per2* Uhrengene den Beginn der Pubertät unter konstanten Licht Bedingungen verlangsamen (*Per1*) bzw. beschleunigen (*Per2*). Unter LD (12 Licht : 12 Dunkel) Bedingungen beschleunigen beide Gene die sexuelle Reife. Diese Tatsache könnte die geringe Fertilität sowie Fekundität der altwerdenden *Per* mutierten Weibchen erklären. Darüber hinaus haben wir gezeigt, dass *Per2* Uhrengene wahrscheinlich einen stimulierenden Effekt auf die Corticosteroidsekretion haben. Dabei zeigten die *Per2* mutierten jung adulten Weibchen weder einen täglichen Rhythmus in der Corticosteroidausschüttung noch eine Variabilität in der Laufaktivität während des Östruszykluses.

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8. Erklärung

Hiermit erkläre ich, dass ich die Dissertation „Impact of *Per1* and *Per2* clock genes on the reproductive outcome and physiological functions in female mice“ selbständig verfasst habe. Bei der Anfertigung wurden folgende Hilfen Dritter in Anspruch genommen:

Ich habe keine entgeltliche Hilfe von Vermittlungs- bzw. Beratungsdiensten (Promotionsberater oder anderer Personen) in Anspruch genommen. Niemand hat von mir unmittelbar oder mittelbar entgeltliche Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Ich habe die Dissertation an folgenden Institutionen angefertigt:

Institut für Zoologie/ Tierärztliche Hochschule Hannover

Die Dissertation wurde bisher nicht für eine Prüfung oder Promotion oder für einen ähnlichen Zweck zur Beurteilung eingereicht.

Ist die Dissertation in einer auswärtigen Institution angefertigt worden, so ist zugleich eine Erklärung des betr. Leiters beizufügen, dass er mit der Einreichung der Arbeit als Dissertation an der Tierärztlichen Hochschule Hannover einverstanden ist.

Ich versichere, dass ich die vorstehenden Angaben nach bestem Wissen vollständig und der Wahrheit entsprechend gemacht habe.

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eigenhändige Unterschrift

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