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

Violetta Pilorz and Stephan Steinlechner

Department of Zoology, University of Veterinary Medicine Hannover, Buenteweg 17, D-30559 Hanover, Germany

Correspondence should be addressed to V Pilorz; Email: violetta2@gmx.de

Abstract

Recent studies on mice with mutations in the Clock gene have shown that this mutation disrupts oestrus cyclicity and interferes with successful pregnancy. In order to determine whether two other molecular components of the main clock, namely the period genes, Per1 and Per2, have an effect on the length of the oestrous cycle and the reproductive success, we used Per1- 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 oestrous cycle of the middle-aged mutants. Besides, we demonstrate that Per mutant females have significantly more embryonal implantations in the uterus than successfully delivered offspring. The reproductive deficits of the middle-aged Per mutant females are comparable with those seen in aged wild-type mice. This suggests that Per1 and Per2 mutations cause an advanced ageing.


Introduction

In mammals, the suprachiasmatic nuclei of the hypothalamus (SCN) play an important role in coordinating circadian rhythms of numerous physiological functions as well as of behaviour. The Per1 and Per2 clock genes (Zheng et al. 1999, 2001) together with Clock (Vitaterna et al. 1994), Bmal1 (Bunger et al. 2000), as well as Cry1 and Cry2 (Vitaterna et al. 1999) belong to the essential molecular components of the pacemaker. To date, all investigations regarding the effects of Per genes on physiological functions and behaviour were made on male mice only, and there are no reports about the impact of these genes on the physiological functions and the reproductive success in female mice. 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 oestrous cycle, pregnancy and lactation, is characterised by rhythms coordinating these functions so that they occur at the most advantageous time (Turek & Van Cauter 1994, Johnson & Day 2000, Kennaway 2005, Dolatshad et al. 2006). This rhythmic behaviour of pregnant and lactating females also forms the cyclic environment for foetuses and neonates before their development enables photic entrainment of the circadian pacemaker (Weaver & Reppert 1995). Thus, maternal condition, health and intact rhythmicity all affect the 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 Clock in mice have caused a disrupted oestrous cyclicity and poor reproductive outcome associated with increased foetal absorption during pregnancy and high pregnancy failure (Kennaway 2005, Dolatshad et al. 2006, Hoshino et al. 2006). Clock mutations may even affect negatively the growth of pups (Miller et al. 2004). Such a decline in fertility and fecundity is also characteristic for ageing females. This decline is generally reflected by smaller litters in polytocous species, increasing intervals between litters or individual births, increased resorptions, abortions and stillbirths (Meites et al. 1976, Talbert 1977, Lu et al. 1979, Matt et al. 1987). Changes in these parameters may give information about age-related changes in the hormonal control of oestrous cycles (Meites et al. 1976, Lu et al. 1979, Matt et al. 1987). Generally, the periods of pregnancy and lactation are characterised by an increased metabolic rate and a preference for high protein food (Trojan & Wojciechowska 1968, Lewis et al. 2001, Kasparian & Millar 2004). Thus, energy expenditure is an important factor during reproductive stages and decreases in ageing females (Luz & Griggs 1998). Since recent studies on Clock and Bmal1 mutants have revealed that clock genes play a role in the regulation of metabolic rate (Shimba et al. 2005, Turek et al. 2005, Kornmann et al. 2007), it is likely to assume that also Per genes may be involved in metabolic regulation.
In the present study, we investigated the impact of \textit{Per1} and \textit{Per2} clock genes on reproductive functions associated with maternal behaviour and the food consumption during pre- and postnatal phases as well as the oestrous cycle length in middle-aged females, using \textit{Per1} and \textit{Per2} mutant mice. In addition, the body weights of the offspring were recorded to monitor their development.

\section*{Results}

\subsection*{Reproductive success}

The result of reproductive outcome of all three strains of middle-aged (9–12 months of age) and young adult females (2–6 months old) are presented in Table 1. All primiparous (first parturition) and multiparous (second parturition) females of all three strains were successful in becoming pregnant (Table 1). In contrast to \textit{Per1} and \textit{Per2} mutant females, almost every pregnant wild-type female gave birth and was successful in nursing their pups until weaning (Table 1). Primiparous wild-type and \textit{Per1} mutant females produced the same litter size as multiparous females, i.e. they did not differ in their reproductive outcome (\textit{U}-test: \textit{P}=0.61). However, only 33.3\% of multiparous \textit{Per1} mutant females bred successfully, whereas 90\% of the wild-type multiparous females bred. By contrast, \textit{Per2} mutant females as primiparous females did not raise a single pup successfully (Table 1). The pups were obviously cannibalised by their mothers. As multiparous females they produced significantly smaller litter sizes than wild-type females did (\textit{U}-test: \textit{P}>0.05). Young adult \textit{Per1} and \textit{Per2} mutants produced significantly larger litter sizes – on average 7.9 and 7 respectively than the middle-aged (9–12 months of age) \textit{Per} mutants (\textit{U}-test: \textit{Per1}(-/-): \textit{P}>0.05; \textit{Per2}(-/-): \textit{P}>0.05). Furthermore, the litter size of young adult \textit{Per} mutants did not differ from the litter size of the adult wild-type (Kruskal–Wallis ANOVA: \textit{P}=0.15). Hence, primiparous as well as multiparous middle-aged mutant female mice \textit{Per1}(-/-) and \textit{Per2}(-/-) are characterised by a low reproductive success in comparison with the middle-aged wild-type females.

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

\subsection*{Oestrous cycle}

All wild-type females exhibited a regular cycling oestrus (100\% over 6 weeks). A 4-day oestrous cycle in wild-type females occurred significantly more often than in \textit{Per} mutant females (Kruskal–Wallis ANOVA: \textit{P}<0.001; Fig. 1). However, \textit{Per1}(-/-) and \textit{Per2}(-/-) females were acyclic in 37.07 and 44.85\% of this time respectively. The remaining time was characterised by cyclicity of 4, 5 and longer than 6 days, while \textit{Per1} mutants exhibited more often 4-day cycles than prolonged cyclicity (ANOVA: \textit{P}<0.05, \textit{post hoc} test: \textit{P}=0.04).

\subsection*{Implantations in uteri and live offspring}

\textit{Per1}(-/-) and \textit{Per2}(-/-) females had a significantly higher number of embryonic scars in the uterus compared with the total number of their live offspring from the first and second parturitions (Fig. 2; Wilcoxon test: \textit{Per2}(-/-): \textit{P}<0.05, \textit{N}=10; \textit{Per1}(-/-): \textit{P}<0.05, \textit{N}=6). Wild-type females, however, did not differ significantly between the number of implantations and the number of live offspring (Wilcoxon test: \textit{P}>0.05, \textit{N}=7). Moreover, \textit{Per1}(-/-) females showed a significantly lower number of implantations than the control group (\textit{U}-test: \textit{P}<0.05).

\subsection*{Body mass of reproductive female mice}

Both as primiparous and as multiparous, the wild-type and \textit{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 multiparous and primiparous females of each strain were pooled for each time point by averaging data of primiparous and multiparous females.

\begin{table}[h]
\centering
\caption{Reproductive outcome of middle-aged and multiparous (second parturition) young adult wild-type, \textit{Per1}(-/-) and \textit{Per2}(-/-) female mice (each group \textit{N}=10), and the number of young at birth equals the number of alive weaned young in young adult reproducing females.}
\begin{tabular}{lcccc}
\hline
\textbf{Wild-type} & \textbf{\textit{Per1}(-/-)} & \textbf{\textit{Per2}(-/-)} \\
\hline
\textbf{Middle-aged females} & & & & \\
Parturition & 1st & 2nd & 1st & 2nd & 1st & 2nd \\
\textit{Pregnant (n)} & 10 & 10 & 10 & 9 & 10 & 8 \\
\textit{Gave birth (n)} & 6 & 10 & 5 & 5 & 7 & 5 \\
\textit{Successful breeder (n)} & 6 & 9 & 5 & 5 & 0 & 4 \\
\textbf{Mean litter size at birth (±s.d.)} & 6.8±1.9 & 6.2±1.98 & 5.2±2.5 & 7±1.7 & 3.5±1.3 \\
\textbf{Mean litter size at weaning (±s.d.)} & 6.3±2.2 & 5.1±1.8 & 4.6±2.5 & 5.7±2.9 & 2.8±1.7 \\
\textbf{Young adult females} & & & & \\
\textit{Pregnant (n)} & 10 & 10 & 10 & 10 \\
\textit{Gave birth (n)} & 10 & 10 & 10 & 10 \\
\textit{Successful breeder (n)} & 10 & 10 & 10 & 10 \\
\textbf{Mean litter size (±s.d.)} & 8.0±2.3 & 7.9±1.9 & 7.0±1.9 \\
\hline
\end{tabular}
\end{table}
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 non-reproductive (rep. ANOVA: \(P<0.05\)) and reproductive phases (rep. ANOVA: pregnancy: \(P<0.05\); lactation: \(P<0.05\)). Per1 mutant females had a lower body mass than the wild-type females during the non-reproductive (rep. ANOVA: \(P<0.05\)) and pregnant stages (rep. ANOVA: first stage: \(P<0.05\); middle stage: \(P<0.05\)) but not when they are lactating (Fig. 3). On the day before parturition, all three genotypes had a significantly increased body mass (one-way ANOVA: wild-type \(P<0.05\); Per1\(^{−/−}\) \(P>0.05\); Per2\(^{−/−}\) \(P>0.05\); Fig. 3). The loss of body mass after parturition was less pronounced in Per1\(^{−/−}\) and Per2\(^{−/−}\) females compared with the wild-type mice. Lactating wild-type females showed significant changes in their body mass (ANOVA: \(P>0.05\)) from the first day up to the last day of lactation. These changes were characterised by a decrease in body weight during the first 3 days, an increase till the 16th day of lactation (\(t\)-test: \(P>0.05\)) 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\(^{−/−}\) \(P=0.32\); Per2\(^{−/−}\) \(P=0.99\)).

**Protein preference and food consumption**

In contrast to non-reproductive wild-type females that preferred 20% protein content (rep. ANOVA: \(P<0.05\)), Per1\(^{−/−}\) and Per2\(^{−/−}\) females did not show any preference to a certain protein content (rep. ANOVA: Per1\(^{−/−}\) \(P=0.79\); Per2\(^{−/−}\) \(P=0.79\)). During pregnancy and lactation period, 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). During pregnancy, wild-type females increased their food consumption progressively (rep. ANOVA: \(P<0.001\)) correlating positively with the body mass (Spearman’s correlation: \(P<0.05\)). Per1 mutant females increased their daily food consumption by 20% up to midterm pregnancy but without any relationship with body mass. Pregnant Per2 mutant females show a tendency for an increased food intake (Fig. 4); however, due to the low sample size and thus high standard error, they do not increase their food consumption significantly (rep. ANOVA: \(P>0.05\)).

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).

**Average daily metabolic rate (ADMR)**

The ADMR of Per1 mutant females increased from the non-reproductive period to the lactation period (ANOVA: P<0.001; Fig. 5). However, during pregnancy the ADMR of Per1 mutant females was lower than that of the control group (U-test: P<0.001) but similar to Per2 mutant females (U-test: P=0.43). Per2 mutants did not show any significant variance in ADMR (ANOVA: 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-reproductive: P<0.05, pregnancy: P<0.001, lactation: P<0.05).

**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 for both genotypes (Wilcoxon; wild-type: first stage: P=0.73; middle stage: P=0.75; last stage: P=0.93; ANOVA; Per1<sup>+/−</sup>: P=0.93). On the day after birth, all three strains spent ~60% of their time in the nest, presumably nursing (Table 2). During the middle and last stages of lactation, wild-type and Per1<sup>+/−</sup> females spent significantly less time in the nest (ANOVA: Per1<sup>+/−</sup>: P<0.001; wild-type: P<0.001; Table 2; Fig. 6A and B). This decrease in duration in the nest correlated negatively with an increase in feeding (Spearman’s correlation; Per1<sup>+/−</sup>: P<0.001) and resting outside the nest (Spearman’s correlation; Per1<sup>+/−</sup>: P<0.001; wild-type: P<0.001). By contrast, Per2<sup>+/−</sup> females spent the same time in the nest during the three lactating stages (ANOVA: P=0.77; Table 2).

**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<sup>+/−</sup> females produced significantly smaller litters than Per1<sup>+/−</sup> and wild-type females (Table 1).

**Discussion**

**Reproductive success in young adult and middle-aged females**

Young adult Per1 and Per2 mutant females have the same reproductive success (i.e. success in fertility and fecundity) as wild-type females, confirming experiences by other laboratories with breeding these strains (U Albrecht & S Daan, personal communication). Young adult Per mutant females show the same oestrous cycle pattern lasting 4–5 days as wild-type females (unpublished data). This regularity in oestrous cyclicity provides a hint for the regularity in the rhythmicity of the luteinizing hormone (LH) surge and ovarian hormones facilitating fertility and embryonal development (Day et al. 1989, Diaz et al. 2000, Wise et al. 2002, Miller et al. 2004, Nelson 2005). Contrary to this, adult Clock mutant females reveal prolonged irregular cycles associated with elevated rates of foetal reabsorption and pregnancy failures (Miller et al. 2004, Kennaway 2005, Dolatshad et al. 2006). The oestrous cycle defects in Clock mutants have been explained by disruption in the timing or coordination of gonadotrophin-releasing hormone release and thus of LH surge on prooestrus leading to reduced levels of oestrogen and progesterone (Miller et al. 2004).
Normally, these ovarian hormones are vital for promoting fertilisation and maintaining uterine receptivity to developing foetuses during pregnancy (Itskowitz & Hodge 1988). Since young adult Per1 and Per2 mutant females do not show any degradation in oestrus cyclicity and failures during pregnancy, we can assume that the Per genes, at least at that age, seem not to have any effect on the reproductive system in the young adult females as it has been shown in adult Clock mutant females. This is surprising in view of the present pacemaker model in which the CLOCK:BMAL1 heterodimers regulate the expression of the Per genes.

In contrast to young adult Per1 and Per2 mutant females, middle-aged Per mutant females have a significantly lower reproductive rate. They produce either fewer litters 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 oestrous cycles in Per mutants compared with 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 oestrous cycle of 4 days occurred significantly more often in the control group. Acyclic phenomena, e.g. persistent vaginal cornification or leukocytosis, characterise the age-related decline of cyclicity in rats and mice (Nelson et al. 1982, Matt et al. 1987, Markowska 1999). The initial phases of declining in cyclicity from the 13th month of age onwards are prolonged and irregular cycles and cause a delay in the preovulatory rise of oestradiol (Nelson et al. 1981, Wise et al. 2002). These prolonged oestrogen secretions in mice and rats have been explained as being a result of incomplete or delayed luteinisation (Nelson et al. 1982, Matt et al. 1987). Ageing female rats displaying irregular cyclicity and acyclicity (permanent oestrus or permanent dioestrus) are unable to perform successful gestation (Matt et al. 1986). Even regularly cycling mutant females also often failed to reproduce successfully. Since only 33% of multiparous pregnant Per1 and 50% of Per2 mutants were successful in breeding, they seem to be characterised by lower fecundity compared with the control group where almost all pregnant females were successful breeders. Genetic background may have an enormous effect on the phenotype and thus, in terms of reproduction, on the fertility. Therefore, it is important to bear in mind that genetic divergences may occur in the successive generations. Ideally, to make comparisons in inbred mouse strains, it is advantageous to use litter mates (Dolatshad et al. 2006). In the present study, however, we were not able to use litter mates. As the second

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>Per1(−/−)</th>
<th>Per2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>7.09±0.07</td>
<td>7.31±0.09</td>
<td>5.72±0.34</td>
</tr>
<tr>
<td>Middle</td>
<td>4.00±0.15*</td>
<td>4.97±0.26*</td>
<td>6.27±0.16*</td>
</tr>
<tr>
<td>Last</td>
<td>4.02±0.09*</td>
<td>5.09±0.21*</td>
<td>6.42±0.25*</td>
</tr>
</tbody>
</table>

First, day 1 of lactation; middle, day 11 of lactation; last, day 21 of lactation.
*Significance within the groups versus first stage of lactation.
†Significance between Per2(−/−) and wild-type.
‡Significance versus the other genotypes.

![Figure 6](image_url) Frequency of mean resting outside of nest (A) and mean food consumption (B) during the dark phase in middle-aged lactating females (±S.E.M).

![Figure 7](image_url) Mean body mass (±S.D.) of offspring of middle-aged females from the 1st day to 31st day of age. Day 1 represents the day of birth.
best choice, we used backcrossed wild-type females consisting of B6 and 129S7 as controls for the Per mutants with the same background. The recent studies on Clock mutant females (Miller et al. 2004, Dolatshad et al. 2006) revealed discrepancies with regard to the light condition under LD conditions. In the study of Dolatshad et al. (2006), however, the same changes occurred only under constant darkness. The differences in results regarding fertility in Clock mutant females in both studies may be associated with background effects (as discussed by Dolatshad et al. 2006) or, as can be assumed from our results, with differences in age of the females in the studies by Miller et al. (2004) and Dolatshad et al. (2006).

Comparisons of the number of implantation scars with the number of 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 & Butcher 1966, 1971, Butcher & Pope 1979, Fossum et al. 1989). Thus, the uterus of aged animals appears to be responsible for post-implantation loss in those females with implantation sites (Parkening et al. 1978). Moreover, it has been demonstrated recently that clock genes including Per1 and Per2 genes are expressed in oocytes and in the early pre-implantation conceptus (Johnson et al. 2002), indicating timing mechanisms that drive both developmental transitions and cell cycles (Johnson & Day 2000). Hence, it is likely to assume that mutations in these clock genes, in our case Per1 and Per2 genes, may affect timing of fertility and embryonal development. Since young adult Per mutant females reproduced as successful as wild-type females, we can almost exclude an impact of Per genes on the embryonal development itself. The low fertility in both middle-aged Per mutant females was characterised by a high number of implantation scars and low total number of surviving pups. Thus, Per mutants seem to suffer from post-implantation loss. Previous studies on rabbits (Larson & Foote 1972) and hamsters (Parkening & 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- to 12-month-old Per1 and Per2 mutant females are qualitatively similar to those of the 13- to 16-month-old C57BL/6j female mice that were investigated by Nelson et al. (1982).

**Energetic investment of females and maternal behaviour**

The period of pregnancy is characterised by an increase in metabolic rate related to rapid growth of the embryos, causing an enhancement in energy intake by the female (Trojan & Wojciechowska 1968, Migula 1969, Johnson et al. 2002, Degen et al. 2002, Johnston et al. 2006). However, Per2 mutant females did not significantly increase their food intake during pregnancy, while Per1 mutant females exhibited an increase in food consumption during midterm pregnancy. The lack of an increased food intake during pregnancy in Per2 mutant females may be explained by their high rate of post-implantation embryonic death, but we cannot exclude a statistical error due to the low sample size leading to a high standard error. The control group showed a continuous increase in food consumption in all three stages, similar to reports for pregnant common voles (Migula 1969).

The fact that both young adult Per mutants have the same body mass as wild-type females (unpublished data), whereas middle-aged Per1 and Per2 mutant females are lighter and heavier than the control group respectively is a further indicator that Per1 and Per2 clock genes in ageing females regulate the metabolic rate differently. An additional explanation for the lower body mass in middle-aged Per1 mutant females may be their wheel-running activity, which is higher than that of middle-aged Per2 mutant and wild-type females (unpublished data). An influence of the circadian clock on metabolic function is well established (for reviews see, Kohsaka & Bass 2006, Wijnen & Young 2006) and mutations in other clock genes such as Bmal1 and Clock have previously been reported to influence energy balance and metabolism (Shimba et al. 2005, Turek et al. 2005, Kornmann et al. 2007). On the molecular level, mammalian circadian oscillators rely on the complex interaction of activators and repressors (Reppert & Weaver 2002, Sato et al. 2006). Therefore, a lack of some clock genes, e.g. Per genes may cause changes in the transcription of Bmal1 and Clock genes that are involved in regulating metabolic processes. To date, the interdependency of clock genes regarding their transcription, particularly when one of the genes is lacking, is largely unknown. Also, in our study, we cannot say whether the lack of either Per1 or Per2 clock genes may affect the transcription of Bmal1 and Clock genes. We can show differences in metabolisable energy intake between reproducing middle-aged Per mutant and wild-type females. This fact provides a further explanation for the failures of middle-aged Per mutant females during pregnancy, which 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 & Markl 1987). However, the maternal care can vary with the size of the litter (Priestnall 1972, König & Markl 1987). Females caring for large litters are known to produce more milk than females caring for small litters (Kumaresan & Turner 1967). 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 & Markl 1987). 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 & 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. 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 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 than wild-type and Per1 mutants, the body mass of the offspring of the three genotypes did not differ from each other. Thus, the total energetic investment of Per2 mutant females into only 2.8 pups must certainly be lower than that of wild-type females with larger litter sizes. 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.

In conclusion, the function of Per1 and Per2 clock genes has been studied up to now only in adult male mice (Albrecht et al. 2001, Steinlechner et al. 2002). In the present study, we show that Per1 and Per2 clock genes seem not to have any influence on the reproductive outcome in young adult females. However, between 9 and 12 months of age, the Per mutant females are clearly less fertile than wild-type females. Furthermore, their oestrous cycles, characterised by prolongation and acyclicity, were comparable with the cyclicity of ageing 13- to 16-month-old C57BL/6j female mice. Thus, these results suggest that Per clock genes cause an accelerated ageing resulting in poor reproductive fitness. Furthermore, studies are needed in order to clarify the impact of Per genes on the ageing process and the hormonal level in association with SCN functionality.

Materials and Methods

**Animals**

For our experiments, we used homozygous null mutants: 129S7-Per1tm1Brd (Per1<sup>−/−</sup>; Zheng et al. 2001) and B6, 129S7-Per2tm2Shd (Per2<sup>−/−</sup>; Zheng et al. 1999) with undefined background consisting of B6 and 129S7. Because all genotypes were maintained homozygous, no littermates of the transgenic animals were available as controls. Instead, a backcrossed strain with background B6 and 129S7 was used as a control group. Thirty virgin females ranging in age from 7 to 9 months at the beginning of the experiment (=middle-aged females) were housed individually in polycarbonate transparent cages type III containing wood shavings as bedding material. The animals were divided into three groups of ten individuals: in a control group represented by wild-type B6, 129S7, in Per1<sup>−/−</sup> and Per2<sup>−/−</sup> mutant females. For comparison of reproductive success, another 30 females (10 of each genotype, 2–6 months of age (=young adult), were used. They were maintained at 22 ± 1°C under a 12 h light:12 h darkness cycle with lights on at 2200 h and off at 1000 h. The room was equipped with two red lamps that were permanently on and provided <6 lux at cage level during the dark phase. Food and water were given ad libitum. Ten males of each strain were kept in the same room as the females. For copulation, each female in prooestrus was placed in the cage of a male of the same genotype for 1 day and then put back into their own cage. During the non-reproductive and reproductive phases, all females were weighed daily. Pregnancy was recognised by progressively increasing body mass following copulation. 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 parturitions 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 females that became pregnant after first mating were considered as primiparous. Primiparous females that gave birth or became pregnant successfully after the second mating were considered as multiparous. For the first and second matings, we always used the same females. 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 oestrous cycle**

To distinguish the different phases and the length of the oestrous cycle, vaginal smears were taken daily in the mice's activity phase between 1100 and 1300 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 deluting loop into the vagina, not further than 1 mm so as to minimise the possibility of inducing pseudo-pregnancy (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 10×60. The vaginal smears were taken daily for 6 weeks in order to identify the length of oestrous cycle and to monitor the oestrus status for successful copulation with a male at the end of this period. After 1.5 months, each female in prooestrus was placed for 24 h in a cage with a male of the same genotype and was then checked for a vaginal plug to verify copulation.

The smears were classified into different oestrous stages, according to the description of Nelson et al. (1982). Prolonged dioestrus and permanent oestrus for at least 15 days were considered as anoestrous. An oestrous 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 CO₂ 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, Lage, 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 (Caesar & 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 husk and pieces of food in bedding) and separating according to diet (colour). Food was replaced after weighing, and at the end all bedding was replaced. The leftover 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 constant body energy content to represent ADMR; (Degen et al. 1998). Measurements were conducted on non-reproductive and twice on reproductive primiparous and multiparous females (Per1(−/−), Per2(−/−) and wild-type) in the middle stage of pregnancy (days 11–12) and on the 5th day of lactation (second 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. The gross energy content of the isocaloric dry diet was 12.67 MJ/kg. After 24 h, all food and faeces were removed and stored frozen at −20 °C. The faecal samples were dried at 65 °C for 24 h, weighed and homogenised, crushing the faeces with a mortar to powder. The gross energy content (kJ/g) of about 0.85 g homogenised faeces of each sample was determined by adiabatic bomb calorimetry (automatic O₂ 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:

\[ \text{ADMR} = \frac{\text{gross energy intake kJ} - \text{faecal energy output kJ}}{\text{body mass (g)}} \]

**Maternal behaviour**

To analyse maternal behaviour, the lactation that took 21 days was divided into three stages: (1) early stage of lactation – 1 day after parturition, (2) middle stage of lactation – on the 11th day of lactation and (3) last stage of lactation – 1 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 1000 to 2200 h under infrared light. The behaviours of lactating primiparous (six wild-type and five Per1(−/−)) and multiparous (nine wild-type, three Per1(−/−) and four Per2(−/−)) females were recorded continuously and analysed using The Observer program (Noldus, Wageningen, Netherlands). The following behaviours were scored: (1) maternal care, i.e. behavior directed at the young-licking and grooming pups, and (2) nursing – mother lying in nest either on her side or over the pups while nursing; self-directed activities: (1) feeding – mother going to the food containers and eating or drinking water from a water bottle, (2) resting – mother standing or lying outside the nest without any body contact to any offspring, (3) grooming – self-grooming and (4) locomotor activity – walking, running and climbing on the cage lid.
Statistical analysis

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 Tukey’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. Due to various time points of abortions during pregnancy (days 16–20 of pregnancy), the body mass and ADMR of pregnant females that did not produce any alive young during pregnancy (days 16–20 of pregnancy), the body mass and ADMR were not included in the dataset of successfully reproducing females. However, they are included in Table 1 and Fig. 2. Results are presented as means ± S.E.M. or means ± S.D. The differences were considered significant at P<0.05.

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