CIRCADIAN RHYTHMS AND FERTILITY

David J. Kennaway, Michael J. Boden and Tamara J. Varcoe

Robinson Institute,

Research Centre for Reproductive Health

University of Adelaide,

Adelaide, South Australia, 5005

Corresponding author:

Associate Professor David Kennaway,
Robinson Institute,
Research Centre for Reproductive Health
University of Adelaide,
Adelaide, South Australia, 5005

Email: david.kennaway@adelaide.edu.au
Fax: 61-8-83034090

Abstract

Circadian rhythms impact on a wide range of physiological systems and this impact extends to fertility, such that disruptions to timing systems can impact upon reproductive capacity. This is highlighted most obviously in mutant mouse models whereby deletion or mutation of single genes results not only in disrupted circadian rhythmicity, but also compromised male and female reproductive function. In this review we discuss the presence of circadian clocks in female and male reproductive tissues and the role these clocks play in the generation of oestrus cycles, ovulation, sperm generation, implantation and the maintenance of pregnancy. Given the increased incidence of shiftwork and international travel which disrupt circadian rhythmicity, and the increasing prevalence of reproductive technologies whereby early embryo development occurs without external time cues, it is important for us to consider the role of circadian rhythms in fertility.

Successful reproduction is an obvious fundamental aspect of survival for a species and it needs to be efficient. Females in particular invest considerable energy in sustaining pregnancy and in the care and nursing of the offspring. Reproductive efficiency extends to ensuring that post natal development occurs at a time of year when the nutritional needs of the offspring can be met. To meet these demands, physiological systems have evolved that integrate daily and seasonal environmental signals to coordinate the various processes required for ovulation, mating, embryo development and parturition. Thus light is detected by the retina and information about its wavelength, intensity and timing is processed by the retina and the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. The SCN then signals to the pineal gland to commence nocturnal production of melatonin and shut down its synthesis in the morning. It also sends signals to other organ systems including the hypothalamic/pituitary/gonadal axis. The circadian timing system is a hierarchical system with endogenous rhythmicity generated through clock gene transcription factors in the SCN, and entrained by light. Other organs systems also express cellular rhythmicity generated by the same genes which are entrained either directly or indirectly by the SCN. In this review we discuss the role of circadian rhythms in fertility and early embryo development. Other reviews in this issue cover the role of rhythms in the later fetal period and parturition. We have concentrated on studies which have been published since our previous reviews on this topic (Boden and Kennaway, 2006; Kennaway, 2005).

Hypothalamus/Pituitary

The reproductive biology of laboratory rodents has been extensively studied over many decades and as a consequence our understanding of the role of circadian rhythmicity in fertility is most developed in rats and mice. Temporal gating of ovulation in rodents has been known for over 60 years (Everett and Sawyer, 1950). For example in studies of the neural

basis of the luteinising hormone (LH) surge, it was found that the stimulus for the release of the hormone begins 9 hours after light onset on the day of pro-estrus and lasts for only a few hours. Administration of pentobarbital (a short-acting barbiturate hypnotic which acts on GABA-A receptors) prior to this time prevents ovulation while the same drug administered after the "critical period" is ineffective. Interestingly, if the LH release was blocked by pentobarbital, the surge subsequently appeared on the following day at the appropriate time of day, providing compelling evidence for the involvement of a timing system in this process (Everett and Sawyer, 1950). Intensive blood sampling across the 4 day cycle of rats and mice revealed that the LH surge commences during the late afternoon of pro-estrus peaking around 12 midnight, with ovulation and mating occurring several hours later (Murr et al., 1973). Subsequent studies indicated the critical role of the SCN in the timing of the LH surge and ovulation (Wiegand et al., 1980). The neural connections between the SCN and other brain regions involved in the control of ovulation are discussed elsewhere in this issue.

Ovary

Like the majority of tissues that have been studied, the ovary expresses the core oscillator genes rhythmically (Fahrenkrug et al., 2006), such that *Bmal1* mRNA expression is highest around the time of light onset and *Per2* mRNA is highest at the time of lights off in the rat (Karman and Tischkau, 2006). There is an 8 hour difference in phase between the ovary and SCN, with the SCN rhythm preceding the ovary. The timing of the gene expression rhythm in the rat ovary is unaffected by the stage of the cycle (diestrus 1 versus proestrus) (Fahrenkrug et al., 2006). In more intensive studies in mice, *Per2* mRNA expression peaks were slightly advanced at diestrus and proestrus, compared with metestrus and estrus (Nakamura et al., 2010).

As discussed above, the timing of the LH surge and ovulation are under tight SCN/circadian control, but until recently the role of cellular rhythms in ovulation has not been understood. To address this question, gene expression in the ovaries of hypophysectomised juvenile rats which had never experienced an LH surge or ovulated was studied. The ovaries did not express *Bmal1* or *Per2* mRNA rhythmically (Karman and Tischkau, 2006), but when equine chorionic gonadotropin was administered to these rats, there was induction of *Per2* mRNA within 4 hours, which was followed 8 hours later by a peak in *Bmal1* mRNA expression. The conclusion drawn was that the LH surge therefore may play an important role in timing ovarian rhythmicity. This suggestion was addressed further in a set of elegant experiments by Yoshimura and colleagues using several different approaches (Yoshikawa et al., 2009). First they showed that sectioning of the superior ovarian nerve failed to impact on the rate of entrainment of ovarian *Per2-luc* expression following a phase shift of the light/dark cycle, suggesting that sympathetic input is not necessary for controlling ovarian rhythms. On the contrary using a transplant and culture approach it was determined that LH and FSH signalling may synchronise the ovarian clock. Nevertheless there is also a role for ovarian rhythms in determining *when* the organ will respond to LH. When endogenous LH secretion was suppressed by a long acting GnRH antagonist, the ovulatory response to exogenous gonadotropin varied markedly across 24 hours with peak recovery of oocytes occurring on the night of proestrus (Sellix et al., 2010).

Both granulosa and thecal cells of the ovary have been shown *ex vivo* to rhythmically express *Bmal1* and *Per2* mRNA and protein. (Fahrenkrug et al., 2006; Karman and Tischkau, 2006). When granulosa cells were cultured for 48 hours and then treated with dexamethasone to synchronise clock gene expression in the cells, *Per2* mRNA was induced within 4 hours, decreased to low levels and then peaked again approximately 24 hours later, but there were

no further cycles after that (He et al., 2007). By contrast, luteal cells sustained at least 3 cycles of *Per2* expression following dexamethasone stimulation. The *Per2* oscillations could be restored by a medium change or stimulation with forskolin and the authors speculated that the failure to sustain rhythmicity was due to their differentiating state.

Oocytes/early embryos

There have been several studies addressing the question of whether oocytes and early embryos express clock genes and express them rhythmically. The first, a qualitative study, was conducted in mice at a single time of day (mid light) and reported that *Per1*, *Cry1*, *Clock* and *Bmal1* mRNA was detectable in unfertilised oocytes and day 1 pronuclear zygotes, 2 cell and 8 – 16 cell embryos and blastocysts (Johnson et al., 2002). Quantitative RT PCR studies of clock gene expression at multiple time points in oocytes and pre-implantation mouse embryos confirmed the presence of *Per1, Per3, Cry1, Clock*, and *Bmal1* mRNA at various stages of development (Amano et al., 2009). In all cases, the expression of these genes was highest in the oocytes and 1 and 2 cell zygotes until about 30 hours post insemination when the expression decreased to very low levels. There was no indication of a rhythm of expression and the authors concluded that the transcripts were probably maternal mRNAs that were not being translated and thus the clock was not ticking in the embryos. Similar patterns of expression were reported by the same authors in cattle and rabbit oocytes and pre-implantation embryos (Amano et al., 2010). Embryonic stem cells also lack circadian rhythms of gene expression as demonstrated by the failure to detect bioluminescence rhythmicity from *Bmal1*-promoter driven luciferase activity (Yagita et al., 2010). Examination of rhythmicity in later embryonic development again failed to show evidence of the expected antiphase rhythm of *Per2* and *Bmal1* mRNA expression in whole embryos at embryonic days E10-E11, E14-E15 or E18-E19 (Dolatshad et al., 2010). Furthermore no

rhythmicity in *Per2* or *Bmal1* mRNA expression was detected in liver kidney or heart of E18-E19 embryos. Surprisingly liver, kidney and heart from *Per2-luc* mice did show circadian oscillations in light emissions but the timing was dependent upon the time of day that the cultures were established. The failure to express gene rhythmicity during embryo development is unlikely to be due to the absence of melatonin rhythmicity in the C57Bl/6 mice since the melatonin proficient C3H strain also lacked embryonic rhythmicity (Dolatshad et al., 2010). As stated, embryonic tissues appear poised to express circadian regulatory cycles but for some reason do not. These results are all the more interesting since the gravid uterus, placenta and fetal membranes of E16 embryos display robust rhythms of clock gene expression (Ratajczak et al., 2010).

Oviduct, uterus and placenta

The oviduct plays an essential role in the transport of the developing embryo into the uterus while at the same time providing essential nutrients and growth factors to ensure optimal development. We have shown previously that the rat oviduct expresses clock genes rhythmically together with a putative clock controlled gene, *plasminogen activator inhibitor 1* which has been implicated in embryo development (Kennaway et al., 2003). The non-pregnant and pregnant uterus also expresses clock genes rhythmically (Nakamura et al., 2010; Ratajczak et al., 2010), at mid and late gestation, and in light/dark and constant darkness conditions (Akiyama et al., 2010). Additionally, all layers of the mid and late gestation rat placenta were found to express *Per1* mRNA, although only the maternally derived decidua expressed this transcript rhythmically (Akiyama et al., 2010).

Clock gene disruption and fertility

With such a key role for the central biological clock (SCN) in the timing and/or success of ovulation it might be expected that the fertility of animals with a disrupted cellular timing system would be compromised. This has been found to be the case, but the extent of the impact on fertility is not as extensive as might be predicted, suggesting that compensatory mechanisms may play a significant role in maintaining reproductive function when the circadian timing system is altered. The first studies on animals with genetically altered rhythmicity were conducted on *Clock* mutant mice (Vitaterna et al., 1994) which produce a truncated CLOCK protein which is capable of dimerising with its partner BMAL1, but not bind to the regulatory E-box sequences in the promoters of *Per1*, *Per2*, *Cry1*, *Cry2* and other genes (King et al., 1997). The *Clock* mutant mice kept in a 12L:12D photoperiod generally confine their activity to the dark period (ie., are entrained) (Kennaway et al., 2003; Vitaterna et al., 1994) and sustain rhythmicity, albeit with a very long period of approximately 27 hours, for at least a few cycles in continuous darkness. Moreover they can actually be entrained to a skeleton photoperiod (0.5L:23.5D) suggesting that light dependent entrainment mechanisms are retained (Kennaway et al., 2003). When maintained on a CBA background which has the capacity to synthesise melatonin, the *Clock* mutants rhythmically secretes this hormone (Kennaway et al., 2003). The apparent reason for this retention of centrally driven rhythmicity is the partial rescue of the cellular timing system by a *Clock* paralog, *Npas2* in the SCN (Debruyne et al., 2007; Kennaway et al., 2006). No such rescue has been reported in other tissues of the *Clock* mutant mice, including the liver, muscle, fat and heart. There are no studies on the rhythmicity of ovaries in the *Clock* mutants but given the lack of rhythmicity in other tissues, this is unlikely.

Clock mutant mice have normal timing of the onset of vaginal opening (a surrogate measure of the onset of puberty) and ovulate, although there is evidence of higher proportions of

irregular estrus cycles (Dolatshad et al., 2006; Kennaway et al., 2004; Miller et al., 2004). They produce normal amounts of estradiol and progesterone and undergo normal follicular development and corpora lutea formation following ovulation. Curiously no LH surge was detected during the late afternoon and evening of proestrus in the *Clock* mutants (Miller et al., 2004). Since only 50% of the wild type controls had an LH surge during the sampling period and the experimental design did not include confirmation that ovulation actually occurred, it is perhaps premature to imply that *Clock* mutant mice do not have an LH surge. A likely explanation is that the stress of blood sampling and replacement of blood lost with donor blood prevented ovulation in both groups and this stress had a greater impact on the mutants. *Clock* mutant mice did, however, also fail to respond to estradiol administration with an increase in LH which does imply disruption to gonadotropin releasing hormone release. The mutants were also shown to have decreased *Avp* (arginine vasopressin*)* mRNA expression in the SCN, but 50% of the mice did respond to the intra cerebroventricular injection of AVP with LH surges suggesting that there are deficits in hypothalamic function (Miller et al., 2006). Despite the apparently altered hypothalamic/pituitary function, *Clock* mutant mice are fertile and carry pregnancies to term (Dolatshad et al., 2006; Kennaway et al., 2004; Miller et al., 2004). There are some reports of parturition difficulties (Miller et al., 2004) and lactation problems (Hoshino et al., 2006) in *Clock* mutants on different background strains, but it is quite feasible to maintain viable homozygous *Clock* mutant colonies (Kennaway et al., 2004).

Mice which have had either the *Per1* (Zheng et al., 2001) or *Per2* (Zheng et al., 1999) genes disrupted can be entrained to 12L:12D and both show a shorter than normal free running period of 22.6h and 22.1h respectively in constant darkness (*Per2* mutant mice eventually become arrhythmic). Both mutants were reported to be fertile as young adults (2 – 6 months of age) and produce litter sizes similar to wild type mice of the same strains. As primiparous

"middle aged" mice, more than 80% of the *Per1* and *Per2* mutants became pregnant, but successful parturition and the numbers of pups weaned was reduced compared to wild type mice (Pilorz and Steinlechner, 2008). The authors suggested that disruption of these genes may have accelerated reproductive aging. Recently Daan and colleagues reported the results of a study of reproductive performance of the same *Per2* mutants in a semi-natural environment (Daan et al., 2011). They found that the reproductive fitness of the mutants was not impaired and the frequency of the mutant allele was maintained throughout the 2 year study.

Bmal1 null mice are poorly entrained to the light dark cycle and when placed in continuous darkness display immediate arrhythmicity (Bunger et al., 2000). Studies on gene expression in various tissues have shown a lack of *Per* 2, *Nr1d1* or *Dbp* gene expression rhythms (Kondratov et al., 2006; McDearmon et al., 2006). While there is evidence that *Bmal1* null mice can develop ossified ligaments and tendons (Bunger et al., 2005) and have a reduced lifespan (Sun et al., 2006), they reach puberty only 4 days later than wild type mice (Boden et al., 2010). Early reports suggested that there was no impact on reproductive performance, the mice being described as "viable and fertile" (Cowden and Simon, 2002). *Bmal1* null mice do also have estrus cycles although they tend to be irregular (Boden et al., 2010; Ratajczak et al., 2009), the ovaries are smaller than wild type mice (Boden et al., 2010) and the embryos fail to develop and/or implant with the result that full term pregnancies were never observed. Nevertheless treatment with PMSG/HCG resulted in large numbers of follicles and corpora lutea, although fewer (approximately one third) embryos were recovered compared to the wild type mice. As expected, rhythmic gene expression in the ovaries (e.g., *Dbp*, and *Nr1d1* mRNA) was lost in the *Bmal1* null mice (Boden et al., 2010). Furthermore there were other changes in ovarian gene expression across the estrus cycle including increased expression of

Hsd3b1, *Vegfa*, *Igf1*, *Igf2*, *Prlr* (prolactin receptor), *Lep*, *Il5*, *Il10* and *Lhcgr* (LH receptor) mRNA (Boden et al., 2010) and decreased *Star* mRNA (Boden et al., 2010; Ratajczak et al., 2009). Interestingly progesterone supplementation resulted in implantation sites being detected at 10.5 days post insemination, but whether pregnancies could be sustained is still not known. These and the other studies highlight potential multiple sites of clock gene influences on reproduction from failure of sufficient LH secretion to stimulate the ovary at ovulation to altered luteal progesterone synthesis and events leading to implantation.

Clock genes in male fertility

The presence of clock gene expression in the testis was first reported for *Per1* (Sun et al., 1997; Tei et al., 1997), and subsequently for *Per2* (Shearman et al., 1997; Zylka et al., 1998), *Per3* (Zylka et al., 1998), *Cry1* and *Cry2* (Miyamoto and Sancar, 1999) although there was no evidence of rhythmic expression of *Per1*, *Cry1* or *Cry2* mRNA in the mouse (Miyamoto and Sancar, 1999). In *Cry2* knockout mice there was no alteration in testicular expression of *Cry1* or *Per1* mRNA (Miyamoto and Sancar, 1999). Interestingly high levels of *Cry1* mRNA and lower levels of *Cry2* mRNA were found to be restricted to spermatogonia as opposed to other “spermatoid” cells or Leydig cells (Miyamoto and Sancar, 1999).

An apparent rhythm in *Per1* and *Per3* mRNA expression was detected in mouse testis by Northern analysis, but there was no indication of the numbers of mice studied or the variance in the results. Subsequent more focussed and careful studies showed that neither *Per1* nor *Bmal1* mRNA expression (analysed by RNA protection assay) varied in rat testis across 24 hours (Morse et al., 2003). Interestingly *Per1* tended to be localised in spermatids while *Clock* mRNA was enriched in spermatogonia and pachytene spermatocytes which suggests they may be developmentally controlled. Alvarez and colleagues extended the information to

include *Clock*, *Per2* and *Npas2* as non-rhythmically transcribed genes and showed that while PER1 protein was readily detected in spermatogonia and condensing spermatids, it did not vary across the day (Alvarez et al., 2003). Bebas and colleagues reported contradictory results for *Per1*, *Per2* and *Bmal1* mRNA expression rhythms in mouse testis, with peak expression during the night for all 3 genes. Not only is this pattern of expression unusual with respect to the lack of an inverse relationship between the *Period* genes and *Bmal1*, but there was no change in PER1 or BMAL1 protein across 24 hours either. Nevertheless changes in mRNA and protein of clock genes were evident in certain parts of the epididymis (corpus), the vas deferens, prostate and seminal vesicles (Bebas et al., 2009).

In hamsters there is some evidence that there is indeed rhythmic clock gene expression in the testis, with *Per1* expression peaking at the end of the subjective night (Tong et al., 2004) or remaining high from the mid-light to mid-dark period (Klose et al., 2011), although these are not the normal patterns seen in other tissues. Furthermore in one study there was a simultaneous increase in *Bmal1* mRNA (Tong et al., 2004), while in another there was no change in *Bmal1* mRNA expression (Klose et al., 2011). Moreover *Per1* mRNA expression was confined to seminiferous tubules but not all tubules expressed the gene and *Bmal1* mRNA was more widespread across the tubules. In this photoperiodic species, long term constant darkness induced gonadal regression and eliminated the apparent oscillations of *Per1* mRNA but increased the amplitude of the *Bmal1* changes (Tong et al., 2004).

What are the potential functional roles of clock genes in male fertility? To our knowledge there have been no systematic studies on the fertility of mutant or null clock gene mice except for the *Bmal1* null mouse. Presumably these former mice do not present with an obvious detrimental phenotype. By contrast male *Bmal1* null mice are infertile (Alvarez et al., 2008),

with smaller testes and seminal vesicles, reduced seminiferous tubule diameter and sperm counts 70% lower than normal males. Interestingly, however, the sperm from *Bmal1* null mice were shown to be motile, could be capacitated and were capable of fertilising eggs in vitro (Alvarez et al., 2008). LH and testosterone levels were reduced by approximately 50% and 70% respectively in *Bmal1* null mice, whereas FSH levels were approximately 3.5 fold higher than wild type mice. Steroidogenic enzyme gene expression was reduced in the *Bmal1* null mice, with *Star* mRNA expression being very low. The very low *Star* mRNA expression in the testis is similar to that found in the ovary (Boden et al., 2010; Ratajczak et al., 2009). In our studies on *Bmal1* null mice at 1, 2, 6 and 9 months of age we did not observe significant decreased testicular weights post puberty but seminal vesicle weights, plasma testosterone and sperm count were all significantly reduced (Figure 1; (Boden, 2008)). Sperm motility was less affected with 27 – 40% motility in the *Bmal1* null mice compared to 39 – 53% motility in the wild type mice (Figure 1; (Boden, 2008)).

Alvarez and colleagues reported that they had never observed successful copulation (i.e. vaginal plugs) in partners of male *Bmal1* null mice (Alvarez et al., 2008) and suggested that there may be an inability to mate or disturbed mating behaviour. In our studies (Boden, 2008) we paired ten 2 month old *Bmal1* null males 1:3 with wild type females and observed that 10/10 successfully mated with females (as detected by a vaginal plug or presence of sperm in the vagina), but there was only 1 term pregnancy out of 30 potential pregnancies. In this litter of 11 pups, only 4 survived to weaning.

In summary, clock genes are expressed and translated in the testes of animals, but it is clear that the regulation of the expression is not the same as that in other tissues and genes like *Bmal1* may have other roles, for example the control of *Star*. A lack of rhythmicity of clock

gene expression was noted in the thymus (Alvarez et al., 2003) and it was suggested by these authors that this may be a feature of tissues undergoing rapid differentiation. This was also a conclusion drawn for the lack of rhythmicity in granulosa cells of the ovary.

Circadian influences on human fertility

The circadian timing system that is evident in animals is also operative in humans. Thus there are endogenous rhythms of core body temperature, melatonin, cortisol, among others. Furthermore the clock gene transcription system is also operative as expected in blood cells (Boivin et al., 2003), buccal cells (Cajochen et al., 2006), muscle (Zambon et al., 2003), adipose (Gomez-Abellan et al., 2008) and hair follicle cells (Akashi et al., 2010). It may be expected therefore that the same circadian influences on reproduction observed in animals will be present in women, but even from the limited studies in the area, this does not appear to be the case. Remarkably there have been few studies addressing the involvement of circadian rhythms in human reproduction since our review in 2005 (Kennaway, 2005) and large gaps in our knowledge still exist. We know that the LH surge occurs between midnight and 0800h (Cahill et al., 1998; Kerdelhue et al., 2002), but it is still not clear what time of day the oocytes are released. We do not know whether there are any implications for embryo development for cultured embryos in IVF procedures where they are maintained in a static environment away from the circadian influences of the oviduct. Given that there are some important fertility consequences in those instances where circadian rhythmicity is seriously disrupted (e.g. *Bmal1* null mice), it is important to consider whether the environmental disruption to rhythmicity provoked by shiftwork may impact on human fertility. Again this is a poorly studied area. Bisanti and colleagues reported an association between shiftwork and prolonged waiting time to pregnancy (Bisanti et al., 1996), while in another study it was suggested that the sleep (and inherent circadian rhythm) disturbances of shiftwork may lead

to menstrual irregularities (Labyak et al., 2002). Recently, the reproductive capacity of women possessing polymorphisms in *Bmal1*, *Bmal2*, *Clock* and *Npas2* genes have been assessed (Kovanen et al., 2010). Interestingly, a single-nucleotide polymorphisms in the *Bmal1* gene was found to be associated with increased number of pregnancies, although number of miscarriages for this polymorphism were also greatly increased. Alternatively, polymorphisms in the *Npas2* gene were associated with a decreased number of miscarriages.

Conclusion

This review has focused on the role of circadian clocks in fertility, with particular focus on the generation of oestrus cycles and ovulation, implantation, the maintenance of pregnancy and male fertility. Other reviews within this issue focus on the role of circadian clocks during embryo development and in the timing of parturition. Together, there is growing evidence that circadian rhythmicity and fertility are interconnected. The large body of data demonstrating reproductive complications in clock gene mutant animals highlights the importance of these genes and the rhythms they generate in the initiation and maintenance of successful pregnancies. However, there has been very little progress in understanding the role of these clocks in human fertility. Particularly, there is scant understanding of the role rhythmic clock and functional gene expression in maternal reproductive tissues plays for the developing oocyte/embryo, or the impact temporal disruption during this period has on implantation and pregnancy outcomes. Given the high incidence of shiftwork and the increased use of IVF procedures in our society, a greater understanding of circadian rhythmicity in the generation and maintenance of pregnancies is required.

Legend for figure

Figure 1

Effects of the loss of Bmal1 gene expression on testicular wand seminal vesicle weight, plasma testosterone and sperm count and motility at 1, 2, 6 and 9 months of age. Wild-type mice are shown as white columns and Bmal1 null mice as black columns. (a) relative testicular weight (mean SEM; mg/g body weight; n = 8 – 11 animals per genotype for each age group). * indicates there was a significant difference between the genotypes by t-test, $P < 0.05$. Note that the increase in relative testicular weight in Bmal1 null mice at 9 months of age is a reflection of the severe decrease in body weight that occurs at this age. (b) Relative seminal vesicle weight for the same animals (c) Plasma testosterone levels (ng/ml) in n = 7 – 9 animals per genotype for each age group. (d) Sperm count and (e) percentage of motile sperm. Data is from the thesis of Boden (2008).

References

Akashi, M., Soma, H., Yamamoto, T., Tsugitomi, A., Yamashita, S., Yamamoto, T., Nishida, E., Yasuda, A., Liao, J.K. and Node, K., 2010. Noninvasive method for assessing the human circadian clock using hair follicle cells. Proc. Natl. Acad. Sci. U.S.A. 107, 15643-15648.

Akiyama, S., Ohta, H., Watanabe, S., Moriya, T., Hariu, A., Nakahata, N., Chisaka, H., Matsuda, T., Kimura, Y., Tsuchiya, S., Tei, H., Okamura, K. and Yaegashi, N., 2010. The uterus sustains stable biological clock during pregnancy. Tohoku J. Exp. Med. 221, 287-298.

Alvarez, J.D., Chen, D., Storer, E. and Sehgal, A., 2003. Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. Biol. Reprod. 69, 81-91.

Alvarez, J.D., Hansen, A., Ord, T., Bebas, P., Chappell, P.E., Giebultowicz, J.M., Williams, C., Moss, S. and Sehgal, A., 2008. The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. J. Biol. Rhythms 23, 26-36.

Amano, T., Matsushita, A., Hatanaka, Y., Watanabe, T., Oishi, K., Ishida, N., Anzai, M., Mitani, T., Kato, H., Kishigami, S., Saeki, K., Hosoi, Y., Iritani, A. and Matsumoto, K., 2009. Expression and functional analyses of circadian genes in mouse oocytes and preimplantation embryos: Cry1 is involved in the meiotic process independently of circadian clock regulation. Biol. Reprod. 80, 473-483.

Amano, T., Tokunaga, K., Kakegawa, R., Yanagisawa, A., Takemoto, A., Tatemizo, A., Watanabe, T., Hatanaka, Y., Matsushita, A., Kishi, M., Anzai, M., Kato, H., Mitani, T., Kishigami, S., Saeki, K., Hosoi, Y., Iritani, A. and Matsumoto, K., 2010. Expression analysis of circadian genes in oocytes and preimplantation embryos of cattle and rabbits. Anim. Reprod. Sci. 121, 225-35.

Bebas, P., Goodall, C.P., Majewska, M., Neumann, A., Giebultowicz, J.M. and Chappell, P.E., 2009. Circadian clock and output genes are rhythmically expressed in extratesticular ducts and accessory organs of mice. FASEB J. 23, 523-533.

Bisanti, L., Olsen, J., Basso, O., Thonneau, P., Karmaus, W., Juul, S., Fletcher, Bolumar, F., Figatalamanca, I., Pantelakis, S., Spinelli, A., Schaumburg, Wulff, M. and Biczysko, R., 1996. Shift work and subfecundity - a european multicenter study. J. Occup. Environ. Med. 38, 352-358.

Boden, M.J. 2008. The role of circadian rhythms in reproduction: Development and fertility in the *bmal1* null mouse. University of Adelaide PhD Thesis, pp 187, http://digital.library.adelaide.edu.au/dspace/handle/2440/48491

Boden, M.J. and Kennaway, D.J., 2006. Circadian rhythms and reproduction. Reproduction 132, 379-392.

Boden, M.J., Varcoe, T.J., Voultsios, A. and Kennaway, D.J., 2010. Reproductive biology of female *Bmal1* null mice. Reproduction 139, 1077-90.

Boivin, D.B., James, F.O., Wu, A., Cho-Park, P.F., Xiong, H. and Sun, Z.S., 2003. Circadian clock genes oscillate in human peripheral blood mononuclear cells. Blood 102, 4143-4145.

Bunger, M.K., Walisser, J.A., Sullivan, R., Manley, P.A., Moran, S.M., Kalscheur, V.L., Colman, R.J. and Bradfield, C.A., 2005. Progressive arthropathy in mice with a targeted disruption of the Mop3/Bmal-1 locus. Genesis 41, 122-132.

Bunger, M.K., Wilsbacher, L.D., Moran, S.M., Clendenin, C., Radcliffe, L.A., Hogenesch, J.B., Simon, M.C., Takahashi, J.S. and Bradfield, C.A., 2000. Mop3 is an essential component of the master circadian pacemaker in mammals. Cell 103, 1009-1017.

Cahill, D.J., Wardle, P.G., Harlow, C.R. and Hull, M.G., 1998. Onset of the preovulatory luteinizing hormone surge: diurnal timing and critical follicular prerequisites. Fert. Steril. 70, 56-59.

Cajochen, C., Jud, C., Munch, M., Kobialka, S., Wirz-Justice, A. and Albrecht, U., 2006. Evening exposure to blue light stimulates the expression of the clock gene PER2 in humans. Eur. J. Neurosci. 23, 1082-1086.

Cowden, K.D. and Simon, M.C., 2002. The bHLH/PAS factor MOP3 does not participate in hypoxia responses. Biochem. Biophys. Res. Comm. 290, 1228-1236.

Daan, S., Spoelstra, K., Albrecht, U., Schmutz, I., Daan, M., Daan, B., Rienks, F., Poletaeva, I., Dell'omo, G., Vyssotski, A. and Lipp, H.P., 2011. Lab mice in the field: unorthodox daily activity and effects of a dysfunctional circadian clock allele. J. Biol. Rhythms 26, 118-29.

Debruyne, J.P., Weaver, D.R. and Reppert, S.M., 2007. CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock. Nat.Neurosci. 10, 543-545.

Dolatshad, H., Campbell, E.A., O'Hara, L., Maywood, E.S., Hastings, M.H. and Johnson, M.H., 2006. Developmental and reproductive performance in circadian mutant mice. Hum. Reprod. 21, 68-79.

Dolatshad, H., Cary, A.J. and Davis, F.C., 2010. Differential expression of the circadian clock in maternal and embryonic tissues of mice. PLoS ONE 5, e9855.

Everett, J.W. and Sawyer, C.H., 1950. A 24-hour periodicity in the "LH-release apparatus" of female rats, disclosed by barbiturate sedation. Endocrinology 47, 198-218.

Fahrenkrug, J., Georg, B., Hannibal, J., Hindersson, P. and Gras, S., 2006. Diurnal rhythmicity of the clock genes Per1 and Per2 in the rat ovary. Endocrinology 147, 3769-3776.

Gomez-Abellan, P., Hernandez-Morante, J.J., Lujan, J.A., Madrid, J.A. and Garaulet, M., 2008. Clock genes are implicated in the human metabolic syndrome. Int. J. Obes. (Lond) 32, 121-128.

He, P.J., Hirata, M., Yamauchi, N., Hashimoto, S. and Hattori, M.A., 2007. The disruption of circadian clockwork in differentiating cells from rat reproductive tissues as identified by in vitro real-time monitoring system. J. Endo. 193, 413-20.

1 Hoshino, K., Wakatsuki, Y., Iigo, M. and Shibata, S., 2006. Circadian Clock mutation in
2 dams disrupts nursing behavior and growth of pups. Endocrinology 147, 1916-1923.

3 Johnson, M.H., Lim, A., Fernando, D. and Day, M.L., 2002. Circadian clockwork genes are
4 expressed in the reproductive tract and conceptus of the early pregnant mouse. Reprod.
5 Biomed. Online 4, 140-145.

6 Karman, B.N. and Tischkau, S.A., 2006. Circadian clock gene expression in the ovary:
7 Effects of luteinizing hormone. Biol. Reprod. 75, 624-632.

8 Kennaway, D.J., 2005. The role of circadian rhythmicity in reproduction. Hum. Reprod.
9 Update 11, 91-101.

10 Kennaway, D.J., Boden, M.J. and Voultsios, A., 2004. Reproductive performance in female
11 Clock $^{\Delta 19}$ mutant mice. Reprod. Fertil. Develop. 16, 801-810.

12 Kennaway, D.J., Owens, J.A., Voultsios, A. and Varcoe, T.J., 2006. Functional central
13 rhythmicity and light entrainment, but not liver and muscle rhythmicity, are Clock
14 independent. Am. J. Physiol. Regul. Integr. Comp. Physiol. 291, R1172-R1180.

15 Kennaway, D.J., Varcoe, T.J. and Mau, V.J., 2003. Rhythmic expression of clock and clock-
16 controlled genes in the rat oviduct. Mol. Hum. Reprod. 9, 503-507.

17 Kennaway, D.J., Voultsios, A., Varcoe, T.J. and Moyer, R.W., 2003. Melatonin and activity
18 rhythm responses to light pulses in mice with the Clock mutation. Am. J. Physiol. Regul.
19 Integr. Comp. Physiol. 284, R1231-1240.

Kerdelhue, B., Brown, S., Lenoir, V., Queenan, J.T., Jr., Jones, G.S., Scholler, R. and Jones, H.W., Jr., 2002. Timing of initiation of the preovulatory luteinizing hormone surge and its relationship with the circadian cortisol rhythm in the human. Neuroendocrinology 75, 158-163.

King, D.P., Zhao, Y., Sangoram, A.M., Wilsbacher, L.D., Tanaka, M., Antoch, M.P., Steeves, T.D., Vitaterna, M.H., Kornhauser, J.M., Lowrey, P.L., Turek, F.W. and Takahashi, J.S., 1997. Positional cloning of the mouse circadian Clock gene. Cell 89, 641-653.

Klose, M., Grote, K. and Lerchl, A., 2011. Temporal control of spermatogenesis is independent of the central circadian pacemaker in Djungarian hamsters (Phodopus sungorus). Biol. Reprod. 84, 124-9.

Kondratov, R.V., Shamanna, R.K., Kondratova, A.A., Gorbacheva, V.Y. and Antoch, M.P., 2006. Dual role of the CLOCK/BMAL1 circadian complex in transcriptional regulation. FASEB J. 20, 530-532.

Kovanen, L., Saarikoski, S.T., Aromaa, A., Lönnqvist, J. and Partonen, T., 2010. *ARNTL* (*BMAL1*) and *NPAS2* gene variants contribute to fertility and seasonality. PLoS ONE 5, e10007.

Labyak, S., Lava, S., Turek, F. and Zee, P., 2002. Effects of shiftwork on sleep and menstrual function in nurses. Health Care Women Int. 23, 703-714.

McDearmon, E.L., Patel, K.N., Ko, C.H., Walisser, J.A., Schook, A.C., Chong, J.L., Wilsbacher, L.D., Song, E.J., Hong, H.K., Bradfield, C.A. and Takahashi, J.S., 2006.

Dissecting the functions of the mammalian clock protein BMAL1 by tissue-specific rescue in mice. Science 314, 1304-8.

Miller, B.H., Olson, S.L., Levine, J.E., Turek, F.W., Horton, T.H. and Takahashi, J.S., 2006. Vasopressin regulation of the proestrous luteinizing hormone surge in wild-type and Clock mutant mice. Biol. Reprod. 75, 778-784.

Miller, B.H., Olson, S.L., Turek, F.W., Levine, J.E., Horton, T.H. and Takahashi, J.S., 2004. Circadian clock mutation disrupts estrous cyclicity and maintenance of pregnancy. Curr. Biol. 14, 1367-1373.

Miyamoto, Y. and Sancar, A., 1999. Circadian regulation of cryptochrome genes in the mouse. Brain Res. Mol. Brain Res. 71, 238-243.

Morse, D., Cermakian, N., Brancorsini, S., Parvinen, M. and Sassone-Corsi, P., 2003. No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. Mol. Endocrinol. 17, 141-151.

Murr, S.M., Geschwind, I.I. and Bradford, G.E., 1973. Plasma LH and FSH during different oestrous cycle conditions in mice. J. Reprod. Fert. 32, 221-230.

Nakamura, T.J., Sellix, M.T., Kudo, T., Nakao, N., Yoshimura, T., Ebihara, S., Colwell, C.S. and Block, G.D., 2010. Influence of the estrous cycle on clock gene expression in reproductive tissues: Effects of fluctuating ovarian steroid hormone levels. Steroids 75, 203-212.

1 Pilorz, V. and Steinlechner, S., 2008. Low reproductive success in Per1 and Per2 mutant
2 mouse females due to accelerated ageing? Reproduction 135, 559-568.

3 Ratajczak, C.K., Boehle, K.L. and Muglia, L.J., 2009. Impaired steroidogenesis and
4 implantation failure in Bmal1-/- mice. Endocrinology 150, 1879-1885.

5 Ratajczak, C.K., Herzog, E.D. and Muglia, L.J., 2010. Clock gene expression in gravid uterus
6 and extra-embryonic tissues during late gestation in the mouse. Reprod. Fertil. Develop. 22,
7 743-750.

8 Sellix, M.T., Yoshikawa, T. and Menaker, M., 2010. A circadian egg timer gates ovulation.
9 Curr. Biol. 20, R266-7.

10 Shearman, L.P., Zylka, M.J., Weaver, D.R., Kolakowski, L.F., Jr. and Reppert, S.M., 1997.
11 Two period homologs: circadian expression and photic regulation in the suprachiasmatic
12 nuclei. Neuron 19, 1261-1269.

13 Sun, Y., Yang, Z., Niu, Z., Wang, W., Peng, J., Li, Q., Ma, M.Y. and Zhao, Y., 2006. The
14 mortality of MOP3 deficient mice with a systemic functional failure. J. Biomed. Sci. 13, 845-
15 851.

16 Sun, Z.S., Albrecht, U., Zhuchenko, O., Bailey, J., Eichele, G. and Lee, C.C., 1997. RIGUI, a
17 putative mammalian ortholog of the Drosophila period gene. Cell 90, 1003-1011.

1 Tei, H., Okamura, H., Shigeyoshi, Y., Fukuhara, C., Ozawa, R., Hirose, M. and Sakaki, Y.,
2 1997. Circadian oscillation of a mammalian homologue of the Drosophila period gene.
3 Nature 389, 512-516.

4 Tong, Y., Guo, H., Brewer, J.M., Lee, H., Lehman, M.N. and Bittman, E.L., 2004.
5 Expression of haPer1 and haBmal1 in Syrian hamsters: Heterogeneity of transcripts and
6 oscillations in the periphery. J. Biol. Rhythms 19, 113-125.

7 Vitaterna, M.H., King, D.P., Chang, A.M., Kornhauser, J.M., Lowrey, P.L., McDonald, J.D.,
8 Dove, W.F., Pinto, L.H., Turek, F.W. and Takahashi, J.S., 1994. Mutagenesis and mapping of
9 a mouse gene, Clock, essential for circadian behavior. Science 264, 719-725.

10 Wiegand, S.J., Terasawa, E., Bridson, W.E. and Goy, R.W., 1980. Effects of discrete lesions
11 of preoptic and suprachiasmatic structures in the female rat. Alterations in the feedback
12 regulation of gonadotropin secretion. Neuroendocrinology 31, 147-157.

13 Yagita, K., Horie, K., Koinuma, S., Nakamura, W., Yamanaka, I., Urasaki, A., Shigeyoshi,
14 Y., Kawakami, K., Shimada, S., Takeda, J. and Uchiyama, Y., 2010. Development of the
15 circadian oscillator during differentiation of mouse embryonic stem cells in vitro. Proc. Natl.
16 Acad. Sci. U.S.A. 107, 3846-51.

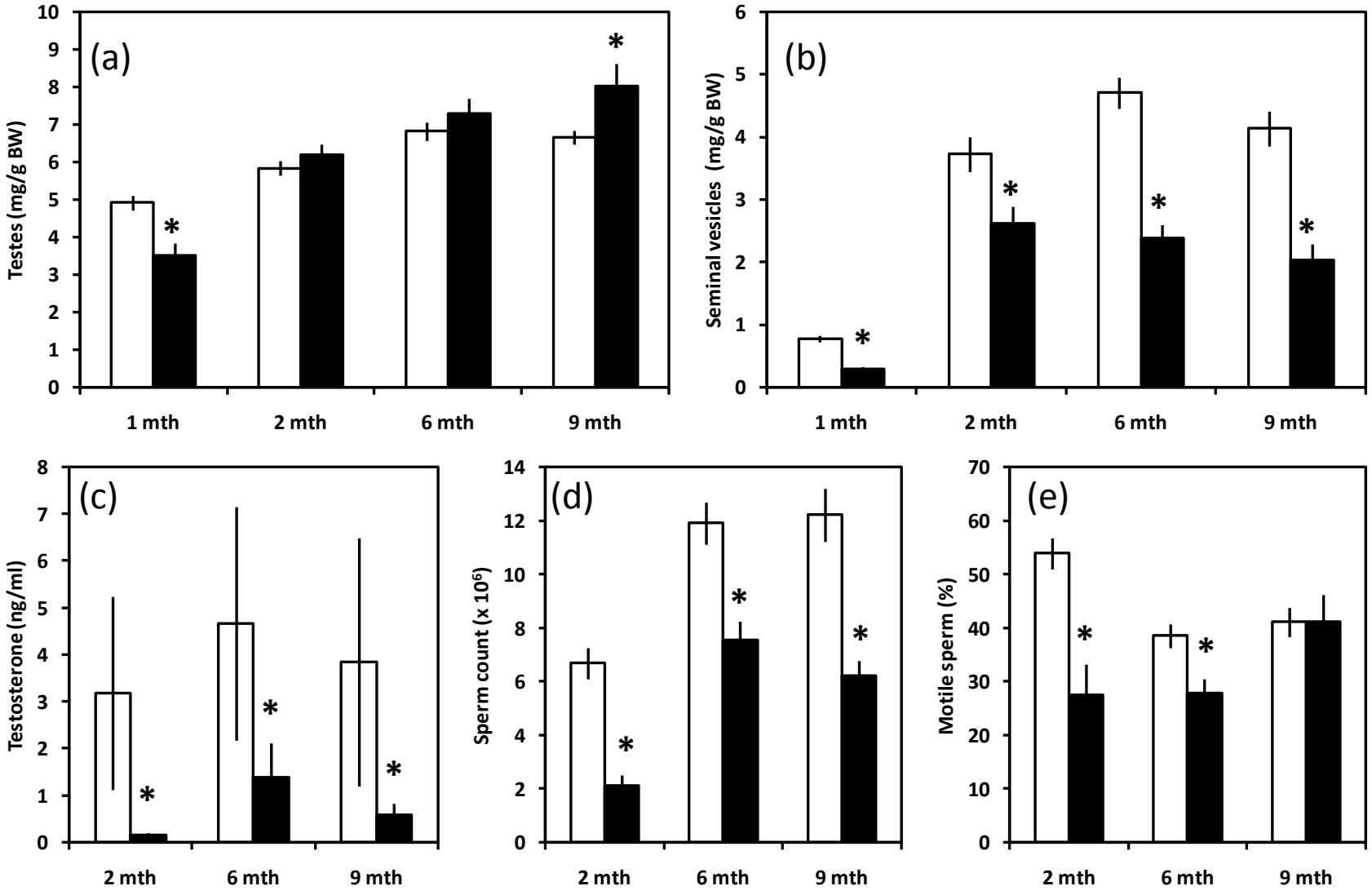
17 Yoshikawa, T., Sellix, M., Pezuk, P. and Menaker, M., 2009. Timing of the ovarian circadian
18 clock is regulated by gonadotropins. Endocrinology 150, 4338-4347.

Zambon, A.C., McDearmon, E.L., Salomonis, N., Vranizan, K.M., Johansen, K.L., Adey, D., Takahashi, J.S., Schambelan, M. and Conklin, B.R., 2003. Time- and exercise-dependent gene regulation in human skeletal muscle. Genome Biol. 4, R61.

Zheng, B., Albrecht, U., Kaasik, K., Sage, M., Lu, W., Vaishnav, S., Li, Q., Sun, Z.S., Eichele, G., Bradley, A. and Lee, C.C., 2001. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105, 683-694.

Zheng, B., Larkin, D.W., Albrecht, U., Sun, Z.S., Sage, M., Eichele, G., Lee, C.C. and Bradley, A., 1999. The mPer2 gene encodes a functional component of the mammalian circadian clock. Nature 400, 169-173.

Zylka, M.J., Shearman, L.P., Weaver, D.R. and Reppert, S.M., 1998. Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20, 1103-1110.


(a)
Testes (mg/g BW)
10
9
8
7
6
5
4
3
2
1
0
1 mth
2 mth
6 mth
9 mth
(b)
Seminal vesicles (mg/g BW)
6
5
4
3
2
1
0
1 mth
2 mth
6 mth
9 mth
(c)
Testosterone (ng/ml)
8
7
6
5
4
3
2
1
0
2 mth
6 mth
9 mth
(d)
Sperm count (x 10⁶)
14
12
10
8
6
4
2
0
2 mth
6 mth
9 mth
(e)
Motile sperm (%)
70
60
50
40
30
20
10
0
2 mth
6 mth
9 mth