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**Regulation of the rabbit's once-daily pattern of nursing: circadian or hourglass?**

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## ABSTRACT

The European rabbit *Oryctolagus cuniculus* has an unusual pattern of nursing behaviour. After giving birth in a nursery burrow (or laboratory nest box), the mother immediately leaves the young and only returns to nurse for a few minutes once approximately every 24 hours. It has been assumed this schedule, like a variety of other functions in the rabbit, is under circadian control. This assumption has been largely based on findings from mothers only permitted restricted access to their young once every 24 hours. However, in nature and in the laboratory, mothers with free access to young show nursing visits with a periodicity shorter than 24 hours, that does not correspond to other behavioural and physiological rhythms entrained to the prevailing 24-hour light/dark (LD) cycle. To investigate how this unusual, apparently non-circadian pattern might be regulated we conducted two experiments using female Dutch belted rabbits housed individually in cages designed to automatically register feeding activity and nest box visits. In Experiment 1 we recorded the behaviour of 17 mothers with free access to their young under five different LD cycles with long photo and short scotoperiods, spanning the limits of entrainment of the rabbit's circadian system. Whereas feeding rhythms were entrained by LD cycles within the rabbit's circadian range of entrainment, nursing visits showed a consistently shorter periodicity regardless of the LD regimen, largely independent of the circadian system. In Experiment 2 we tested a further 12 mothers under more conventional LD 16:8 cycles but "trained" by having access to the nest box restricted to one hour at the same time each day for the first seven days of nursing. Mothers were then allowed free access either when their young were left in the box ( $n = 6$ ), or when the litter had been permanently removed ( $n = 6$ ). Mothers with pups still present returned to nurse them on the following days according to a similarly advancing pattern to the mothers of Experiment 1 despite the previous seven days of "training" to an experimentally enforced 24 hour nursing schedule as commonly used in previous studies of rabbit maternal



behaviour. Mothers whose pups had been removed entered the box repeatedly several times on the first day of unrestricted access, but on subsequent days did so only rarely, and at times of day apparently unrelated to the previously scheduled access. We conclude that the pattern of the rabbit's once-daily nursing visits has a periodicity largely independent of the circadian system, and that this is reset at each nursing. When nursing fails to occur nest box visits cease abruptly, with mothers making few or no subsequent visits. Together, these findings suggest that the rabbit's once-daily pattern of nursing is regulated by an hourglass-type process with a period less than 24 hours that is reset at each nursing, rather than by a circadian oscillator. Such a mechanism might be particularly adaptive for rhythms of short duration that should end abruptly with a sudden change in context such as death or weaning of the young.

**KEYWORDS:** Circadian rhythm; entrainment; hourglass; interval timer; nursing rhythm; rabbit; *Oryctolagus cuniculus*; zeitgeber

## Introduction

Of the many rhythmic processes that underlie the organization of physiological and behavioural phenomena, among the best studied are circadian rhythms, that is, those with a periodicity of approximately 24 hours and regulated by endogenous cyclic processes (so-called clocks, oscillators or pacemakers) set by or entrained to environmental stimuli such as the ubiquitous 24-hour cycle of night and day (Aschoff, 1960; Moore-Ede et al., 1982; Pittendrigh & Minis, 1964; Takahashi & Zatz, 1982). However, not all diurnally rhythmic phenomena are necessarily governed by such endogenous “clock-like” mechanisms.

Although receiving less attention compared to endogenous circadian rhythms, chronobiologists have long known that daily rhythmicity may also arise as the result of direct stimulation, that is, as the result of hourglass-like mechanisms reset to run for a certain period following a recurring triggering stimulus (review in Rensing et al., 2001). Such mechanisms could, for example, be particularly adaptive for organizing the timing of regularly occurring behavioural and physiological functions that only persist across relatively short time spans and might be terminated abruptly, necessitating animals to quickly adjust to a new set of behavioural and/or physiological contingencies.

In contrast to a circadian oscillator, an hourglass mechanism measures a time interval without being self-sustaining (Aschoff, 1960; Bradshaw et al., 2003; Dad & Keone, 1981; Lees, 1973; Lewis & Saunders, 1987; Page, 1985; Saunders & Lewis, 1987). That is, the timer does not reset in the absence of periodic environmental stimuli, and the periodicity of the timed event is driven directly by events in the external environment (Aschoff, 1960). For example, under a light/dark (LD) cycle an hourglass timer may be re-activated each day by an external time cue, such as the beginning of the dark phase (Dad & Keone, 1981; Lees, 1973; Reebs & Lague, 2000). Hourglass timers responsive to the dark phase of LD cycles

have been suggested to control diapause in the aphid *Megoura viciae* (Lees, 1987), the spider mite *Tetranychus urticae* (Vaz Nunes & Veerman, 1982), the butterfly *Pieris brassicae* (Claret, 1985) and the pitcher-plant mosquito *Wyeomyia smithii* (Bradshaw et al., 2003). To our knowledge, however, hourglass timers have not been demonstrated in the regulation of diurnal biological functions of mammals.

The pattern of once-daily nursing visits by rabbit mothers to their young provides an unusually good opportunity to distinguish between circadian and hourglass-type mechanisms in the regulation of a naturally occurring behaviour of clear biological significance. In the European rabbit (*Oryctolagus cuniculus*), including its domesticated (laboratory) form, the altricial young are born into a nest of dry grass and fur constructed by the mother in a nursery burrow (or laboratory nest box). Immediately after giving birth the mother leaves the young and only returns for a few minutes approximately once every 24 hours to nurse (Deutsch, 1957; Hudson & Distel, 1982; Broekhuizen et al., 1986; Hudson et al., 1999; Rödel et al., 2012; Zarrow et al., 1965; reviews in Hudson & Distel, 1989; Jilge & Hudson, 2001). This continues until the young, under naturalistic conditions, are abruptly weaned by the mother, usually around one month of age (Hudson et al., 1996; Lincoln, 1974). If the mother has mated again during postpartum estrus, common in many small mammals (review in Martínez-Gómez et al., 2004), she will repeat the cycle of nest building and daily nursing a few days later (Hudson et al. 1996; Lincoln 1974; Martínez-Gómez et al., 2004).

For various practical reasons it has been common in laboratories to restrict rabbit mothers' access to their young to a specific time each day. Mothers appear to accept this well, successfully raising their young and anticipating the time of nursing with changes in behaviour and in physiological and neurobiological processes (reviews in González-Mariscal et al., 2016; Jilge & Hudson, 2001). This has led to the use of the term "circadian" when referring to the rabbit's daily pattern of nursing visits, and to the assumption that this is part

of, and is regulated by this species' well-studied and robust circadian system (Caba et al., 2018; González-Mariscal et al., 2013, 2016; Hudson & Distel, 1989; Jilge & Hudson, 2001; Meza et al., 2011).

And yet, the rabbit's diurnal pattern of nursing has not actually been demonstrated to be circadian as it has not been shown to be self-sustaining, that is, to persist in the absence of nursing stimuli. In fact, there are at least two reasons to question the assumption of circadian control of the rabbit's daily pattern of nursing. First, both in the laboratory (Hudson et al., 1995; Jilge, 1995) and nature (Rödel et al., 2012; review in Hudson & Distel, 1989), rabbits with free access to their young usually return to the nest to nurse them somewhat earlier each day, at least during the first one to two weeks of lactation. As parturition usually takes place during the dawn or early daylight hours (Hudson et al., 1995, 1999; reviews in Hudson & Distel, 1989; Ninomiya-Alarcón et al., 2004) this results in the mothers' nursing visits advancing steadily back into the night (Hudson & Distel, 1989; Hudson et al., 1995; Rödel et al., 2012). The second reason is that if the nursing mother is pregnant with a another litter as a result of postpartum mating (review in Martínez-Gómez et al., 2004), she will resist nursing at the experimentally scheduled time during the daylight hours, and if forced to do so, may have difficulty giving birth to the second litter. This gradual separation of the time of nursing and of parturition is presumably to avoid the surge in the release of oxytocin into the mother's blood stream during nursing from provoking premature parturition at a time of day when the (pregnant) uterus is maximally sensitive to oxytocin (Hudson et al., 1995; cf. Ninomiya-Alarcón et al., 2004).

We therefore conducted two experiments designed to address the question whether the daily pattern of nursing by the rabbit corresponds to an hourglass-type model or is indeed better considered part of this species' endogenous circadian system. In Experiment 1 we examined the relation between females' circadian-regulated daily feeding rhythm and their

pattern of daily nursing visits with free access to their young under a range of LD cycles within and outside the rabbit's known range of LD entrainment (Kennedy & Hudson, 2016). In Experiment 2 we asked if mothers' daily pattern of nursing visits would persist for two or more cycles after nursing was prevented by permanently removing the pups, as would be expected if nursing visits were controlled by an endogenous circadian oscillator.

## **General methods**

### ***Animals and apparatus***

We used a medium-size (2.5–3.0 kg) Dutch belted rabbit breed with pigmented eyes, purchased from a commercial breeder (Nanowie Small Animal Production Unit, Melbourne, Australia) and vaccinated against rabbit haemorrhagic disease virus. Females were nulliparous at the start of each of the two experiments. They were housed individually in cages modified for chronobiological studies in medium-size mammals (Kennedy et al., 1990a, b; 1994; 1995). Each cage was an acrylic box 120 x 63 x 43 (height) cm with opaque sides and back and a transparent front and floor. The top of the cage was covered with wire mesh, and the floor was perforated to drain the cage into sawdust-filled trays below. The cage contained an opaque acrylic nest box 46 x 24 x 42 (height) cm with a removable lid and with rye grass hay for nest building. It could be accessed by the mothers via an opaque foyer 19 x 25 x 31 (height) cm via two doorways 19 cm apart. The foyer provided mothers with a refuge in an enclosed area other than the nest box itself. To keep the pups in the nest box during the early postnatal period (see below) the box was separated from the foyer by a 10-cm high barrier. The doorway between the foyer and the cage was fitted with a vertical sliding door with a handle that extended above the cage to allow mothers' access to the nest to be regulated from outside. Cages were fitted with an open-face acrylic running-wheel (details in Kennedy et al., 1994) to comply with ethics requirement of providing opportunity for

activity. Data from the running-wheels were analysed but not used because there was too much variability across rabbits in levels of use. Containers for food and water 12.5 x 12.5 cm each, were attached to the front of the cage and could be accessed by the females via acrylic swing doors (Figure 1). The frequency and duration of visits to the food container and to the nest box were automatically recorded (see *Procedure* below).

--- Figure 1 about here ---

We used a total of 12 such cages located in two separate rooms. They were placed on racks, two rows one above the other, with 45 cm between them. Cage cleaning was limited to the renewal of sawdust in the trays under the cages twice a week, and took approximately 15 min for all cages. Timing of visits to replenish food and water, for cage cleaning and for general laboratory maintenance were randomized across the day to avoid the rabbits entraining to extraneous stimuli accompanying laboratory entry. Room temperature was maintained at  $21 \pm 2$  °C. Animals were fed a mixture of pasture replacement pellets (Barastoc®, Ridley Corporation Ltd, Australia), lucerne chaff, oats and bran (Northern Valley Stockfeeds, Australia), supplemented daily with a piece of apple or carrot. Food and tap water were available continuously and replenished daily. Breeding males were housed individually in standard acrylic holding cages 62 x 36 x 43 (height) cm under LD 12:12 (LD schedules for females are given for each of the experiments below) in a separate room, and otherwise under similar general conditions to the females.

Each cage was lit by a 60 W incandescent bulb mounted above it. The bulbs were connected to a digital 7-day programmable timer and light intensity regulator (Clipsal IP 56, Gerard Industries, Melbourne, Australia) providing central control for the illumination of all cages. Light intensity was set to be similar to that used in previous studies of circadian rhythms in rabbits (e.g. Bobbert et al., 1994; Jilge & Stähle, 1993; review in Jilge & Hudson,

2001) using a Tektronix J 17 light meter (Beverton, USA). Light intensity measured at the floor level of each cage was on average 140 Lx. During the dark phase a 4 W red light torch was used to check for parturition. The torchlight gave intensity readings of 0.4 to 0.6 Lx, and in its absence no light was detected by the light meter.

### ***Procedure***

Females were habituated to the experimental cages for 2 to 3 weeks prior to mating. Average litter size for this breed is four to five pups, approximating litter sizes in wild rabbits (Eccard & Rödel, 2011). To provide comparable nursing conditions across females and experimental treatments, litter sizes were adjusted by culling and cross-fostering pups within 10 hours of birth so that females nursed four to five pups each. The following behaviours were recorded:

*Feeding.* This, a commonly used measure of general activity in the rabbit and other small mammals (e.g. Bobbert et al., 1992, 1994; Jilge, 1992), was monitored using micro-switches mounted on the swing door of the food trough. Opening or releasing the door activated a switch that registered each opening and closing as a frequency count and the time between them as an indicator of the duration and time of day spent feeding.

*Nest box visits.* These were measured using passive infra-red detectors installed in the doorway between the foyer and the nest box. This system registered when a female entered or left the box, allowing the frequency, time of day and duration of each visit to be recorded.

*Nursing visits.* These could be recorded only from postnatal days 1 – 14 during the period that pups remained reliably in the nest (cf. Hudson & Distel, 1982; Rödel et al., 2017; parturition = day 0). Although females sometimes entered the nest box on multiple occasions within a 24 h period, there was almost always only one daily visit sufficiently long (at least 3 minutes) to be considered a nursing visit.

Data were collected on a custom-built data acquisition computer and transferred to a main frame computer for analysis. For the preparation of actograms, data were collapsed into 5 min bins and the actograms presented as double plots to facilitate viewing of the continuation of activity across days (see Figure 2 A – D and Figure 4 A, B).

The experimental protocol was approved by the Animal Ethics Committee, Department of Psychology, Monash University, Clayton, Australia, where the experiments were carried out. It also conformed to the ethical standards and methods for the use of animals in biological rhythm research (Portaluppi et al., 2010).

### ***Data analysis***

To estimate periodicities of feeding and nest box (nursing) visits during the different stages of the experimental protocols (see Experiments 1 and 2 below), Chi-square periodogram analysis with alpha set at 0.001 and based on the algorithm of Sokolove and Bushell (1978) was used. This analysis has been used previously by Jilge, 1993, 1995 for the analysis of circadian rhythms in rabbits. Sokolove and Bushell's (1978) periodogram analysis has been found effective for the evaluation of biological rhythm data even when based on relatively few days of observations as was the case with the nursing visit data in the present study (Refinetti, 1991). Sokolove and Bushell's Chi-square periodogram analysis can produce period estimates for runs of activity over as little as 7 days with an accuracy of +/- 0.5 h. The feeding activity and nursing rhythms in Experiments 1 and 2 were difficult to interpret by visual inspection alone and therefore this analysis was used for all estimates of period length.

### **Experiment 1: Influence of LD cycles on the nursing pattern**

If the daily pattern of nursing in the rabbit is controlled by the circadian system it could be expected to entrain more readily to LD cycles close to 24 h (Daan & Pittendrigh, 1976;



Kennedy & Hudson, 2016; Pittendrigh & Daan, 1976a, b, c; review in Jilge & Hudson, 2001).

## ***Methods***

We tested 17 females, where possible in five LD cycles with long light and short dark periods within (22:2, 23:2) and close to or outside the rabbit's range of circadian entrainment (18:2, 20:2, 27:2) (Kennedy & Hudson, 2016). We used short, 2 h dark periods as we thought this might constrain nursing to a narrower time period and make it easier to discern any separation of the nursing rhythm from the general circadian system, e.g. as expressed by the daily pattern of feeding. Where possible, we presented these in randomized order (Table 1). For various reasons (e.g. unsuccessful mating, limitations of laboratory space) it was not possible to test all females in all LD cycles. In addition, to check the reliability of findings we retested 7 of the 13 females tested in LD 22:2 at various times in the sequence of trials (Table 1) and obtained essentially the same results on both occasions (Tables 2 and 3).

--- Table 1 about here ---

## ***Results and discussion***

*Periodicity of feeding.* In the initial phase of the LD cycles during gestation (e.g. Figure 2A – D; Figure 3), the feeding rhythm was synchronised to the LD cycle in all females in the two LD 22:2 trials and in almost half in 23:2, but not in LD 18:2, 20:2 and 27:2. Synchrony was defined as periodicity of  $\leq 30$  min difference from the experimentally imposed LD cycle (Figure 3, Table 2). In the second phase of the experiment when females had pups, a similar although weaker pattern of synchronization to LD 22:2 and 23:2 was seen, suggesting that the feeding rhythm may have been modulated somewhat by the nursing rhythm. But again, no

evidence of synchrony to the LD cycle was seen in LD 18:2, 20:2 and 27:2 (Figure 2A–D; Figure 3; Table 2).

--- Figures 2 and 3 and Table 2 about here ---

Thus, using feeding as an indicator of general circadian activity, the females showed a limited range of entrainment to the LD cycles, with the strongest synchronization to cycles close to 24 hours and failure to entrain to cycles several hours shorter or longer than this. That only half the rabbits were judged to have entrained to LD 23:2 might be explained by individual variability in tau length. While Kennedy and Hudson (2016) found that LD 23:2 is within the rabbit's overall range of entrainment, half the animals had a tau of less than 24 h. Such animals as judged from the phase response curves in the previous study might have struggled to entrain to the LD 23:2 cycle. Nevertheless, the general range of entrainment found here is consistent with that estimated from the phase response curve in response to brief light pulses in the rabbit (Kennedy & Hudson, 2016), and with reports of the circadian regulation of the rabbit's daily pattern of feeding (Jilge, 1992; Jilge & Stähle, 1993).

*Periodicity of nursing.* After giving birth, mothers left the nest box almost immediately and only returned and stayed long enough once a day ( $M$  4.7,  $SD$  3.0 min) for this to be considered a nursing visit (e.g. Figure 2A – D). This was the case for all LD cycles and all pups survived to weaning. These visits had a period between 21.78 and 22.85 h for all five LD cycles (Figure 2A – D; Figure 3; Table 3). Thus, there was little evidence that the LD cycles influenced the nursing rhythm. The only time the two rhythms clearly coincided was when the LD cycle (20:2) was closest to the mothers' natural nursing rhythm. While this may appear to be synchrony, it may also just be coincidence due to that period of LD cycle (20:2) being close to that of the nursing rhythm, which in the present study was between 21.78 and 22.85 (Table 3; see also Hudson & Distel, 1989; Jilge, 1993; Jilge et al., 2001; Rödel et al.,

2012). No evidence of entrainment was seen under any of the other LD cycles (18:2, 22:2, 23:2 and 27:2; Figure 3; Table 3).

Most notable was the consistent separation of the timing of nursing visits from the circadian feeding rhythm, suggesting a considerable degree of independence between the two (Figure 2A – D; Figure 3; Tables 2 and 3). This, in turn, suggests that the rabbit's pattern of daily nursing visits may result from processes largely independent of its well-demonstrated light- and food- entrainable circadian system. To examine the nature of this apparent discrepancy in more detail we conducted a second experiment designed to investigate the possible role of endogenous and exogenous stimuli in regulating the rabbit's pattern of once-daily nursing.

--- Table 3 about here ---

## **Experiment 2: Influence of restricted nest access and absence of pups on nursing visits**

Our main interest in conducting this experiment was to determine if rabbit mothers' daily pattern of nursing would be self-sustaining, at least for a minimal number of cycles, in the absence of the stimuli normally accompanying nursing. Specifically, in a first step our aim was to determine if the rabbit's non-24 h daily pattern of nursing could be synchronized to an experimentally imposed 24-h nursing schedule. Our second aim was then to examine if the pattern of nursing visits would continue (be self-sustaining) following the removal of the stimuli accompanying nursing (the pups).

### ***Methods***

A further 12 nulliparous females were maintained under the same conditions as the females of Experiment 1 except they were kept under LD 16:8, and for the first seven days

postpartum nest access was restricted to one hour a day during the second half of the dark phase, that is, during the mothers' active period and roughly corresponding to the natural time of nursing visits described above. Restricted nest access began on the day after parturition (visible as a broad band of activity in red under an asterisk in Figure 4A, B). In Figure 4A restricted nest access was permitted from 23 to 24 h, while in Figure 4B restricted access was permitted from 01 to 02 h. Following nursing on day 7 of restricted nest access, the nest box was left open and the mothers were randomly allocated to two groups: Group 1 ( $n = 6$ ); pups and nest material permanently removed, and Group 2 ( $n = 6$ ); pups and nest material left in the box undisturbed.

### ***Results and discussion***

Inspection of the plots for feeding activity of females in both groups showed that this was entrained to the LD 16:8 cycle, particularly around dusk and dawn, across gestation and nursing. As would be expected in rabbits, greater levels of feeding activity occurred during the crepuscular and dark phase (e.g. Figure 4 A, B; cf. Jilge, 1991, 1992).

--- Figure 4 about here ---

*Group 1: pups and nest material permanently removed on day 7.* For the six mothers of this group brief nest box (nursing) visits occurred during the scheduled opportunity for the first seven days and all pups survived. When the restriction schedule ended on day 7 after nursing at 01 h and the pups and nest material were permanently removed, all six mothers entered the box at the previously scheduled nursing time but only two remained for a duration corresponding to a typical nursing visit (including the mother in Figure 4A). Then, on the second day, although some mothers (but not the mother shown in Figure 4A) entered the nest box there was no evident pattern for any of them in the timing of these visits, and on the

remaining days there were virtually no nest box entries that could be construed as nursing visits (e.g. Figure 4A).

*Group 2: pups and nest material left permanently in the box on day 7.* As for Group 1, for the six mothers of this group, nest box (nursing) visits occurred during the scheduled opportunity for the first seven days postpartum and all pups survived. Mothers entered the box almost immediately they were allowed access, and remained in the box successfully nursing for the approximately 3 – 4 minutes typical for rabbits. When the 7-day restriction schedule ended, all mothers nursed on the following day at approximately the same time as the previously scheduled nursing opportunity. Then, over the next five days, nursing visits advanced with a mean periodicity of 23.49 *SD* 0.26 min in all mothers (e.g. Figure 4B). This is consistent with the findings in Experiment 1 where for all mothers the time of nursing visits also showed a daily advanced shift (Figure 3; Table 3).

In conclusion, the results of this experiment further confirm the independence of the rabbit's pattern of nursing visits from its circadian system as seen by the lack of coupling of unrestricted nursing visits to the 24-h feeding rhythm and even after forcing ("training") mothers to nurse during the first week postpartum according to a commonly used 24-h LD schedule. Furthermore, the abrupt cessation of nest box (nursing) visits by the mothers of Group 1 after the pups and nest material had been permanently removed suggests that the daily pattern of nursing visits by the rabbit is not controlled by an endogenous oscillator. Rather, it appears to be the product of an hourglass mechanism that is re-set with a period of less than 24 h at each nursing and ceases to run almost immediately the stimuli associated with nursing (e.g. suckling by the pups) are no longer present.

## General discussion

In the past it has been largely assumed that the European rabbit's unusual pattern of brief, once-daily nursing visits to the young represents a circadian rhythm controlled by similar mechanisms to its other well studied circadian rhythms such as the daily pattern of feeding, drinking, hard faeces excretion, and locomotor activity (reviews in Hudson & Distel, 1989; Jilge & Hudson, 2001). To our knowledge, however, this assumption has never been directly tested using standard chronobiological procedures such as examining the pattern of entrainment to the 24-h light/dark cycle of nursing visits, and whether these persist in a self-sustaining, endogenous manner in the absence of the natural zeitgeber of once-daily suckling stimulation by the young. Results of the two experiments reported here using such methods suggest that the rabbit's nursing rhythm is to a considerable degree independent of its circadian system, and appears to be regulated largely by an hourglass-type, interval-timing process rather than by an endogenous circadian oscillator (see Rensing et al., 2001). There are several arguments in support of this proposition based on the present findings.

In Experiment 1 the females, both when pregnant and lactating, showed a daily pattern of feeding activity entrained by LD cycles with periodicities close to 24 hours, corresponding to this species' phase response curve (Kennedy & Hudson, 2016) and consistent with previous evidence for the operation of a circadian oscillator (review in Jilge & Hudson, 2001). This was in contrast to the pattern of daily nursing visits, which showed a period consistently shorter than 24 hours (ranging from 21.78 to 22.85 h), and which appeared to be largely unaffected by the experimentally imposed LD cycles, and apparently running independently of and cutting across the feeding rhythm (Figure 2A–D).

In Experiment 2 also, and consistent with the results of Experiment 1 for LD cycles within the range of entrainment, females' rhythm of feeding activity entrained to the 16:8 LD

cycle across both gestation and nursing. In Group 1 immediately after giving birth, mothers were given restricted daily nest box access for a week. When this restriction was lifted and the pups and nest material were permanently removed, mothers stopped returning to the nest at the previously scheduled or any other time already by the second day after restriction (e.g. Figure 4A). This suggests that without the stimuli accompanying nursing the “hourglass” was not reset and motivation to nurse quickly abated, with long (nursing) visits to the nest box ceasing almost immediately. In contrast, in Group 2 when restricted access to the nest was lifted, mothers returned to nurse somewhat earlier each day with a periodicity significantly shorter than 24 h (e.g. Figure 4B), and similar to that of females in Experiment 1.

Despite the modest sample sizes the findings appear reliable. First, evidence for the circadian regulation of the mothers’ daily pattern of feeding activity in both Experiments 1 and 2 is consistent with previous reports for the rabbit (review in Jilge & Hudson, 2001). Second, the apparent failure of the daily pattern of nursing to be entrained by LD cycles within the rabbit’s range of entrainment as indicated by its phase-response curve (Kennedy & Hudson, 2016), and showing a periodicity notably less than the approximately 24-h periodicity characteristic of a circadian rhythm, is consistent with previous observations of the daily pattern of unrestricted nursing both in the laboratory and nature (Hudson et al., 1995; Jilge, 1993; 1995; Jilge et al., 2001; Rödel et al., 2012; review in Hudson & Distel, 1989). Third, the small number and crisper patterning of nest box entries qualifying as nursing visits between this and some previous laboratory reports may be partly due to differences in housing conditions (see also Coureaud et al., 2000). In the present study, providing a running wheel as an additional opportunity for motor activity (cf. Kennedy et al., 1994; Kennedy & Hudson, 2016), and an opaque foyer to the nest box where females had a protected area to which they could retreat other than the nest box itself, may partly account for such differences. In addition, the Dutch belted breed used here with pigmented eyes,

greater general activity (Kennedy et al., 1994), and litter sizes closer to wild rabbits than other domestic breeds commonly used in laboratory research might also partly account for differences between reports. Nevertheless, in this, as in other studies of rabbit circadian biology, considerable individual variation within and between litters in mothers' daily pattern of nursing visits should be noted.

Assuming, then, that the findings of the present study are valid, what proximate mechanisms might serve to time the interval between the rabbit's nursing visits? In a series of elegant experiments involving systematic manipulation of the presence or absence of stimuli accompanying suckling, it has been shown that it is not the emptying and refilling of the mammary glands with milk that is important, but rather the degree of stimulation of the nipples by the suckling pups (Findlay & Roth, 1970; Findlay & Tallal, 1971). This is consistent with reports that both the duration of nursing visits and the interval between them is modulated by litter size. Mothers with larger litters and thus presumably receiving greater stimulation of nipples during nursing than mothers with smaller litters, show correspondingly longer nursing intervals (González-Mariscal et al., 2013; Rödel et al., 2012). Similarly, early in lactation when newborn pups are often slower to attach to nipples and provide suckling stimulation, longer nursing times are consistently reported in the literature (Hudson & Distel, 1982; Hudson et al., 1996; Lincoln, 1974; Venge, 1963).

Indirect support for the independence of the rabbit's daily nursing rhythm from its circadian system also comes from neurobiological studies. These include both the reported lack of nursing-induced expression of the "clock" gene protein PER1 (Caba et al., 2018; Meza et al., 2008) or of enhanced c-Fos expression (González-Mariscal et al., 2009) in the SCN of the hypothalamus, generally considered the master clock regulating circadian rhythms in mammals (review in Moore-Ede et al., 1982). Clearly, however, further studies of the neural mechanisms regulating the rabbit's nursing rhythm in comparison with those



regulating its well-documented circadian pattern of other functions are needed in order to establish the degree of independence between the two.

Finally, although the operation of hourglass-type mechanisms has been reported across the 24-h day for a variety of functions in invertebrates, to our knowledge the regulation of the rabbit's pattern of once-daily nursing is presently the only example of a diurnal behavioural rhythm in a mammal regulated by the operation of an hourglass-like mechanism with a periodicity apparently independent of the circadian system. And yet, for certain functions an hourglass mechanism, allowing an immediate stop or go response to rapidly changing short-term contingencies may be more adaptive than an enduring, self-sustaining circadian mechanism. In the rabbit such contingencies include the abrupt cessation of nursing visits at weaning by mothers pregnant with a further litter (Lincoln, 1974; Hudson et al., 1996), or in response to nest mortality, in which mothers often lose an entire litter due, for example, to predation, infanticide or flooding (Palomares, 2003; Rödel et al., 2009; Wood, 1980). Under such circumstances it would be presumably maladaptive for mothers to repeatedly return to raided or flooded nests, and when they should resume breeding as soon as possible.

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## **Declaration of interest**

The authors declare no conflicts of interest for this report.

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**Table 1.** The order of exposure to each LD cycle for each female rabbit in Experiment 1.

Note the repetition of LD 22:2 in some females (in bold).

Rabbit #	LD18:2	LD20:2	<b>LD22:2</b>	<b>LD22:2</b>	LD23:2	LD27:2	Trial <i>n</i>
			Trail 1	Trail 2			
1		3	2	1	4	5	5
2	5	3	2	1	4	6	6
3			1		2	3	3
4		3	1		2	4	4
5		2	1				2
6		1			2		2
7		1			2		2
8		1	4		2	3	4
9	3	1			2		3
10	2		4		1	3	4
11	2		3	5	1	4	5
12	2		3	5	1	4	5
13	2		3	5	1	4	5
14	1		2	3		4	4
15	1		3	2		4	4
16			1				1
17		1					1
Total <i>n</i>	8	9	13	7	12	11	

**Table 2.** Summary of mean feeding rhythm period lengths in Experiment 1 during gestation and nursing, and the number of rabbits synchronised to the five different LD cycles. Note the repetition of LD 22:2 in some females (in bold).

Condition	Statistics	LD18:2	LD20:2	<b>LD22:2 Trail 1</b>	<b>LD22:2 Trail 2</b>	LD23:2	LD27:2
Feeding (Gestation)	<i>M(SD)</i>	24.22(0.34)	24.34(0.56)	24.01(0.04)	24.00(0.00)	24.90(0.27)	24.14(0.23)
	<i>CI</i>	23.98-24.46	23.97-24.71	23.98-24.03	0	24.74-25.05	24.00-24.28
	Total <i>n</i>	8	9	13	7	12	11
	Synchronised	0	0	13	7	5	0
Feeding (Nursing)	<i>M(SD)</i>	24.15(0.42)	24.12(0.61)	23.68(0.62)	24.01(0.43)	24.69(0.43)	23.44(1.21)
	<i>CI</i>	23.86-24.44	23.72-24.52	23.34-24.01	23.69-24.33	24.44-24.93	22.73-24.15
	Total <i>n</i>	8	9	13	7	12	11
	Synchronised	0	0	6	4	2	0

**Table 3.** Summary of mean nursing visit period lengths in Experiment 1 and the number of rabbits synchronised to the five different LD cycles. Note the repetition of LD 22:2 in some females (in bold).

Statistics	LD18:2	LD20:2	<b>LD22:2 Trail 1</b>	<b>LD22:2 Trail 2</b>	LD23:2	LD27:2
<i>M(SD)</i>	21.78(1.14)	22.07(0.97)	22.30(1.64)	22.85(1.11)	22.29(2.35)	22.82(0.94)
<i>CI</i>	20.99-22.57	21.44-22.70	21.41-23.19	22.03-23.67	20.96-23.62	22.27-23.37
Total <i>n</i>	8	9	13	7	12	11
Synchronised	2	4	2	1	0	0

## Figure legends

**Figure 1.** Schematic of a test cage, not drawn to scale. See General methods for details.

**Figure 2.** Representative examples of activity plots for mothers during 4 of the 5 LD cycles showing feeding activity (dark bars) during gestation and nursing, and nest box visits (red bars) indicating nursing. Actograms are double plotted with two 24-h records shown side by side, and with the record on the right moved up one day to facilitate visualization of rhythm continuity. The two-hour dark phase is indicated by a black bar at the top of each plot and by the grey stippling that extends through the plot. Data are presented in 5-min bins with the height of the bars representing the duration of activity within that interval. Animals were pregnant at the beginning of the plots and parturition is indicated by an asterisk. A). Example of a mother in LD 18:2 showing 24 h periodicity of feeding during gestation and 24.42 h periodicity of feeding during nursing, but a periodicity of nursing visits of 23.33 h. B). Example of a mother in LD 22:2 showing 24 h periodicity of feeding during gestation and 23.92 h periodicity of feeding during nursing, but a periodicity of nursing visits of 21.92 h. C). Example of a mother in LD 23:2 showing 24.50 h periodicity of feeding during gestation and 24.67 h periodicity of feeding during nursing, but a periodicity of nursing visits of 20.33 h. D). Example of a mother in LD 27:2 showing 24.08 h periodicity of feeding during gestation and 23.58 h periodicity of feeding during nursing, but a periodicity of nursing visits of 22.92 h.

**Figure 3.** Summary of the mean feeding period lengths during gestation and nursing (Table 2) and the mean period lengths of the nursing rhythm (Table 3). The standard deviations for mean period lengths are shown in Tables 2 and 3 and are not shown here. This figure illustrates the independence of the nursing visit rhythm from the feeding rhythm. The 5 different LD cycle period lengths are plotted on the X axis, and on the Y axis the mean period

lengths for feeding activity during gestation and nursing, and nursing visits to the nest box. Note that feeding activity during gestation and nursing only appeared to be synchronized to the 22:2 and the 23:2 LD cycles, whereas nursing visits only appeared to be synchronized to the 20:2 LD cycle. Trials 1 and 2 in LD 22:2 have been combined and *M* and *SD* for each point are shown in Tables 2 and 3.

**Figure 4.** Representative examples of activity plots for nursing mothers in LD 16:8 showing feeding activity during gestation and nursing, and nest box visits indicating nursing (for details of data representation see Figure 2). Animals were pregnant at the beginning of the plots and an asterisk indicates parturition and below it as a red band of activity in the nest box. Subsequent nest box visits for nursing are shown as the red bars. Vertical broken lines late in the dark phase indicate restricted access (one hour) to the nest box for 6 days immediately following parturition. Restricted access ended when the nest box was left permanently open, allowing the mother free access to it from that time onwards. A). Example where the pups and nest material were removed on day 7 and the box left permanently open following nursing at 23 h. Nest box visits (red bars) continued on this day, but in the absence of the pups ended abruptly and did not continue on any of the subsequent days for the rest of the experimental period. B). Example where the pups were not removed when restricted access ended on day 7 after nursing at 01 h. The mother continued to visit the nest to nurse until the end of the experiment, with nursing visits (red bars) drifting to the left with a periodicity of less than 24 h. Note that on day 4 of restricted access the mother forced her way under the closed nest box door shortly before the scheduled access, demonstrating her strong motivation to nurse at that time.









