

Setting the main circadian clock of a diurnal mammal by hypocaloric feeding

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Key points

- Timed hypocaloric feeding alters the main circadian clock, the suprachiasmatic nucleus (SCN), in nocturnal rodents.
- The endogenous oscillatory mechanism in the SCN is similar between nocturnal and diurnal mammals.
- In this study we report that in the diurnal rodent *Arvicanthis ansorgei*, a timed hypocaloric feeding (HF) entrains and shifts behavioural and molecular circadian rhythms in the SCN. Nevertheless, instead of phase advancing the clock as in nocturnal rodents, HF phase delays the *Arvicanthis* SCN pacemaker.
- Thus, HF modifies the circadian system of the diurnal rodent *Arvicanthis ansorgei* differentially from nocturnal rodents.
- The present results will help us to better understand the circadian system in diurnal species and how feeding cues can synchronize daily rhythms.

Abstract Caloric restriction attenuates the onset of a number of pathologies related to ageing. In mammals, circadian rhythms, controlled by the hypothalamic suprachiasmatic (SCN) clock, are altered with ageing. Although light is the main synchronizer for the clock, a daily hypocaloric feeding (HF) may also modulate the SCN activity in nocturnal rodents. Here we report that a HF also affects behavioural, physiological and molecular circadian rhythms of the diurnal rodent *Arvicanthis ansorgei*. Under constant darkness HF, but not normocaloric feeding (NF), entrains circadian behaviour. Under a light–dark cycle, HF at midnight led to phase delays of the rhythms of locomotor activity and plasma corticosterone. Furthermore, *Per2* and vasopressin gene oscillations in the SCN were phase delayed in HF *Arvicanthis* compared with animals fed *ad libitum*. Moreover, light-induced expression of *Per* genes in the SCN was modified in HF *Arvicanthis*, despite a non-significant effect on light-induced behavioural phase delays. Together, our data show that HF affects the circadian system of the diurnal rodent *Arvicanthis ansorgei* differentially from nocturnal rodents. The *Arvicanthis* model has relevance for the potential use of HF to manipulate circadian rhythms in diurnal species including humans.

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Abbreviations AL, *ad libitum*; Avp, vasopressin; CORT, corticosterone; CT, circadian time; DD, constant darkness; FAA, food anticipatory activity; HF, hypocaloric feeding; 5-HT, serotonin; LD, light–dark cycle; NF, normocaloric feeding; SCN, suprachiasmatic nucleus; ZT, *zeitgeber* time.

Introduction

Circadian clocks confer adaptive advantages to an organism, enabling it to anticipate daily environmental changes (Pittendrigh, 1993). In mammals, the principal circadian clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (Moore & Eichler, 1972; Stephan & Zucker, 1972). Whereas photic stimuli entrain the SCN clock (Albrecht *et al.* 1997; Meijer & Schwartz, 2003), restricted feeding schedules in constant darkness conditions (DD) have been reported to affect behavioural rhythms and the molecular clockwork of the SCN in a few mammalian species (Kennedy *et al.* 1991; Mistlberger, 1994; Stephan, 2002; Castillo *et al.* 2004; Lamont *et al.* 2005; Mendoza *et al.* 2005a). Restricted feeding schedules are also able to synchronize behavioural and physiological rhythms independently of the SCN clock, and under the control of a circadian food-entrainable oscillator (FEO); however, the locus of FEO is still unidentified (Mistlberger, 1994; Stephan, 2002).

Otherwise, when feeding/fasting cycles comprise a hypocaloric feeding (HF), strong effects on the SCN molecular activity are observed (Caldelas *et al.* 2005; Mendoza *et al.* 2005b). Under DD, behavioural rhythms and clock gene oscillations in the SCN are entrained by a daily HF in rats (Caldelas *et al.* 2005). Moreover, in mice entrained to a light–dark (LD) cycle, HF presented during the day causes phase advances of behavioural and physiological rhythms as well as of clock gene expression in the SCN (Mendoza *et al.* 2005b). Despite a considerable literature on food entrainment in nocturnal rodents, relatively little is known about circadian phase resetting by feeding cues in diurnal mammalian species. In squirrel monkeys, 3 h of food access (without caloric restriction) has no effect on the locomotor activity rhythm under a LD cycle or in constant light (LL) conditions (Aschoff & von Goetz, 1986; Boulos *et al.* 1989). However, there is one report in the same species showing that in LL, 3 h of food access entrains the free-running rhythm of locomotor activity (Sulzman *et al.* 1977). In rodents, to our knowledge, there are very few reports showing that free-running behavioural rhythms of diurnal species are entrained to restricted feeding schedules (squirrels; Chandrashekar, 1982). Recently it has been reported that the diurnal rodent *Octodon degus* may develop a nocturnal phenotype when scheduled feeding is imposed at night (Vivanco *et al.* 2010).

The small rodent Sudanian grass rat (*Arvicanthis ansorgei*) expresses a diurnal-like activity pattern in a crepuscular fashion (Challet *et al.* 2002). The phase responses to light and melatonin in *Arvicanthis ansorgei* do not differ from those in nocturnal rodents, with similar phase delays and advances in the early and late night, respectively, for light, and entrainment to daily melatonin pulses when they occur at the end of the subjective

day (Caldelas *et al.* 2003; Slotten *et al.* 2005). However, the circadian times when serotonergic and dark cues can phase shift the *Arvicanthis* SCN clock are opposite to those of nocturnal rodents (Mendoza *et al.* 2007a; Cuesta *et al.* 2008). This suggests that, at least for these non-photoc stimuli (serotonin and dark pulses), there are differences in the circadian windows of sensitivity in the SCN between diurnal and nocturnal species. Nevertheless, in other diurnal species (ground squirrels), phase advances to non-photoc stimulation (activity-inducing stimulus) occur when stimulation coincide with the end of the (subjective) day, similarly to nocturnal rodents (Syrian hamsters). This study suggests that phase response to non-photoc stimulation depends on the phase of the clock and not on the phase of the activity cycle (Hut *et al.* 1999). Therefore, it may be possible that phase responses to non-photoc cues in diurnal rodents are species dependent.

To gain a better knowledge in non-photoc entrainment in diurnal species, the aim of the present study was to examine the effect of restricted feeding schedules with and without a HF regimen on circadian behaviour and expression of clock genes in the SCN of *Arvicanthis*. Moreover, we investigated the modulation of the synchronization to the LD cycle and phase shifting responses to light in *Arvicanthis* subjected to a daily HF.

Methods

Animal housing

Adult male Sudanian grass rats (*Arvicanthis ansorgei*) from our breeding colony in Strasbourg, weighing 100–120 g at the beginning of the study, were maintained in a 12:12 h light–dark cycle (LD, lights on at 07.00 h; light intensity about 200 lux), regulated temperature ($22 \pm 1^\circ\text{C}$), free access to regular laboratory diet (Ref. 105, SAFE, 89290, Augy, France) and tap water unless otherwise stated. Animals were acclimated to environmental conditions for at least 2 weeks before starting the experimental procedures. Animal handling was conducted in accordance with *The Journal of Physiology's* standards and advice (Drummond, 2009) and the *Guide for the Care and Use of Laboratory Animals* (NIH Pub. No. 86-23, revised 1985) and the French Department of Agriculture (licence no. 67–378 to J.M.).

Experimental design

Effects of a daily hypocaloric feeding in *Arvicanthis* under constant darkness conditions. At a first stage of the study, we explored the effect of a timed hypocaloric feeding (HF) in the wheel-running activity rhythms of *Arvicanthis* ($n = 8$) exposed to constant darkness (DD) conditions. As a control group we used animals exposed to daily

normocaloric feeding (NF, $n = 8$). Free-running circadian rhythms of the animals were monitored at least 15 days in DD before the onset of feeding schedules. Since no reports exist about protocols of caloric restriction in this species, HF was induced by reducing progressively the time of food access. Therefore, HF animals were exposed first to 6 h of food access during 10 days (the amount of food eaten at this schedule was 8.9 ± 0.6 g), then to 4 h (8.2 ± 0.5 g) and finally to 2 h (6.6 ± 0.2 g) of food access during 7 and 10 days, respectively. In the control NF group, the time of daily food access was restricted to 6 h (8.8 ± 0.4 g) during the whole experiment. Since in this experiment animals were under DD conditions, mealtime was at 18.00 h geographical time for both NF and HF *Arvicanthis*. Animals were weighed weekly to verify body weight loss by feeding conditions.

Timed hypocaloric feeding in *Arvicanthis* entrained to a light–dark cycle. For this experiment, animals entrained to a LD cycle (12–12 h) received a NF or HF ($n = 6–8$ per group) at the *zeitgeber* time 18 (considering ZT-0 as lights on; ZT-18 corresponds to the middle of the *Arvicanthis* rest period) for 5 weeks. The caloric restriction protocol was different in this experiment compared to the previous one. For this first group of animals, the 100% (e.g. 9 g) of food intake was determined in the first 2 weeks of baseline under food *ad libitum* (AL) conditions, and was the amount of food given to the control NF group daily at ZT-18. The HF consisted of the 66% (e.g. 5.6 g) of the daily food intake for each animal. The order of these protocols was chosen to assess the global effect of caloric restriction on circadian behaviour with different methods. Finally animals were re-fed *ad libitum* and behaviour was recorded for at least 3 weeks.

To evaluate the effects of HF on the phase of wheel-running activity rhythms avoiding the masking effect of light, another group of *Arvicanthis* ($n = 10–12$ per group) was exposed, under a LD cycle, to AL and HF (66%) conditions during 3 weeks. On the last day of food restriction, animals were released in DD conditions (at least for 3 weeks) to evaluate behavioural phase shifts. *Arvicanthis* were weighed weekly.

Clock gene expression in the SCN of *Arvicanthis*: differences between *ad libitum*, normocaloric and hypocaloric feeding conditions. New animals under a 12–12 LD cycle were exposed to AL, or timed NF (100%) and HF (66%) conditions. Food was delivered at ZT-18 (6 h after lights off) similar to the behavioural experiments under LD cycle. Three weeks after the food restriction period, animals were killed at four different *zeitgeber* times ($n = 4–6$ per group and time point); ZT-0 (lights on), ZT-6, ZT-12 (lights off) and ZT-18 (mealtime) with an overdose of isoflurane and brains were rapidly removed

and blood samples were obtained for *in situ* hybridization and hormonal assays, respectively.

Light resetting: behavioural phase shifts, genes and protein expression in the SCN clock induced by light. To evaluate the effect of HF in photic resetting, two groups of animals (*ad libitum*, AL vs. HF, $n = 8$ per group) under DD conditions were exposed to a 30 min light pulse (200 lux) at the circadian time 0 (CT-0; activity onset) and CT-12. At the end of baseline conditions (LD 12:12 for 2 weeks), animals were transferred to DD and fed *ad libitum* for 2 weeks. The control group remained under food *ad libitum* conditions and the HF group received food (66%) at 12.00h (geographical time) for 3 weeks. After the light pulse manipulation, all animals were fed *ad libitum* on the same day and remained on DD for 3 weeks. Another group of animals was used under the same feeding conditions (AL vs. HF) and was exposed to a light stimulation (30 min) only at CT-12. Animals were killed 1 h (CT-13; $n = 8$ per group) after light exposure. Dark control animals were killed at CT-13 ($n = 8$ per group) without light stimulation. Brains were removed for *in situ* hybridization and immunohistochemistry experiments.

Behavioural recordings and analysis

For behavioural recordings, animals were housed singly in plastic cages ($35 \times 22 \times 40$ cm) equipped with running wheels (30 cm diameter). Each running wheel was equipped with a magnetic microswitch connected to a PC. Wheel-running activity was continuously recorded with a data acquisition system (CAMS; Circadian Activity Monitoring System, University of Lyon, France) and analysed with ClockLab software (Actimetrics, Evanston, IL, USA). Activity data were displayed as actograms and average waveforms using ClockLab. The following parameters were quantified: 24 h mean of activity for animals under a LD cycle, and the endogenous period (τ) of behavioural rhythms for animals in DD. The endogenous period (τ) was assessed for each animal before, during and after food entrainment using a χ^2 periodogram analysis and by a regression line fitted to activity onsets (ClockLab software, Actimetrics, Evanston, IL, USA). The duration of nocturnal and diurnal activity was calculated using the onset and offset of light. In addition, we measured the magnitude of food anticipatory activity (FAA) as the total daily activity occurring during the 3 h preceding mealtime in experiment 1. For light resetting experiment, the magnitude of phase shifts was determined by measuring the phase difference, based on the activity onset as a phase reference point, between regression lines fitted to activity onsets of the 10 days before the light pulse and at least 10 consecutive activity-onset times after the light pulse.

In situ hybridization

Animals were deeply anaesthetized with isoflurane and decapitated. Brains were rapidly excised and frozen on dry ice. Coronal sections of 18 μm were cut in a cryostat for *in situ* hybridization at the level of the SCN. Antisense RNA probes were transcribed in the presence of $\alpha^{35}\text{S}$ -UTP (1250 Ci mmol^{-1} , PerkinElmer, Milan, Italy) according to the manufacturer's protocol (MAXIScript, Ambion, Austin, TX, USA). Here we used riboprobes for *rPer1* (bases 638–1618 from GenBank accession no. NM001034125), *rPer2* (bases 1170–1930 from NM031678; *Per1* and *Per2* plasmids were kindly donated by H. Okamura, Department of Systems Biology, Kyoto University, Japan), *rAvp* (vasopressin, bases 68–539 from M25646) and *rRev-erba* (bases 908–1890 GenBank accession NM145775). Brain sections were fixed in 4% phosphate-buffered paraformaldehyde, rinsed twice with phosphate-buffered saline (PBS) and then acetylated twice in 0.1 M triethanol-amine, washed again with PBS and dehydrated in a graded ethanol series. Sections were hybridized overnight with either denatured antisense riboprobes (1.107 c.p.m. per ml of hybridization medium buffer) in a humid chamber at 54°C with radio-labelled probe in a solution containing 50% deionized formamide, 10% sulfate dextran, 1 \times Denhardt's solution, 2 \times SSC (sodium citrate saline), 0.5 mg ml^{-1} salmon sperm DNA, 0.25 mg ml^{-1} transfer RNA, and 10 mM dithiothreitol. Sections were then rinsed with SSC, treated with ribonuclease A (Sigma), rinsed with stringency washes of SSC and dehydrated in a graded ethanol series. Slices and radioactive standards were exposed for 5 days to an autoradiographic film (Biomax MS-1 Kodak, Sigma). Standards were included in each cassette to verify that the measured values of optical densities were in the linear response range of the film. Densitometric analysis of hybridization signals was performed using ImageJ (NIH). The optical density of specific signal was calculated by subtracting the intensity of staining background area measured in surrounding (i.e. anterior) hypothalamic area where no hybridization signal was detected from that measured in the SCN. Measures were made bilaterally on three slices and averaged for a given brain. Data were expressed as relative optical density values.

Immunohistochemistry

For immunohistochemistry experiments, animals were killed with an isoflurane overdose and perfused transcardially with 100 ml of 0.9% saline followed by 100 ml of cold 4% paraformaldehyde (PAF) in 0.1 M phosphate buffer (PB; pH 7.4). Brains were removed, post-fixed (overnight in 4% PAF at 4°C) and transferred to a cryoprotectant buffered sucrose solution (30%) for 72 h at 4°C. Brains were then frozen in isopentane at -30°C

and then stored at -80°C . Coronal cryosections through the SCN of 30 μm were prepared on a cryostat at -20°C , collected and stored in 0.1 M phosphate-buffered saline (PBS; pH 7.4; Sigma, St Louis, MO, USA) supplemented with 0.01% sodium azide. Free-floating sections were washed in cold PBS and incubated in a solution of 3% of H_2O_2 (Sigma) in PBS for 30 min at room temperature. Brain sections were then rinsed in PBS for 10 min three times, and incubated for 2 h in a blocking solution in 10% normal donkey serum (NDS) in PBS with 0.3% Triton X-100 (0.3% PBS-X), followed by an incubation in the primary antibody (in 0.3% PBS-X plus NDS) for 24 h at 4°C. We used a rabbit polyclonal anti-c-Fos antibody (1:10,000, raised against an epitope mapping the N-terminus of human c-Fos; SC-7724, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit polyclonal anti-p-ERK (rabbit polyclonal antibody, dilution: 1:20000; Cell Signaling Technologies, Danvers, MA, USA). Following incubation in the primary antibody, sections were rinsed in PBS-X and incubated for 2 h at 4°C with a biotinylated anti-rabbit IgG made in donkey (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, diluted 1:2000 with 0.3% PBS-X). Following incubation with secondary antibody, sections were rinsed in PBS-X and incubated for 1 h at room temperature with an avidin–biotin–peroxidase complex (Vectastain Standard Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA). Following incubation with the ABC reagents, sections were rinsed two times for 10 min in PBS, and incubated with 0.025% 3,3'-diaminobenzidine (Sigma) with 0.01% H_2O_2 in 50-mM Tris buffer. After this final incubation, sections were rinsed with PBS, wet-mounted onto gel-coated slides, dehydrated through a series of alcohols, soaked in xylene and coverslipped.

Microscopic quantification

Cells were visualized by using a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). Images were taken (5 \times) standardizing all lighting parameters on the microscope and the camera software (Viewfinder Lite, Olympus) to ensure consistent stable lighting throughout the image capture, and stored on a PC as described previously (Mendoza *et al.* 2008). Positive c-Fos cells (cells were considered positive when the signal represented more than 3 times the background value) were counted in the whole central SCN (2–3 sections/animal), based upon a series of Nissl stained *Arvicanthis* brain sections. The number of immunoreactive (ir) nuclei was expressed as the mean number of immunopositive cells per section. Since p-ERK-positive staining is present not only in the SCN cell bodies but also in fibres, expression of p-ERK was quantified by measuring

staining density as described previously (Mendoza *et al.* 2008). The optical density (OD) of p-ERK-ir in the SCN was expressed in arbitrary units corresponding to grey levels. To calculate the OD, the background intensity of staining was subtracted from the intensity of staining in the middle SCN. The background intensity was measured in an area devoid of p-ERK cell bodies or fibres (lateral hypothalamic area) in the same coronal section as the SCN analysis.

Hormonal and metabolic assays

Blood glucose (mg dl^{-1}) was determined on fresh blood using a digital glucometer (Glucotrend, Roche Diagnostics, Meylan, France). Plasma insulin was determined by an ELISA kit for rats (90060, Crystal Chem, Inc., Downers Grove, IL, USA). The limit of sensitivity of insulin assay was 0.1 ng ml^{-1} . Plasma corticosterone was assayed with a commercial ^{125}I RIA kit for mice and rats (ImmuChem Double Antibody, MP Biomedicals, Orangeburg, NY, USA). The limit of sensitivity of corticosterone assay was 7.7 ng ml^{-1} .

Statistical analysis

The statistical analysis was performed by one- and two-way analyses of variance (ANOVA), for both independent and repeated measures followed by a Fisher's least significant difference (LSD) *post hoc* test. A critical value for significance in all experiments was $P < 0.05$. Statistical analysis was performed with the statistical package Statistica (version 8.0; StatSoft Inc., 2007, France). Values are means \pm SEM.

Results

HF synchronizes circadian rhythms of wheel-running behaviour in *Arvicanthis*

All *Arvicanthis* recorded in DD displayed stable free-running rhythms of wheel-running behaviour (Fig. 1A and 1B). Differentially from NF animals, *Arvicanthis* fed with a daily HF did not show an increase of body weight. This difference was significant when animals had 2 h food access (Fig. 1C; $P < 0.05$). Moreover, food intake was also significantly lower in HF animals compared to NF *Arvicanthis* at the 2 h feeding schedule (Fig. 1C; $P < 0.05$). The amount of food ingested during the 2 h feeding schedule in *Arvicanthis* is quite similar to the amount of food intake in the protocol of the second experiment (66% of the daily food intake; 5.6 g vs. 6.6 g; $P = 0.181$). This suggests that the protocol used in this experiment is reflecting a hypocaloric state in animals and not only the strongest form of restricted feeding.

In animals from the NF group (6 h of food access), no entrainment was observed (Fig. 1A and C). Consequently, the mealtime schedule was timed to overlap with the phase of both rest and activity in *Arvicanthis*. In animals that did not entrain, some slight changes in τ were present (data not shown) but not real entrainment. The meal schedule was continued until all circadian phases were sampled without entrainment (Fig. 1A). The lack of entrainment in NF *Arvicanthis* was confirmed by χ^2 periodogram analysis (Fig. 1C). In all NF *Arvicanthis* free-running period was still longer than 24 h ($24.16 \pm 0.02 \text{ h}$) during synchronization and after the entraining stimulus was removed ($24.26 \pm 0.07 \text{ h}$; Fig. 1A and C), without significant differences (NS) between values before and during entrainment.

In HF animals, following a short time on the 4 and 2 h feeding schedules, the free-running component became entrained to the feeding time (Fig. 1B). In addition, χ^2 periodogram analysis showed values of τ of almost 24 h for HF animals significantly different from those of NF *Arvicanthis* (Fig. 1C; $P < 0.05$). Furthermore, τ values of entrained rhythms remained of almost 24 h after food restriction in the HF group (Fig. 1B and C; $P < 0.05$). These data suggest that under hypocaloric conditions, the behavioural rhythms in *Arvicanthis* can be entrained by a daily feeding schedule.

During the period of food restriction, food anticipatory activity (FAA) was present in both NF and HF groups (Fig. 1D). However, FAA duration and magnitude in HF animals were greater than those in NF *Arvicanthis* (Fig. 1D; $P < 0.05$).

Behavioural and physiological circadian rhythms during food restriction in LD

In all *Arvicanthis*, the actograms and average waveforms revealed that wheel-running activity was exclusively diurnal (with a crepuscular pattern) in LD prior to food restriction (Fig. 2A). For the NF group, when food availability was limited to 100% of their daily food intake at 6 h after dark onset, all *Arvicanthis* showed some evidence of nocturnal wheel-running activity in anticipation to feeding time (Fig. 2A). The duration and magnitude (total number of daily wheel revolutions) of FAA was weak compared to the diurnal activity, which remains entrained to the LD cycle (Fig. 2A). At the end of restricted feeding, when NF animals returned to free access to food, no FAA was present and the diurnal rhythm of activity was not altered (Fig. 2A).

In *Arvicanthis* fed with a HF, FAA was as evident as in NF animals; however, it was most robust during the last days of food restriction. Moreover, the diurnal pattern was altered in almost all *Arvicanthis*, which demonstrated more activity principally during offset of light period,

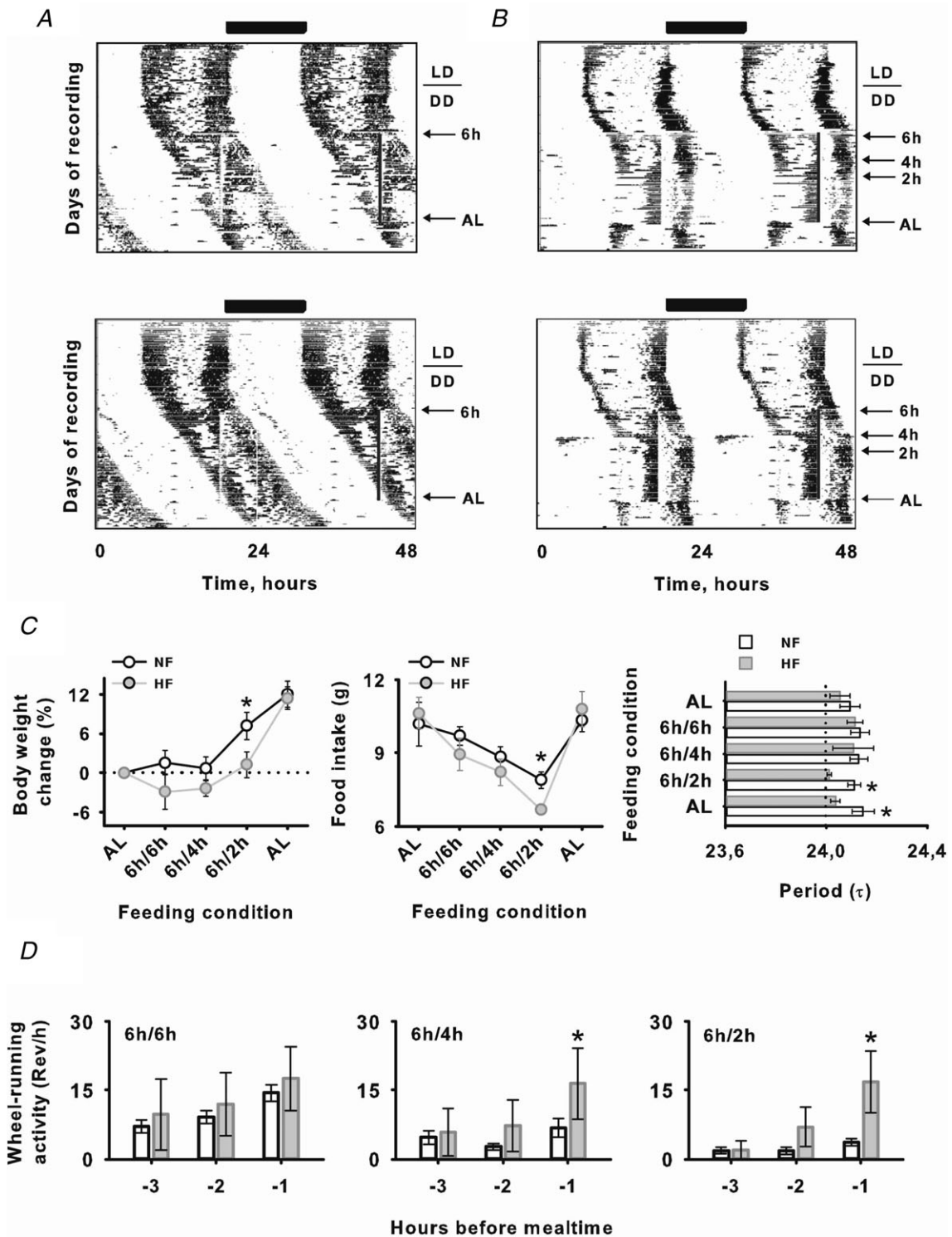


Figure 1. HF entrains behavioural circadian rhythms of *Arvicanthis*

Circadian wheel-running activity of NF (A) and HF (B) fed *Arvicanthis*. In the representative actograms daily NF and HF feeding is indicated by vertical lines. C, left and middle, body weight (left) and food intake (middle) of *Arvicanthis* subjected to NF (fixed, 6 h food access) and HF (progressive, from 6 to 2 h food access) restricted feeding schedules. Right, endogenous period values (τ) in both NF and HF animals during previous food *ad libitum* conditions, during food entrainment and under *ad libitum* conditions after re-feeding. D, food anticipatory activity (FAA) from 3 to 1 h before mealtime in *Arvicanthis* subjected to NF (6 h food access) and HF (from 6 to 2 h food access) conditions. * $P < 0.05$, differences between groups (NF vs. HF).

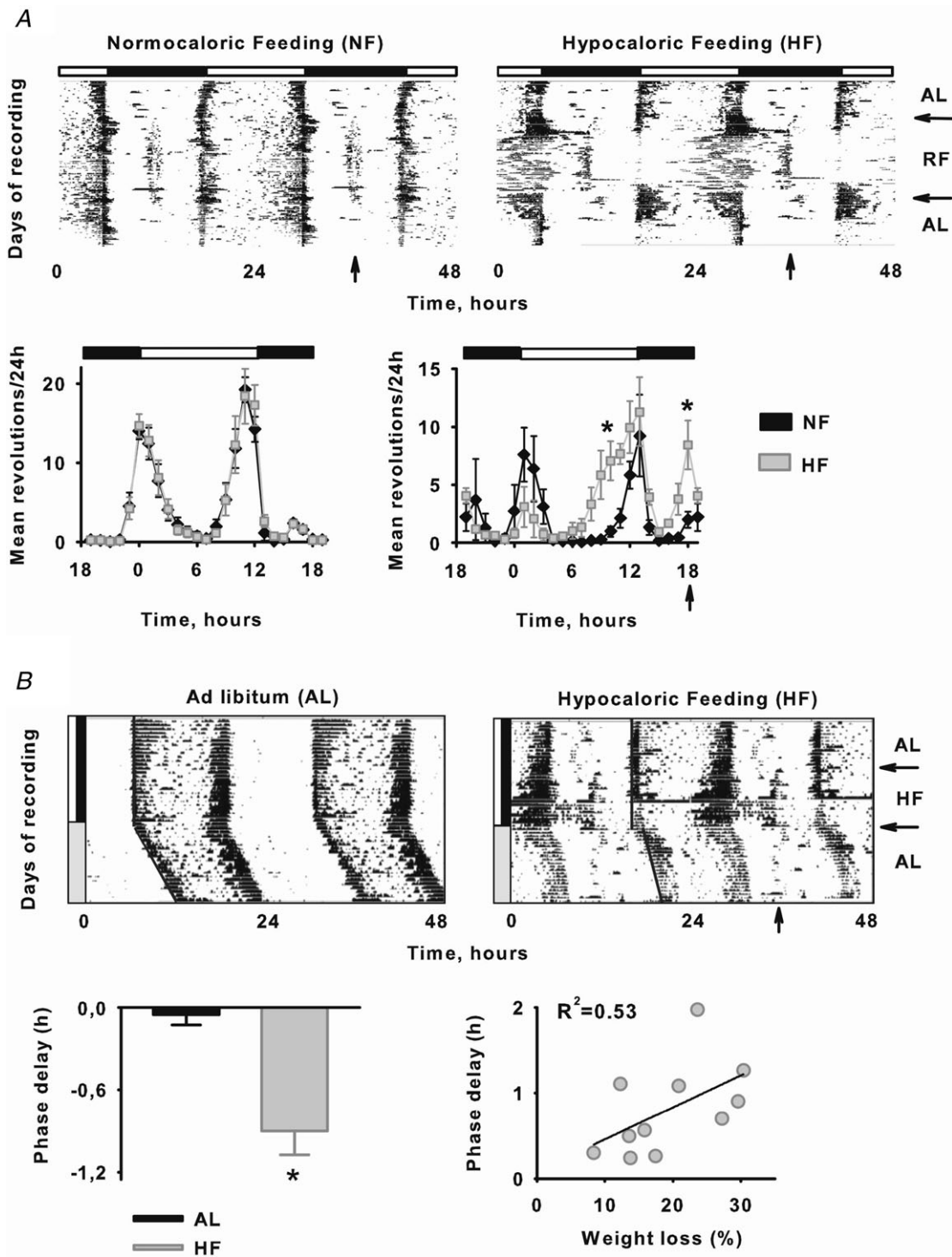


Figure 2. Behavioural rhythms entrained to a LD cycle are affected by HF
 A, daily wheel-running activity of *Arvicantis* entrained to a light–dark cycle (indicated by the top black–white bar) and to a NF or HF at the middle of the night period (vertical arrow). In actograms horizontal arrows on the right indicate transition *ad libitum*–restricted feeding–*ad libitum* feeding conditions. Activity profiles of wheel-running activity over the 24 h cycle of NF and HF *Arvicantis* (means \pm SEM) under food *ad libitum* conditions (left) and during the restricted feeding period (right). B, representative actograms of wheel-running behaviour in *Arvicantis* under AL (left) and HF (right) conditions (ZT-18) during the entrainment to a LD cycle and then released in DD conditions. Arrows on the right indicate the time duration of HF. Group mean (\pm SEM) of phase delays in AL and HF fed animals after to be released in DD conditions. Linear regression of weight loss (%) plotted against phase delays in HF *Arvicantis* ($R^2 = 0.53$). * $P < 0.05$, differences between groups.

leading to a higher day–night variation compared to NF animals (Fig. 2A). During the re-feeding period, the circadian rhythm of locomotion returned to being diurnal as in the previous food *ad libitum* conditions (Fig. 2A).

In a second protocol to evaluate modulation of light entrainment by caloric restriction, and avoid possible masking effects by light in phase resetting, animals exposed to HF under LD cycles were released into DD at the end of the feeding schedule. Differentially to AL animals, HF *Arvicanthis* showed significant phase delays in behaviour after to be released in DD conditions (Fig. 2B; $P < 0.05$). Moreover, a regression analysis revealed a strong correlation ($R^2 = 0.53$) between the body weight loss and the magnitude of phase delays (Fig. 2B; $P < 0.05$). Using comparable protocols previously in nocturnal rodents, we observed phase advances rather than delays in behavioural rhythms (Challet *et al.* 1998; Mendoza *et al.* 2005b).

During baseline, all *Arvicanthis* (AL, NF and HF) under food *ad libitum* conditions showed a daily pattern of food intake with a higher feeding behaviour during the day (day, 9.96 ± 0.6 ; night, 1.93 ± 0.3 ; Fig. 3A, Inset; $P < 0.05$). Nevertheless, when animals from NF and HF groups were given food at ZT-18, feeding behaviour showed some changes. In NF animals, the main amount of food was ingested between ZT-0 and ZT-6 (6.6 ± 0.7 g), and between ZT-18 (mealtime; 5.7 ± 0.4 g) and ZT-0 (Fig. 3A). HF *Arvicanthis*, however, ate almost the whole amount of food from ZT-18 to ZT-0 (5.9 ± 0.13 g) showing a nocturnal feeding behaviour (Fig. 3A; $P < 0.05$).

Corticosterone (CORT) daily profiles were not different between AL and NF animals; both showed a peak at ZT-0, corresponding to the night–day transition (Fig. 3A; $P < 0.05$). However, in HF animals, CORT daily profile was phase shifted with significantly higher levels and with an acrophase at the day–night transition as in nocturnal rodents (Fig. 3A; $P < 0.05$). Daily concentrations of plasma glucose were down-regulated in the HF group compared to those in AL and NF animals (Fig. 3A; $P < 0.05$). Finally, daily rhythms of plasma insulin were shifted in both NF and HF animals compared to AL *Arvicanthis* (Fig. 3A; $P < 0.05$).

SCN clock gene expression during food restriction in LD

In *Arvicanthis* exposed to AL food conditions, daily rhythms in the expression of *Per1–2*, *Rev-erba* and vasopressin (*Avp*) genes were observed with an acrophase at ZT-6 for *Per1–2* and *Avp*, and ZT-0 for *Rev-erba* (Fig. 3B). Daily profiles of *Per1* and *Rev-erba* were similar between AL and NF animals (Fig. 3B). Interestingly, for HF animals whereas *Per1* expression showed a significant increase of the acrophase at ZT-6 compared to the AL

control group (Fig. 3B; $P < 0.05$), a significant phase delay of the rhythmic expression was detected for *Per2* and *Avp* (Fig. 3B; $P < 0.05$). This effect is consistent with the delayed pattern of behaviour in HF *Arvicanthis*, and different from the phase advances in clock genes induced by HF in nocturnal rodents (Mendoza *et al.* 2005b). For *Rev-erba* gene expression in HF animals, a down regulation was observed at ZT-0 leading to a phase delayed pattern (Fig. 3B; $P < 0.05$).

Light-resetting in *Arvicanthis* under hypocaloric food conditions

Light resetting is altered in mice under hypocaloric feeding conditions, showing changes principally in phase advances induced by light (Challet *et al.* 1998; Mendoza *et al.* 2005b). We asked whether a HF can affect light information input to the clock in the SCN of the diurnal rodent *Arvicanthis* in a similar manner as in nocturnal mice. Therefore, we applied light pulses to *ad libitum* and hypocaloric fed *Arvicanthis* that were kept under DD conditions. We found that a light pulse (30 min) applied at circadian time (CT) 0, which corresponds to onset of subjective day, did evoke a phase advance of the clock in both AL and HF animals with no difference in the magnitude of the shift between groups (NS; Fig. 4A). A light pulse applied at CT-12 resulted in a phase delay of the clock in AL animals, and although the phase shift in HF *Arvicanthis* was larger, it was not significantly different from the AL group ($P = 0.08$; Fig. 4A).

We used *in situ* hybridization and immunohistochemistry analysis to examine whether a 30 min light pulse delivered during the onset of subjective night (CT-12) had an effect on *Per1*, *Per2*, c-Fos and p-ERK in the *Arvicanthis* SCN. In AL animals, a light pulse delivered at CT-12 led to a dramatic increase in *Per1*, *Per2*, c-Fos and p-ERK-IR 1 h after exposure different from dark control animals (Fig. 4B; $P < 0.05$). However, the increase of *Per1* and *Per2* expression was lower in HF *Arvicanthis* (Fig. 4B). Similarly to AL animals, c-Fos and p-ERK expression was enhanced after light stimulation in the SCN of HF *Arvicanthis* without significant differences (Fig. 4B; NS).

Discussion

The present study showed that a daily HF affects both the photic resetting and the endogenous mechanisms of the SCN clock in the diurnal rodent *Arvicanthis ansorgei* in a different manner from nocturnal rodents.

Effects of HF in animals under DD conditions

In contrast to a NF, a daily HF is capable of producing entrainment in *Arvicanthis* under DD conditions. These

findings are in accordance with a previous study in rats (Caldelas *et al.* 2005). Entrainment was produced when timed HF fell in either the subjective night or the subjective day, indicating that the sensitivity of the SCN to HF is not dependent on the behavioural state of animals as for other non-photic cues (Mendoza *et al.* 2007a; Cuesta *et al.* 2008).

Behavioural synchronization by feeding (without caloric restriction) in nocturnal rodents under DD conditions has been previously observed (Abe & Rusak, 1992; Marchant & Mistlberger, 1997; Castillo *et al.* 2004). Lighting condition (DD or LL) is a critical variable in the SCN entrainment by feeding schedules (Caldelas *et al.*

2005; Lamont *et al.* 2005). Nevertheless, no entrainment to feeding schedules has previously been observed in diurnal mammals under LL conditions (Aschoff & von Goetz, 1986; Boulos *et al.* 1989), suggesting that HF is necessary to entrain behavioural rhythms.

Behavioural activation can entrain the SCN in diurnal and nocturnal rodents (Mrosovsky, 1996; Marchant *et al.* 1997; Hut *et al.* 1999; Kas & Edgar, 2001). HF *Arvicanthis* showed intense arousal previous to mealtime (food anticipatory activity; FAA). Thus, it is possible that FAA, higher in HF than in NF, would contribute to clock entrainment in *Arvicanthis*. Furthermore, serotonin

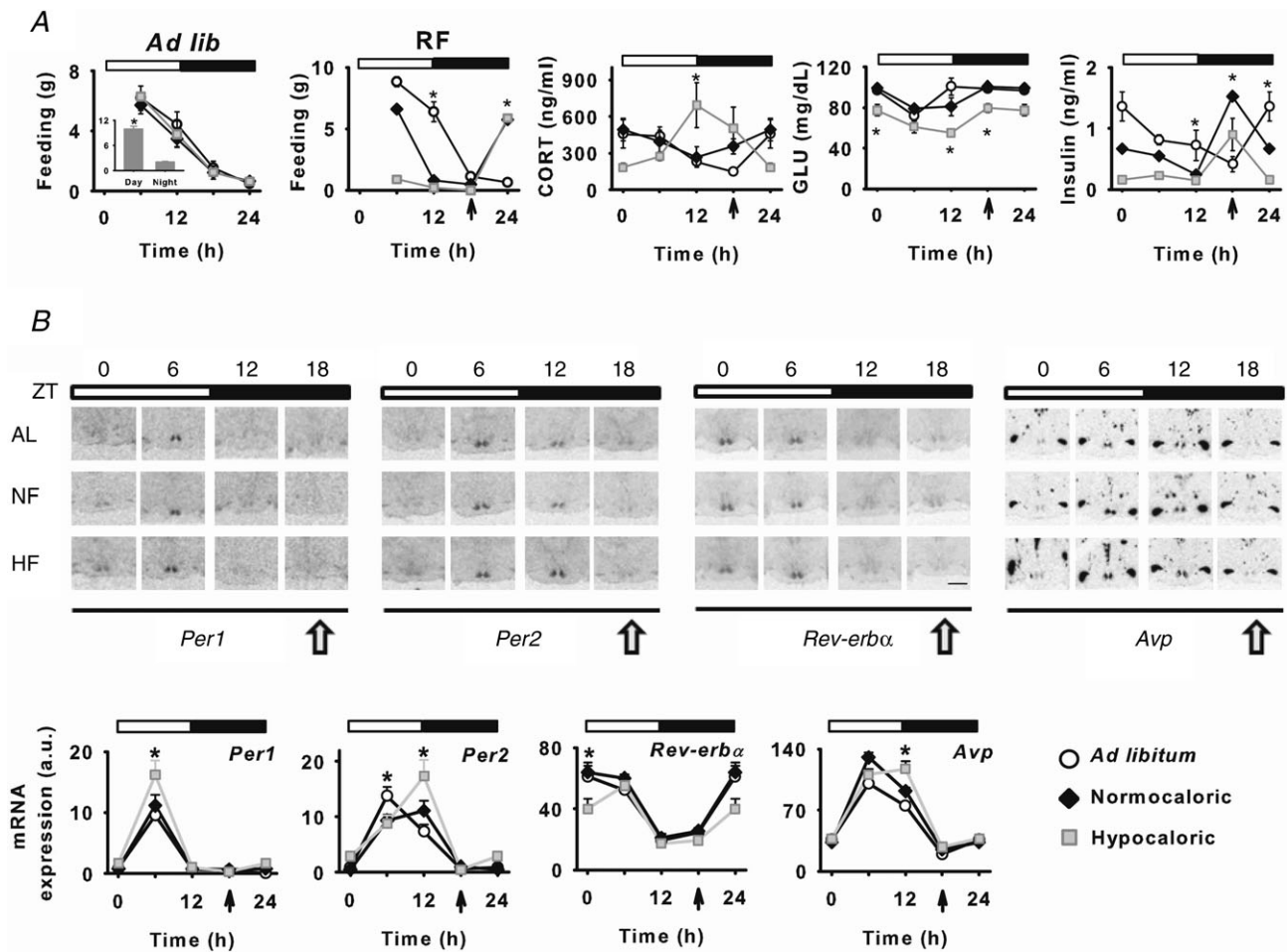


Figure 3. Physiological rhythms and *Arvicanthis* SCN clock gene expression are delayed by HF
 A, daily food intake, metabolic and hormonal profiles in AL, NF and HF fed *Arvicanthis*. Feeding behaviour under food *ad libitum* (Ad lib) conditions, previous to exposure to restricted feeding protocols was significantly diurnal in all animals (inset plot). Daily feeding behaviour patterns changed under restricted feeding schedules (RF), leading to a critical nocturnal feeding behaviour in HF fed *Arvicanthis*. Corticosterone (CORT), glucose and insulin daily profiles in AL, NF and HF fed animals. **P* < 0.05, differences between groups. B, daily profiles of clock genes expression in the SCN of AL, NF and HF fed *Arvicanthis*. Top, representative photomicrographs show coronal sections through the SCN of *Arvicanthis* kept in LD conditions (indicated by white and black bars) and exposed to AL, NF or HF conditions (Scale bar = 1 mm). Animals were sampled every 6 h at different *zeitgeber* times (ZT; 0, 6, 12 and 18). Yellow arrows indicate feeding time. Bottom, daily profiles of *Per1*, *Per2*, *Rev-erbα* and *Avp* in the SCN of AL, NF and HF fed *Arvicanthis*. Data are presented as the mean ± SEM (a.u., arbitrary units) of 4–6 animals per group and time point. LD conditions are indicated by white and black bars. Time of feeding is indicated by vertical arrows, 6 h after lights off. **P* < 0.05, differences between groups.

(5-HT) afferents from the raphe nuclei to the SCN are strongly implicated in entrainment by arousal in nocturnal rodents (Pickard & Rea, 1997; Marchant *et al.* 1997). 5-HT release in the SCN of nocturnal rodents shows a

daily rhythm in freely behaving animals, with an increase in release during late midday, peaking at the light–dark transition (Dudley *et al.* 1998). In the *Arvicantis* SCN, in an opposite manner to nocturnal rodents, rhythmic

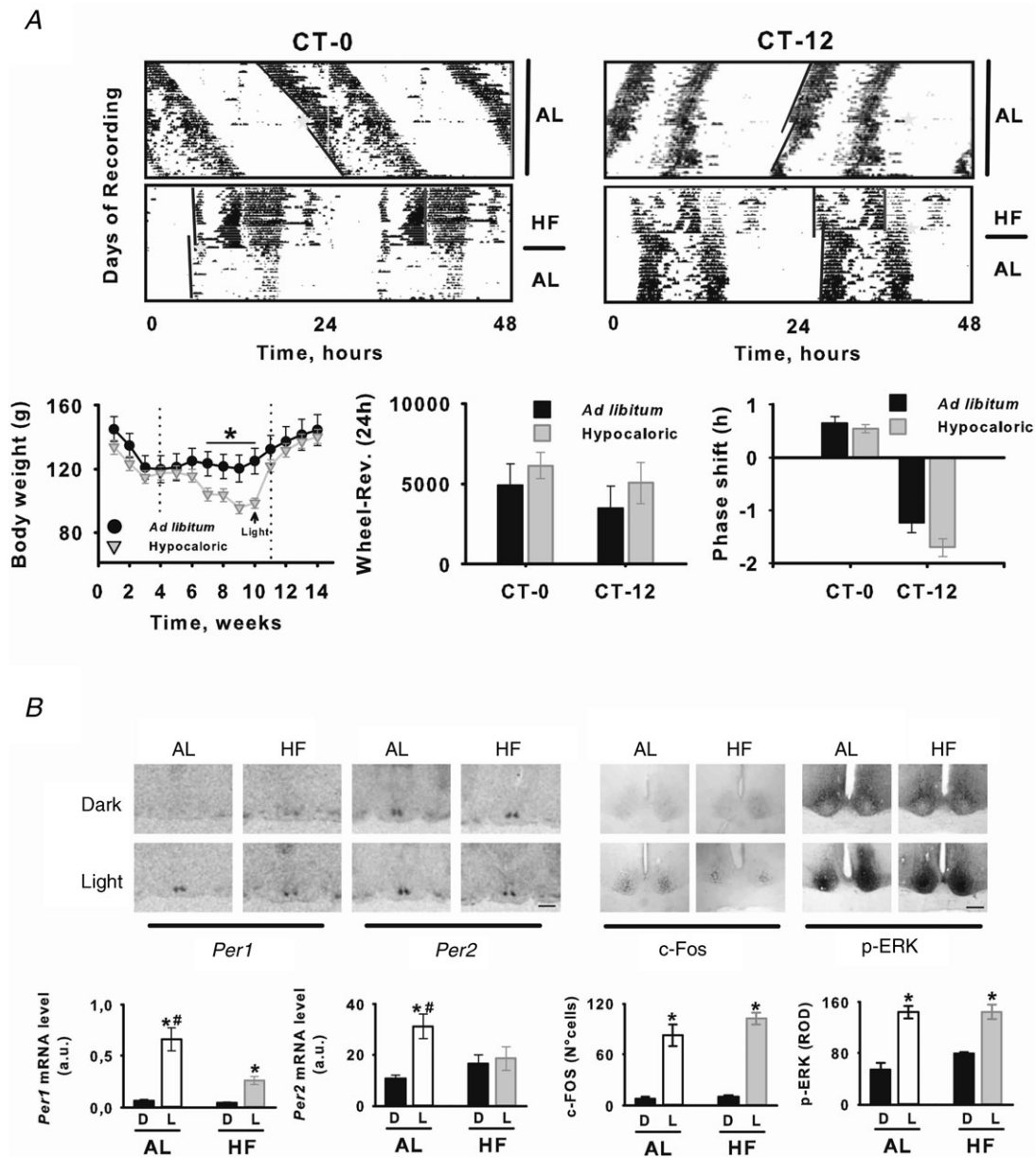


Figure 4. Photoc resetting of the clock is changed in *Arvicantis* fed with a HF

A, top, circadian wheel-running activity from AL and HF *Arvicantis* receiving a 30 min light pulse at CT-0 and CT-12. In double-plotted actograms stars indicate the time of light stimulation. Bottom, body weight (left), total locomotion (middle) and group mean (\pm SEM) phase shifts to light at CT-0 and CT-12 (right) of AL and HF *Arvicantis*. In the body weight plot, vertical dotted lines indicate the time in which animals were exposed to HF condition, and arrow indicates body weight values at the moment of light stimulation. **B**, light exposure induces differential changes on *Per1*, *Per2*, c-Fos and p-ERK in the SCN of AL and HF *Arvicantis*. Top, representative photomicrographs show coronal sections through the SCN of *Arvicantis* kept in constant darkness and exposed to a HF or AL feeding conditions and 30 min light pulse (Light) or a control dark pulse (Dark) at CT-12. Scale bars = 1 mm (*Per1*–*2*) and 200 μ m (c-Fos, p-ERK). Bottom, semiquantitative analysis of *Per1*, *Per2*, c-Fos and p-ERK in the SCN of AL and HF *Arvicantis* after 30 min light exposure (L) or after exposure to darkness (D) at CT-12. Data are presented as the mean \pm SEM of 4 animals per group. * P < 0.05, differences between light conditions (Light vs. Dark). # P < 0.05, differences between groups (AL vs. HF).

5-HT concentrations are higher during the day (Cuesta *et al.* 2009). That suggests that 5-HT (induced by high locomotion during mealtime anticipation) could be an important neurotransmitter implicated in the entrainment of behavioural rhythms in *Arvicanthis* by HF.

Behavioural and molecular changes in HF fed animals under a LD cycle

Arvicanthis exposed to a LD cycle and under food AL conditions showed a clear diurnal behaviour and in some cases a bimodal wheel-running activity pattern, with peaks of activity at the day–night and night–day transitions (Challet *et al.* 2002). Interestingly, CORT daily rhythms peaked with a single peak (ZT-0) in AL *Arvicanthis*, and previously in the same species a two-daily peak rhythm (ZT-23 and ZT-11) of CORT was reported (Verhagen *et al.* 2004). Here, animals were sampled every 6 h (ZT-0, ZT6, ZT-12 and ZT-18) and in the previous study animals sampling was every 2 h starting at ZT-9 (Verhagen *et al.* 2004). Thus, the closer time points of this study to the current data are ZT-1 and ZT-13. Therefore, it is possible that in the present experiments, our sampling just skipped the second peak (ZT-11) of CORT. Moreover, in the present study animals were exposed to a running-wheel access. The size of alpha (i.e. the portion of a daily rest–activity cycle corresponding to activity) is compressed in *Arvicanthis* with access to a running wheel (Challet *et al.* 2002; Cuesta *et al.* 2009). Thus, running-wheel activity could also influence the shape of the rhythm of CORT.

Differentially to AL animals, HF *Arvicanthis* spent more time being active during late day (i.e. last part of the activity period) and early night (i.e. before mealtime). The induction of activity is one of the main responses in organisms exposed to caloric restriction (Bordone & Guarente, 2005). Similarly, in mice exposed to some way of caloric restriction ('work for food' paradigm) there is an increase in activity during day time (Hut *et al.* 2011). This spontaneous diurnal behaviour in mice (animals are not submitted to a kind of feeding time) has been proposed to be important to minimize daily energy expenditure (Hut *et al.* 2011). In our study HF animals are submitted to timed feeding schedules, and therefore a different mechanism seems to increase night time activity in *Arvicanthis*. Increase of activity at night time in HF *Arvicanthis* could be related to the expression of FEO in this species (Mistlberger, 1994). Under food *ad libitum* conditions the SCN and FEO are strongly coupled, but under restricted feeding schedules (with or without caloric restriction), the FEO becomes uncoupled from the SCN clock (Mistlberger, 1994; Stephan, 2002).

Otherwise, a switch of the behavioural rhythms has been reported in another species as a function of wheel access (Kas & Edgar, 1999; Smale *et al.* 2003; Redlin & Mrosovsky,

2004). Comparable effects in behavioural timing, albeit in DD, have been observed in rats as a consequence of a HF (Caldelas *et al.* 2005). Recently a diurnal–nocturnal inversion was reported in the diurnal rodent *Octodon degus* as a function of restricted feeding (Vivanco *et al.* 2010). A two-process account of switching is advocated, involving both a change in clock-controlled outputs and a change in the direct response (i.e. masking) to food (Mrosovsky, 1999; Redlin & Mrosovsky, 2004). However, our molecular data indicate that the behavioural shift in HF *Arvicanthis* is not due only to masking mechanisms. Circadian rhythms of clock gene expression are similar within the SCN of diurnal and nocturnal species (Mrosovsky *et al.* 2001; Caldelas *et al.* 2003). Here we observed that circadian genes are shifted or affected in the SCN of HF fed *Arvicanthis*. In the SCN of control AL animals, *Per* genes peaked around midday (ZT-6; Caldelas *et al.* 2003). In HF animals, however, whereas *Per1* expression was increased around midday at ZT-6, *Per2* and *Avp* were phase delayed. Moreover, a decrease in *Rev-erba* expression at ZT-0 in HF animals suggests a possible phase delay in the daily rhythm of this gene as well. Differential effects have also been observed in nocturnal rodents. In mice, a HF induces an increase in the expression of *Per2* and a phase advance of *Per1* and *Avp* expression in the SCN (Mendoza *et al.* 2005b). The differential responses to HF between diurnal and nocturnal species indeed support the proposition that *Per1* and *Per2* are more related to phase advances and phase delays, respectively (Albrecht *et al.* 2001). Together, this demonstrates that the internal phase relationships between circadian cycles of clock gene expression in the *Arvicanthis* SCN and their phasing relative to the light–dark cycle are phase delayed by HF conditions.

A possible factor influencing the phase delays in behaviour and clock genes in the SCN by HF is the free-running period. Due to the long free-running period in *Arvicanthis*, the circadian clock delays to synchronize to HF. In mice, which show a free-running period shorter than 24 h, the circadian clock advances in response to a HF (Mendoza *et al.* 2005b). However, in nocturnal rats with endogenous periods longer than 24 h, behavioural rhythms advance whatever the time of HF exposure (Caldelas *et al.* 2005). Thus, other mechanisms exclusive to the diurnal species could determine the direction of phase shifts of the *Arvicanthis* clock under HF conditions.

Light resetting in *Arvicanthis* fed with a HF

Light induces phase delays and advances in *Arvicanthis*, regardless of whether they were under AL or HF conditions, similarly to those of a wide variety of nocturnal and diurnal mammals (Daan & Pittendrigh, 1976; Mahoney *et al.* 2001; Caldelas *et al.* 2003). A tendency

to larger phase delays was visible in *Arvicanthis* fed with a HF; however, this difference was not significant. Previous studies have indicated that low-caloric diet conditions modify the circadian responses to light in mice (Mendoza *et al.* 2005b).

Arousal can also influence the magnitude of photic phase shifts in mammals via 5-HT modulation in the SCN (Mrosovsky, 1996). 5-HT stimulation in *Arvicanthis* potentiates light-induced phase shifts (Cuesta *et al.* 2008). In nocturnal rodents, however, 5-HT stimulation decreases phase shifts produced by light (Weber *et al.* 1998; Challet *et al.* 2001). Thus, non-photoc modulation of the SCN clock appears to differ between nocturnal and diurnal species, highlighting the potentiating effects of 5-HT and possibly of caloric restriction on light resetting in diurnal species.

Light resetting of the SCN involves an induction of *Per1–2* expression (Albrecht *et al.* 1997; Shearman *et al.* 1997). Here we observed an increase in the expression of *Per1* in the SCN of both AL and HF animals after light exposure at CT-12. In nocturnal rodents fed with HF, we previously reported a reduction in light-induced of *Per1* transcription at CT-12 (Mendoza *et al.* 2005b). Despite a trend for larger phase delays in circadian behaviour, HF fed *Arvicanthis* also showed significant lower light-induced increases of *Per1* in the SCN. The most likely scenario is that even if *Per1* normally participates in light-induced resetting responses, in the absence of its induction by light in HF animals, other mechanisms may maintain SCN responsiveness. Alternatively, these may be due to a delayed photic response as observed in HF mice (Mendoza *et al.* 2007b).

In mice under HF conditions, light-induced expression of *Per2* in the SCN (at CT-12) is not altered (Mendoza *et al.* 2005b). However, in HF *Arvicanthis* exposed to light at CT-12, no induction of *Per2* in the SCN was observed. In *Arvicanthis* fed with HF under LD cycle, *Per2* was phase delayed compared to AL animals. Therefore, it is possible that the absence of *Per2* light-induction is the consequence of a change in the time course of *Per2* sensitivity to light due to the SCN metabolic state. However, the role of other clock genes (e.g. *Cry*, *Dec*) in light resetting of HF fed *Arvicanthis* remains to be studied.

In conclusion, the present study indicates that feeding is a potent synchronizer for the main circadian clock in the diurnal rodent *Arvicanthis ansorgei*. Importantly, most of these effects are in a different direction from those found in nocturnal rodents.

The use of a diurnal rodent to study the effects of synchronizers in circadian (e.g. jet-lag, scheduled-work rotations) and non-circadian disruptions (e.g. obesity, ageing-related health problems) could be useful for future basic and biomedical research. Caloric restriction has been reported to induce molecular and cellular changes that reduce ageing and prevent metabolic diseases

(Bordone & Guarente, 2005). Notwithstanding, the report of food-induced entrainment in a diurnal rodent could suggest that the use of timed feeding programmes in people (Aschoff *et al.* 1986) would be useful to ameliorate circadian and non-circadian pathologies and for disease prevention and human health.

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Author contributions

All the experiments were conducted at the Institute of Cellular and Integrative Neurosciences, CNRS UPR-3212 and the Chronobiotron Platform (IFR37-CNRS) of the Neurosciences Federation of Strasbourg (IFR-37 France). Conception and design of the experiments: J.M., P.P. and E.C. Collection, analysis and interpretation of data: J.M., S.G., S.D. and D.S.-C. Drafting the article or revising it

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