# Sleep alterations in the course of aging environmental inputs

Panagiotou, M.

# Part 1

## Young data

# Chapter 2

## High-caloric diet and sleep homeostasis in mice

Chronic high-caloric diet modifies sleep homeostasis in mice

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## Abstract

Obesity prevalence and sleep habit changes are commonplace nowadays, due to modern lifestyle. A bidirectional relationship likely exists between sleep quality and metabolic disruptions, which could impact quality of life. In our study, we investigated the effects of a chronic high-caloric diet on sleep architecture and sleep regulation in mice. We studied the effect of 3 months high-caloric diet (HCD, 45 % fat) on sleep and the sleep electroencephalogram (EEG) in C57BL/6J mice during 24-hr baseline (BL) recordings, and after 6-hr sleep deprivation (SD). We examined the effect of HCD on sleep homeostasis, by performing parameter estimation analysis and simulations of the sleep homeostatic Process S, a measure of sleep pressure, which is reflected in the non-rapid-eye-movement (NREM) sleep slow-wave-activity (SWA, EEG power density between 0.5 and 4.0 Hz). Compared to controls (n = 11, 30.7 $\pm$ 0.8 g), mice fed with HCD (n = 9, 47.6 $\pm$ 0.8 g) showed an increased likelihood of consecutive NREM-REM sleep cycles, increased REM sleep and decreased NREM sleep EEG SWA. After SD, these effects were more pronounced. The simulation resulted in a close fit between the time course of SWA and Process S in both groups. HCD fed mice had a slower time constant (Ti = 15.98 hr) for the increase in homeostatic sleep pressure compared with controls (5.95 hr) indicating a reduced effect of waking on the increase in sleep pressure. Our results suggest that chronic HCD consumption impacts sleep regulation.

**Keywords:** EEG, SWA, sleep regulation, sleep deprivation

## Introduction

Modern society is characterized by an increase in obesity prevalence, and concurrently by alterations in sleep habits. A negative relationship between sleep duration and quality, and risk for metabolic disruptions and obesity has been established [1, 2, 3, 4, 5], verifying the role of sleep in the regulation of endocrine functions, glucose metabolism and energy homeostasis.

However, the relationship between sleep, high-caloric diet (HCD) and obesity is likely bidirectional. Obesity is associated with daytime sleepiness and has been characterized as a significant risk factor for sleep disturbances, independent of sleep disorder breathing and age [6]. As obesity is also associated with a widerange of chronic diseases [7, 8], multiple animal models have been developed to study the interaction between sleep and excess body weight.

The majority of these animal models consisted of gene knockout animals, like ubiquitin knockout mice (Ubb-/-) and narcoleptic mice [9, 10, 11, 12] spontaneous mutations in genes like leptin-deficient (ob/ob) or Db/db mice and Obese Zucker rats [13, 14, 15, 16], polygenic models like the OP/OR rat model [17]. Only a rather small amount of studies was conducted on diet-induced obese mice or rats [18, 19, 20, 21], a condition probably closer related to human obesity. Several characteristic changes in sleep noted in humans with obesity were found in these models, for example, increased sleep especially during the active phase, and changes in waking, NREM and REM sleep episode number and duration throughout the 24 hr [4].

In adult humans, the circadian distribution of sleep is monophasic, whereas in rodents, it is polyphasic. Despite this difference, the main homeostatic, circadian and neurochemical modulations of sleep remain essentially similar among species [22]. We consider sleep to be regulated by two main processes, a circadian process governed by the internal biological clock, and a sleep homeostatic process which is dependent on prior waking and sleep [23, 24]. In mammals, the homeostatic sleep process is thought to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA), which is the EEG power density between 0.75 and 4.0 Hz [23, 24]. The dynamics of sleep regulation have been successfully simulated with the use of the two-process model, for the human monophasic sleep-wake pattern [25], as well as for the rodents' polyphasic one [26, 27, 28, 29, 30].

In the aforementioned diet-induced obese models, the regulation of sleep has not been studied. By conducting sleep deprivation, the system is put under elevated homeostatic sleep pressure to better assess the regulation of sleep. This is strengthened by applying parameter estimation analysis and mathematical modeling of the observed homeostatic sleep response visible in the EEG SWA, which is directly associated with the homeostatic sleep process.

We, therefore, investigated the effect of chronic HCD exposure (12 weeks) on baseline (BL) sleep architecture and electroencephalogram (EEG) parameters, as well as after 6 hr of sleep deprivation in C57BL/6J mice. In addition, we performed parameter estimation analysis and simulation of the sleep homeostatic Process S, during the 48-hr recording period, in order to test whether the time course of SWA can be predicted on the basis of temporal organization of sleep. We found altered sleep patterns in mice fed with chronic HCD compared with controls, reflected mainly in increased REM sleep, changes

in absolute SWA and a modulated response to SD. The differences in the dynamics of the SWA levels were consistent with changes found in the estimated time constants of Process S, illustrating first the bidirectional relationship between obesity and sleep, and second, a slower build-up of sleep pressure induced by HCD, leading to an altered sleep homeostasis.

## Materials and Methods

### Animals

Young male C57BL/6JOlaHsd mice (6 months old; n = 20) (Harlan, Horst, the Netherlands) were used for this study. The C57BL/6 mouse strain is known to be vulnerable to altered dietary patterns, for example high-fat diet, where it induces obesity, hyperglycemia, hyperinsulinemia [31, 32], rendering it an appropriate model to study the effects of diet, obesity and metabolic syndrome. At the age of 3 months, mice were partitioned into two groups: the control group, in which mice were fed with normal chow (11% fat, 27% protein, 61% carbohydrate, Special Diet Services, UK) and the HCD fed group, in which mice were fed exclusively with high-caloric food (45% fat mainly derived from lard, 35% carbohydrate, 20% protein; D12451, Research Diet Services, The Netherlands) for 12 weeks. By providing mice with a chronic HCD diet, we attempted to simulate the human condition as closely as possible, including anatomical (weight gain and body composition) and physiological changes in endocrinology and metabolism, which are reported in the literature [31, 32]. The well-being of all mice was controlled for potential side effects of the diet, and it was ascertained that the animals did not develop any movement problems. The mice were individually housed under controlled conditions (12:12-hr light:dark cycle; lights on at 10:00) with food and water *ad libitum* in a temperature controlled room ($\sim 23°C$).
All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

### Surgeries

On the day of the surgery, control animals (n = 11) weighed on average 30.7 g ($\pm$ 0.8), whereas HCD fed animals (n = 9) weighed 47.6 g ($\pm$ 0.8), showing a 65% increase in body weight compared with controls ($p < 0.0001$). Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; and Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One, Roanoke, VA, USA) were implanted as described previously [30, 33]. The wire branches of all electrodes were set in a plastic pedestal fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

### EEG and EMG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [30, 33, 34]. Before each recording, a calibration signal (10 Hz sine wave 300 $\mu$V peak-to-peak) was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for consecutive 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz.

### Data analysis and statistics

To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup, where conditions were similar to the home cage. Before starting the experiment, animals were allowed to adjust to the experimental conditions for a week. Subsequently, a BL day was recorded, starting at lights on. At the start of the second day, six hours of SD, were conducted by gentle handling, which is a mild intervention in order to induce elevated sleep pressure conditions [27, 30, 33]. EEG and EMG were recorded continuously during SD and, subsequently, for 18 hours to investigate sleep characteristics after SD.
Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of EEG and EMG signals as described previously [27, 30, 33, 35]. For each epoch, the EEG power density in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. “Waking” was identified by high EMG and low EEG amplitude as well as high theta activity concomitant with highest EMG values, “NREM sleep” by low EMG and high EEG amplitude as well as high delta activity, and “REM sleep” by low EMG and low EEG amplitude as well as high theta activity. The scores for each 4-s epoch were entered into the PC via the keyboard. Epochs in which the vigilance state could not be identified were excluded and epochs that contained EEG artifacts were marked and excluded from spectral analysis. Subsequently, the vigilance states were expressed as a percentage of artifact-free recording time. The number and duration of the episodes for each state were computed according to the scoring results. Light and dark (L1, D1, L2, D2 for light and dark periods of the first, BL, and second day respectively, after SD) mean values of vigilance states, state episode frequency and duration, transition probabilities and probabilities of repeated NREM-REM sleep cycles' (NRc) were analyzed by three-way repeated measures analysis-of-variance (rANOVA) with main factors ‘treatment’ (between-subject factor), ‘day’ and ‘Light-Dark’ (within-subject factors) to test the effect of SD, and, consequently, by two-tailed unpaired t-tests for each period to determine the effect of treatment. Note that L2 corresponds to the 6-h after SD and is compared to the corresponding 6-h of the BL. An SD effect was considered when an interaction or the factor ‘day’ was significant. The factor‘treatment’ generally refers to the effect of diet. 24-h mean values of vigilance states were analyzed by two-tailed unpaired t-tests. To test the effect of treatment and SD, 2-h values of vigilance states were analyzed by three-way repeated measures ANOVA with main factors

'treatment' (between-subject factor), 'day' and 'time of day' (within-subject factors). To compare NREM sleep EEG power density between the groups across three frequency bins (0.5-4 Hz, 6-9 Hz and 15-25 Hz) two-way ANOVA was performed (main factors 'treatment' and 'frequency'). To test the effect of SD, three-way ANOVA (a repeated measures experimental design was not used due to missing values in both groups across different timepoints) was performed (main factors 'treatment', 'day' and 'time of day') for each frequency bin. Regarding the simulation, two-way repeated measures ANOVA was performed (main factors 'time of day' and 'simulation') for the two experimental groups. The time constants (Ti, Td) and the initial value (iV) were tested with two-tailed unpaired t-tests to determine the effect of treatment. When appropriate (if interaction or main factor effects were significant), post hoc two-tailed paired and unpaired student's t-tests with Bonferroni correction for multiple comparisons were applied to determine the effects of SD or treatment. Correlation coefficient r-values were averaged after Fisher-Z transformation.

**Transition probabilities**

Transition probabilities were calculated on the basis of frequencies of vigilance state episodes, counted for each mouse individually (Table 1), as it was described previously [36]. Transition probabilities (p) were calculated from the following formulas (#W, #N, #R for the number of Waking, NREM and REM sleep episodes respectively and $p_{W\Rightarrow N}, p_{N\Rightarrow R}, p_{N\Rightarrow W}, p_{R\Rightarrow W}, p_{R\Rightarrow N}$ the transition probabilities between Waking (W), NREM (N) and REM (R) sleep states). It is considered that each waking episode is followed by a NREM sleep episode, rendering this transition probability 100% and that, since REM sleep is preceded only by NREM sleep, the probability for REM sleep to occur is equal to the number of REM sleep episodes (#R) divided by the number of NREM sleep episodes (#N):

$$p_{W\Rightarrow N} = 100\%(1)$$
$$p_{N\Rightarrow R} = \#R/\#N(2)$$
$$p_{N\Rightarrow W} = 1 - \#R/\#N(3)$$
$$p_{R\Rightarrow W} = (\#W - \#N * p_{N\Rightarrow W})/\#R(4)$$
$$p_{R\Rightarrow N} = 1 - p_{R\Rightarrow W}(5)$$

**Simulation**

We simulated the time course of Process S iteratively on the basis of the vigilance states, as described previously [26, 27, 28, 29, 30]. In the 4-s epochs scored as waking or REM sleep, S increases as a saturating exponential function with an upper asymptote of 1, while in NREM sleep S decreases as an exponential function with a lower asymptote of 0. Time constants for the decrease (Td) and increase (Ti) were determined for each mouse separately, on the basis of vigilance states of the 24-h BL period, 6-h SD and 18-h recovery period. S was computed according to:

increasing function:

$$S_{t+1} = 1 - (1 - S_t) \cdot e^{-\Delta t/Ti} (1)$$

decreasing function:

$$S_{t+1} = S_t \cdot e^{-\Delta t/Td} (2)$$

where t=4-s intervals, $\Delta t = 4 - s, S_t$ and $S_{t+1}$ values of S for consecutive epochs, and Ti and Td the time constants of the increase and decrease rate of S, respectively. The time constants Ti and Td and the initial value of S (iV=S0) were estimated by optimizing the linear correlation between the hourly values of SWA in NREM sleep and S, in BL and recovery for each animal separately. To test whether the estimated parameters could predict the time course of SWA, a simulation was performed over the entire data set consisting of BL, 6-h SD which immediately followed the BL day, and recovery. The optimized parameters of each individual animal were applied to obtain average curves of process S. To enable the comparison between SWA and S, SWA was linearly transformed according to a linear regression based on the 1-h values.

## Results

### Vigilance states

During undisturbed BL, mice fed HCD showed increased REM sleep during the light period, as compared to control mice, whereas no differences were found in the dark period and only a trend was found in the 24-h values (Fig.1) [REM sleep, post-hoc unpaired t-test for L1, (t,df)=(2.46,72); p=0.016 following significant factor 'treatment' and 24-h value: (t,df)=(1.97,18); p=0.065] (Table 2). No further alterations were detected between the groups in the 12h or 24h NREM sleep and waking values (p>0.05). Compared to BL, decreased waking and increased REM sleep were apparent during the 12-h dark period after SD in the control mice (Fig. 1) [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). In HCD fed mice, decreased waking, as well as increased NREM sleep in both light and dark periods and increased REM sleep in the 12-h dark period were found [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). Differences in the 2-h values of the continuous recordings were mainly evident in REM sleep, where after SD they were more pronounced between the groups, and, additionally, in both groups an effect of SD was apparent on the 2-h values (Fig. 2) [post-hoc two-tailed unpaired t-tests between groups following significant interaction as well as factor 'treatment' and paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2).

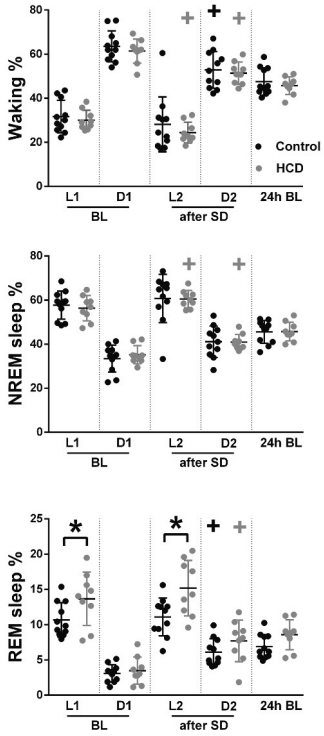


Figure 2.1: Light-dark distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline day (BL: L1 and D1) and after sleep deprivation (after SD: L2 and D2). Scatter plots represent mean (± SD) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for control (n=11, black) and high-caloric diet fed mice (HCD, n=9, gray). Asterisks indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day') and black and gray plus symbols indicate significant differences between recovery and BL day for control and HCD fed mice respectively (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

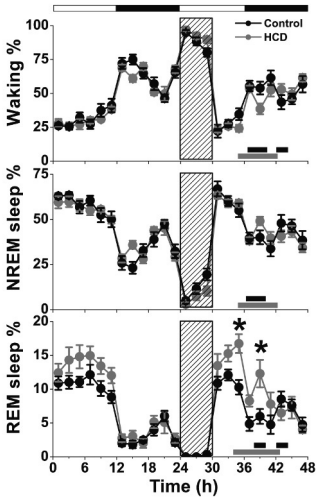


Figure 2.2: Time course of vigilance states, for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for the two groups, control (black circles, n=11) and high-caloric diet (HCD) treated mice (gray circles, n=9). Curves connect 2-h values of Waking, NREM and REM sleep. The black and white bars above each graph indicate the light-dark cycle. Black asterisks at the top of each graph represent significant differences between the groups and black (control) and gray (HCD) bars at the bottom of each graph significant differences between recovery and BL day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'time of day', 'day').

### Episode duration and frequency

We found that diet-induced obesity influenced the 12-h average light and dark NREM and REM sleep episode duration and frequency (Fig. 3). In HCD fed mice, REM sleep episodes were more frequent in both light periods, and they lasted significantly shorter in both dark periods [Frequency, post-hoc unpaired t-test following significant factor 'treatment' for L1: (t,df)=(2.8,72); p=0.0067, L2: (t,df)=(3.8,72); p=0.0003, D2: (t,df)=(2.4,72); p=0.02, Duration, D1: (t,df)=(2.13,72); p=0.037 D2: (t,df)=(2,72); p=0.0485] (Table 2). Under HCD, NREM sleep episodes lasted significantly longer in the BL dark period compared to control [Duration, post-hoc unpaired t-test following significant interaction, D1: (t,df)=(2.43,72); p=0.0176] (Table 2). After SD, HCD fed mice had more NREM sleep episodes in the light period compared to controls [Frequency, post-hoc unpaired t-test following significant factors 'day' and 'Light-Dark' L2: (t,df)=(3.2,72); p=0.002] (Table 2). Compared to BL, SD decreased REM sleep episode frequency in the light period and increased REM sleep episode frequency in the dark period in control animals and increased NREM and REM sleep episode frequency in the dark period in HCD fed animals [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). Additionally, SD shortened waking and NREM sleep episode duration during the dark period and lengthened REM sleep

Table 2.1: Mean numbers (± SEM) of state episodes are shown for Waking, NREM and REM sleep, counted for light and dark periods (L1, D1, D2 for 12-h periods, and L2 for the 6-h recovery period after sleep deprivation) during the 48-h period of recordings for control and high-caloric diet (HCD) fed mice. Asterisks indicate significant differences between groups (unpaired t-tests for each period, $p<0.05$).

| Table 1: Number of vigilance state episodes (per h) | | | |
|---|---|---|---|
| Control mice | Waking | NREM sleep | REM sleep |
| L1 | 20 (1.8) | 24 (1.1) | 10 (1.1) |
| D1 | 16.1 (1.8) | 17.5 (1.8) | 3.3 (0.5) |
| L2 | 16.9 (2.5) | 20.8 (2.2) | 8.5 (0.8) |
| D2 | 17.7 (1.2) | 20.1 (1.1) | 5.7 (0.7) |
| HCD mice | | | |
| L1 | 19 (2.5) | 26.4 (1.8) | 15.2 (2.3)* |
| D1 | 13.3 (2.2) | 15.3 (1.8) | 4.4 (0.8) |
| L2 | 13.3 (1) | 21 (1.5) | 14.1 (1.9)* |
| D2 | 17.6 (2.4) | 21.8 (1.9) | 9.4 (1.4)* |

episode duration during the light period in HCD fed mice [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors ‘day’ and ‘Light-Dark’] (Table 2). The state transition probability analysis showed that, in the HCD treated mice, a NREM sleep episode was more likely to be followed by a REM sleep episode and less likely to be followed by a waking episode, particularly after SD (Fig. 4) [post-hoc unpaired t-test following significant factors ‘treatment’,‘day’ and ‘Light-Dark’ for L2: (t,df)=(3,72); p=0.003, D2: (t,df)=(2.28,72); p=0.025] (Table 2). Moreover, more sleep episodes were terminated after one NREM-REM sleep cycle (NRc) in the control mice compared to the HCD fed mice (L1: 82% vs. 73%, D1: 92% vs. 84%, L2: 79% vs. 66% and D2: 88% vs. 79%) [main effects ‘day’ and ‘Light-Dark’] (Table 2). Consequently, the probability that a NRc was followed by a second NRc was approximately two times larger in the HCD fed mice compared to controls (L1: 18% vs. 27%, D1: 8% vs. 16%, L2: 21% vs. 34% and D2: 12% vs. 22%) (Fig. S1). Together the data suggest that sleep consolidation was higher in HCD treated mice.

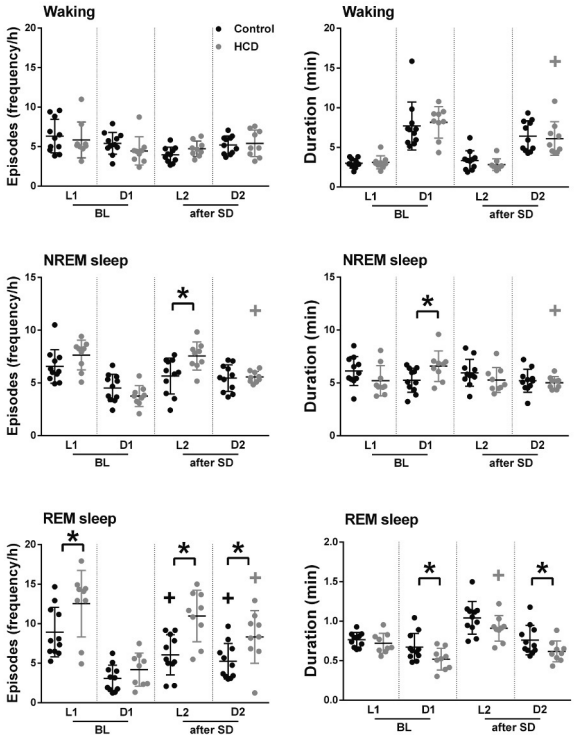


Figure 2.3: Light-dark distribution of episode frequency (frequency/hour, left) and duration (minutes, right) of each behavioral state (Waking, NREM and REM sleep) during the baseline day (BL: L1 and D1) and after sleep deprivation (after SD: L2 and D2). Scatter plots represent mean ($\pm$ SD) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) for Waking, NREM and REM sleep for control (n=11, black) and high-caloric diet fed mice (HCD, n=9, gray). Asterisks indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day'), and black and gray plus symbols indicate significant differences between recovery and BL day for control and HCD fed mice respectively (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

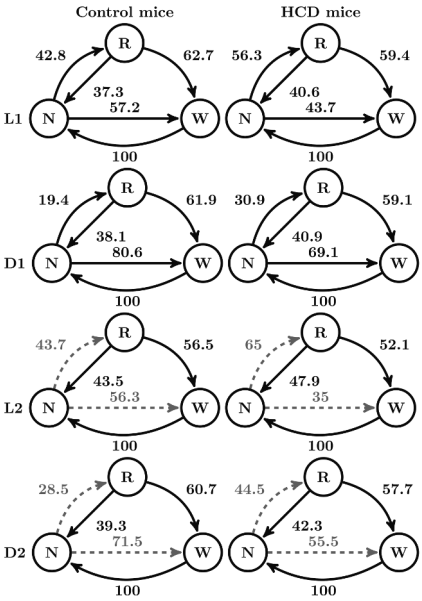


Figure 2.4: State transition probability analysis of the number of vigilance state episode transitions (W = waking, N = NREM sleep, R = REM sleep) for control mice (n=11) and high-caloric diet fed mice (HCD, n=9) (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively). The probability of each state in percent (%) to enter the next state was calculated on the basis of episodes (averages per h shown in Table 1) and is denoted by arrows (see Methods for details). Gray dashed arrows indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

### Absolute EEG power density (0.5-25 Hz)

Regarding the spectral analysis of the EEG, differences in the NREM sleep EEG power density between the groups were found across frequency bins (0.5-4 Hz, 6-9 Hz and 15-25 Hz) [Two-way ANOVA, interaction factors 'treatment*frequency'; $F(2,711)=3.7$, $p=0.025$ with main factors 'treatment' $p=0.057$ and 'frequency' $p<0.0001$], which were specific for the SWA range (0.5-4.0 Hz), whereas no differences between the groups were found in the absolute EEG power density levels of 6-9 Hz or 15-25 Hz [post-hoc one-way ANOVA, factor 'treatment'; $F(1,215)=10.4$, $p=0.001$, for the SWA only] (Fig. 5). HCD fed mice had overall significantly lower absolute SWA levels (Fig. 5, top panel). In the first hours after SD, SWA was significantly increased above BL levels in both groups [post-hoc two-tailed paired t-tests between BL and after SD time points following significant factor 'day'] (Table 2). Compared to BL, HCD treated mice showed lower SWA values in the dark period of the recovery day displaying a negative rebound, similar to the negative rebound found previously in the rat [26]. No differences between groups were found in the other frequencies. In both groups, SD induced alterations compared to BL in the theta but not the faster frequencies [post-hoc two-tailed paired t-tests between

BL and after SD time following significant factor 'day'] (Table 2). The absence of differences between the groups in the absolute EEG power density levels of 6-9 Hz or 15-25 Hz (Fig. 5, middle and bottom panels), showed that the effect on SWA was specific for this frequency range and not caused by a general decrease in EEG power density in the HCD fed mice.

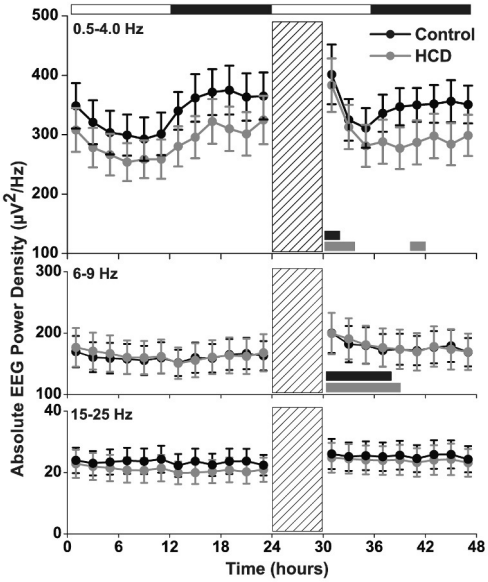


Figure 2.5: Time course of absolute electroencephalographic (EEG) power density ($\mu V^2/Hz$) for the slow-wave activity range (SWA, 0.5-4 Hz) in non-rapid-eye movement (NREM) sleep (upper graph), for 6-9 Hz (middle graph) and 15-25 Hz (lower graph) for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for control (black circles, n=11) and high-caloric diet fed (HCD) mice (gray circles, n=9). Black (control) and gray (HCD) bars at the bottom of each graph indicate significant differences between recovery and BL day (post-hoc paired t-tests with Bonferroni multiple comparisons correction p<0.05, after significant ANOVA main factors 'treatment', 'time of day', 'day'). Overall non-specific decreased EEG SWA levels were revealed in the HCD fed mice compared to control mice, only for the SWA range in BL day (one-way ANOVA, factor 'treatment'; p<0.05).

### Simulation of Process S

The differences in sleep-wake distribution and the lower SWA (Fig.1 and Fig.5) suggest that homeostatic sleep pressure may be lower in HCD treated mice. To investigate this further, we simulated the time course of Process S in both groups. The increasing time constant was found to be significantly higher in the HCD fed mice compared to control, whereas no difference was found in the decreasing time constant [HCD mice: Ti=15.98$\pm$0.64, Control mice: Ti=5.95$\pm$0.21; (t,df)=(16.1966,18); p<0.0001, HCD mice: Td=6.22$\pm$0.84 and Control mice: Td=6.0$\pm$0.41; (t,df)=(0.25,18); p=0.81].

The initial value was found to be significantly lower in the HCD fed mice [HCD mice: iV=0.42±0.02, Control mice: iV=0.65±0.02; (t,df)=(9.549,18); p<0.0001]. The simulation of Process S was applied to the 24-h BL, 6-h SD and 18-h recovery, taking into account the parameters estimated for the two consecutive days of recordings of the individuals, and resulted in a close fit between SWA and process S for both groups (Fig. 6) [Control mice: r=0.733, HCD mice: r=0.775]. Discrepancies between the simulation and SWA were found in the light period and after SD for both the HCD fed as well as control mice, similar to an earlier study [Two-way rANOVA, interaction factors 'simulation*time of day', HCD mice: F(41,330)=7.657; p<0.0001 with main factors 'simulation' p=0.919 and 'time of day' p=0.049, Control mice: F(41,407)=7.994; p<0.0001 with main factors 'simulation' p=0.931 and 'time of day' p<0.0001] [27]. The larger increasing time constant in the HCD treated mice indicates that homeostatic sleep pressure increases slower in these animals.

Table 2.2: Elaborate statistical analysis: three-way repeated measures analysis of variance (rANOVA) or ordinary ANOVA was conducted to test the effect of sleep deprivation and high-caloric diet (treatment) (see text for more details). Asterisks following reported p values indicate significance (* p<0.05).

| Table 2: Detailed statistics | | | |
|---|---|---|---|
| Figure | Vigilance state | 3-way ANOVA (Interaction factors) | Main factors |
| 1 | Waking | 'treatment*day*Light-Dark': F(1,18)=0.365 p=0.553 | 'treatment' p=0.344 'day' p<0.0001 * 'Light-Dark' p<0.0001 * |
| | NREM sleep | 'treatment*day*Light-Dark': F(1,18)=0.511 p=0.484 | 'treatment' p=0.985 'day' p<0.0001 * 'Light-Dark' p=0.004 * |
| | REM sleep | 'treatment*day*Light-Dark': F(1,18)=0.008 p=0.93 | 'treatment' p=0.02 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 * |
| 2 | Waking | 'treatment*day*time of day': F(11,198)=1.704 p=0.075 | 'treatment' p=0.525 'day' p<0.0001 * 'time of day' p<0.0001 * |
| | NREM sleep | 'treatment*day*time of day': F(11,198)=1.236 p=0.265 | 'treatment' p=0.672 'day' p<0.0001 * 'time of day' p<0.0001 * |
| | REM sleep | 'treatment*day*time of day': F(11,198)=3.191 p=0.001 * | 'treatment' p=0.032 * 'day' p<0.0001 * 'time of day' p=0.001 * |
| 3 [episode frequency] | Waking | 'treatment*day*Light-Dark': F(1,18)=0.007 p=0.936 | 'treatment' p=0.832 'day' p=0.635 'Light-Dark' p=0.042 * |
| | NREM sleep | 'treatment*day*Light-Dark': F(1,18)=0.004 p=0.951 | 'treatment' p=0.22 'day' p<0.0001 * 'Light-Dark' p=0.033 * |
| | REM sleep | 'treatment*day*Light-Dark': F(1,18)=0.265 p=0.613 | 'treatment' p=0.012 * 'day' p<0.0001 * 'Light-Dark' p=0.063 |
| [episode duration] | Waking | 'treatment*day*Light-Dark': F(1,18)=0.01 p=0.922 | 'treatment' p=0.91 'day' p<0.0001 * 'Light-Dark' p=0.017 * |
| | NREM sleep | 'treatment*day*Light-Dark': F(1,18)=7.782 p=0.012 * | 'treatment' p=0.751 'day' p=0.658 'Light-Dark' p=0.143 |
| | REM sleep | 'treatment*day*Light-Dark': F(1,18)=1.03 p=0.324 | 'treatment' p=0.021 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 * |

| | | | |
|---|---|---|---|
| 4 | Transition probability NREM-REM sleep | 'treatment*day*Light-Dark': F(1,18)=0.312 p=0.583 | 'treatment' p=0.018 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 * |
| 5 | Slow-wave activity (SWA) in NREM sleep | 'treatment*day*time of day' : F(11,411)=0.093 p=0.99 | 'treatment' p<0.0001 * 'day' p=0.028 * 'time of day' p=0.54 |
| | 6-9 Hz in NREM sleep | 'treatment*day*time of day' : F(11,418)=0.071 p>0.99 | 'treatment' p=0.902 'day' p=0.032 * 'time of day' p=0.995 |
| | 15-25 Hz in NREM sleep | 'treatment*day*time of day' : F(11,411)=0.032 p>0.99 | 'treatment' p=0.223 'day' p=0.093 'time of day' p>0.99 |
| S1 | Probability of repeated NREM-REM sleep cycles | 'treatment*day*Light-Dark': F(1,18)=0.15 p=0.703 | 'treatment' p=0.149 'day' p<0.0001 * 'Light-Dark' p=0.006 * |

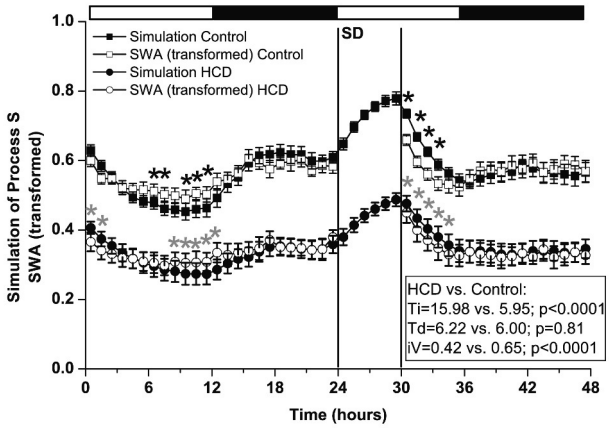


Figure 2.6: Time course of electroencephalographic slow-wave activity (EEG SWA, EEG power density in the range of 0.5-4.0 Hz, linearly transformed according to a linear regression based on the 1-h values) and simulation with the optimized time constants for the increase (Ti), decrease (Td) and initial value (iV) of Process S for the control mice (white and black squares, n=11) and the high-caloric diet fed (HCD) mice (white and black circles, n=9). Curves connect 1-h mean values ($\pm$ SEM) for 24-h baseline (BL), 6-h sleep deprivation (SD) and 18-h recovery. Black and gray asterisks indicate differences between simulation and SWA data for the control and the HCD fed group respectively (paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, factors 'simulation', 'time of day'). The optimized mean values of Ti, Td and iV for each condition are noted on the graph along with the p levels (unpaired t-tests, significance when $p<0.05$).

## Discussion

In this study, we recorded sleep and the sleep EEG during undisturbed 24-h BL in control mice and mice fed on HCD for three months, and applied a 6-h SD to investigate potential differences in sleep regulation under influence of elevated sleep pressure. We found moderate sleep architecture alterations denoted by an increased REM sleep amount. The results of the sleep episode frequency and duration suggest an increase in sleep consolidation in HCD fed mice. HCD treated mice revealed overall lower absolute SWA levels during NREM sleep, as well as a significant slower increasing time constant for the homeostatic sleep process, compared to control mice. A more pronounced effect on sleep architecture was found after SD, characterized mainly by an increased probability to enter REM sleep from NREM sleep in HCD fed mice, resulting in increased total REM sleep amount compared to control mice, and a negative rebound in SWA level in the dark period, as compared to BL. These changes in SWA were corroborated by changes in the levels of the modeling of the putative average level of the homeostatic sleep process and a slower increase rate in sleep pressure. We conclude that chronic HCD modulates the sleep architecture, although without modifying the daily amplitude of the vigilance state rhythms, and alters sleep homeostasis.

### Sleep architecture

Moderate effects of the HCD on sleep architecture were revealed by our results in the BL dark period. Earlier studies showed an increase in the 12-h value of NREM sleep and a decrease in waking during the dark period [19, 20]. These effects were not apparent in our study, however, during the BL dark period, we found longer NREM sleep episodes and shorter REM sleep episodes. Since earlier studies exposed mice to a HCD for a shorter period (usually 8-weeks), a potential explanation for the absence of findings in the amount of waking and NREM sleep in our study could be the longer-term administration of HCD possibly allowing mice to have additional time to adapt to the diet. A clear finding was the increase in REM sleep that was apparent in the HCD fed mice, particularly in the light period compared to control mice, originating from an increase in REM sleep episode frequency, similar to previous studies on HCD in rodents [18, 19, 20, 21]. We conducted further analysis on the number of episodes and showed that an episode of NREM sleep was followed more often by a REM sleep episode in the HCD treated mice, mainly shown after SD, similar to a study in obese compared to lean Zucker rats [14]. Additionally, HCD treated mice revealed an increased probability of an NREM-REM sleep cycle to be followed by a second NREM-REM sleep cycle. Overall these data suggest that sleep consolidation is greater in HCD fed mice compared to controls. The concomitant absence of sleep fragmentation in our data, confirms earlier research in diet-induced obese rodents [18, 19, 20, 37].

The increased transitions into REM sleep can be caused by several different mechanisms. The preoptic area in the hypothalamus is involved in basal metabolism, and has also been associated with REM sleep regulation [38] and thermoregulation [39]. Thermoregulatory control is suspended during REM sleep [40] which is the reason why forced thermoregulatory activation, for instance cold exposure, depresses REM sleep [40, 41]. In contrast, sleeping in thermo-neutral conditions, where active thermoregulation is not necessary, increases REM sleep [42]. Similarly, Djungarian hamsters that reduce body temperature, when exposed to short photoperiods to save energy with reduced thermoregulation, show an increase in NREM-REM sleep transitions when kept at the same ambient temperature, as demonstrated in the present study [36]. In these hamsters, there was a clear relationship between the NREM-REM transition probability and the set point of body temperature. In accordance with this, it was shown that the metabolic efficiency index, an indirect measure for metabolic rate, was lower in high-fat diet-fed mice compared with normal diet-fed mice [43]. Considering the body composition changes in HCD fed mice, which gained weight and have a smaller surface-to-content ratio compared to control mice, it is likely that these mice will cool down slower and will probably show less thermoregulatory activity. This is in congruence with a reduced metabolic rate and an increase in REM sleep episode frequency. Therefore, a mere change in body size and thermoregulatory activity may explain the difference in REM sleep patterns observed.

However, changes in neurotransmitter release, under influence of HCD, may also alter sleep. Obesity has been associated with decreased levels of orexin in humans and mice [44, 45, 46, 47]. Orexin is predominantly found in posterior lateral hypothalamus and is a critical regulator of vigilance states, energy homeostasis, as well as the reward system and feeding [12, 48]. In narcolepsy, which is caused by the loss of neurons that contain orexins or orexin receptors, massive increases in REM sleep are found [49]. Thus,

changes in orexin levels in HCD fed mice may have also influenced sleep architecture in this group. Disentangling the different mechanisms affecting REM sleep in HCD induced obese models may prove to be difficult, as the diet is likely to produce several of these changes in parallel, which may all promote the increase in REM sleep.
We found a remarkably intact day-night modulation of sleep and waking. In other words, the daily amplitude of the vigilance state rhythms did not differ between the two groups, owing to the modest effects of HCD on sleep architecture. HCD is known to disturb circadian organization, particularly in the peripheral clocks, whereas functioning of the central clock seems to remain intact [50]. The present data show only minor changes in the distribution of sleep. This suggests that the day-night distribution of sleep and wakefulness is mainly determined by the central clock in the suprachiasmatic nucleus and only mildly influenced by changes in peripheral clocks.

**NREM sleep EEG SWA: data and simulations**

An overall decrease in absolute EEG SWA during NREM sleep was revealed in the HCD fed mice compared to control. This decrease was specific for the slow wave range, as other frequency ranges (theta, fast frequency activity) were not affected. Additionally, the absolute SWA levels in the HCD fed mice showed a negative rebound in the dark period after SD, similar to a previous study in rats [26], revealing an altered response to SD. A study in rats showed that increased sleep induced by high-fat/high-salt diet (i.e. cafeteria food) was associated with decreased EEG SWA during several days of cafeteria food [37], similar to our findings. However, studies in C57BL/6 mice found no alterations in EEG SWA compared to control across a 6 or 10-week exposure to HCD experiment [19, 20].
Previous findings of the effect of HCD on sleep and the sleep EEG were not always consistent. In our data, we found lower SWA and increased REM sleep that indicate lower homeostatic sleep pressure. In order to elucidate these findings, we further analyzed the effect of HCD on the regulation of sleep by modeling the homeostatic sleep response. We found a significantly higher increasing time constant and a lower initial value of S in the HCD fed mice. The time constants obtained for the two groups could predict the effect of SD, with similar discrepancies between the two groups, consistent with the study of Huber et al. (2000). The discrepancies, as previously indicated, may emerge from the mathematical limitations of the model adjusted for mice data [27]. Importantly, the time constants of the control mice were similar to previous estimations in the C57BL/6J mouse strain [27]. However, the significantly higher increasing time constant in the HCD fed mice (Ti= 15.98h) suggests that the build-up of sleep pressure during waking and REM sleep is slower in these mice. On the contrary, the decrease rate obtained from our data, is similar between the groups, resembling previous reported decrease rates in mice [27], and remains unaffected by the HCD. The decreasing time constant is unaltered among different species, as indicated in mice, rats and humans [27], and it seems that it likely obtains more rigid properties. Therefore a condition such as obesity is probably not expected to alter it significantly. Thus, sleep homeostasis seems to be influenced by HCD, rendering these mice less susceptible to prolonged waking.
The mechanisms underlying HCD influence on the occurrence of SWA or sleep homeostasis remain elusive. High-fat feeding and food restriction are thought to enhance ke-

togenesis [51]. Ketone bodies are generated from the breakdown of fatty acids and have been shown to become major fuels in most tissues during starvation, prolonged exercise, or consumption of a high-fat, low-carbohydrate diet [52]. Ketogenic diet has been applied as a treatment for epilepsy, autism and brain tumours, and it has been shown to induce sleep alterations [51] possibly by a shift of the excitatory/inhibitory (E/I) balance in the cortex to a more inhibitory state [53], which is consistent with the lower SWA found in our analysis [54]. Although less extreme than a pure ketogenic diet, HCD may have an effect on sleep homeostasis through the fatty acids metabolic pathway. Additionally, changes in melanin concentrating hormone (MCH), an appetite-stimulating peptide expressed in the lateral hypothalamus, shown to have an increased expression in adult offspring after prenatal HCD exposure [55], may also mediate SWA and/or sleep homeostasis alterations, since MCH neurons were proposed to be implicated in sleep-wake regulation [56]. In conclusion, multiple pathways are likely to be involved in the case of obesity considering that sleep is governed by complex brain networks. Further research is, hence, needed to elucidate the mechanisms underlying the HCD effects on sleep homeostasis.

### Concluding Remarks

In the current study we show that, although sleep architecture is not strongly affected after chronic HCD, sleep homeostasis and the response to SD is altered. Translated to humans, we could deduce that HCD reduces the effect of prolonged waking on subsequent EEG SWA in NREM sleep. Prolonged waking is known to increase craving for food, especially for high-fat and high-carbohydrate food [57]. This change in diet may have a similar effect on the build-up of sleepiness as caffeine [58], however not by influencing adenosinergic mechanism, but by a change in the E/I balance of neuronal activity in the cortex. Therefore, the increased craving for high-caloric food when sleep deprived may have short-term benefits by lowering the burden of the sleep debt incurred. Nevertheless, a vicious cycle is likely created, in which high caloric intake and prolonged waking strengthen each other, inducing an increase in weight with all the associated health risks. That way, HCD and reoccurring sleep debt are possibly synergistic entities, that in the long-term are detrimental for health and longevity.

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## Author Contributions Statement

T.DB. and J.H.M. designed research; M.P. and T.DB. performed research; M.P. and T.DB. analyzed data; and M.P., J.H.M., and T.DB. wrote the paper.

## Competing financial interests

The authors declare no conflict of interest.

## Data Accessibility Statement

The authors deposit supporting information and datafiles to Figshare for data archiving https://figshare.com/s/f2db8ab6be1ee1b9e875

## Abbreviations

BL, baseline; EEG, electroencephalogram; E/I, excitatory/inhibitory; EMG, electromyogram; HCD, high-caloric diet; NREM, non-rapid-eye-movement; SWA, slow-wave activity (EEG power density in NREM sleep between 0.75-4.0 Hz); SD, sleep deprivation.

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## Supplementary Material

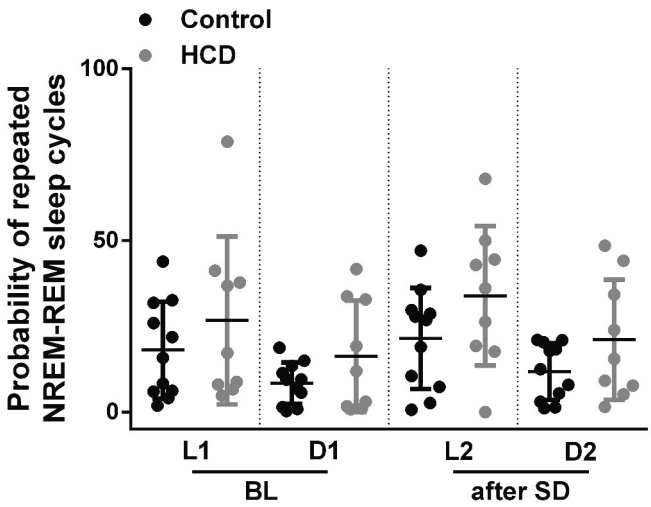


Figure 2.1: Probability of repeated NREM-REM sleep cycles (NRc) for control (n=11) and high-caloric diet fed mice (HCD, n=9) (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively). The probability that a NRc was followed by a second NRc was approximately two times larger in the HCD fed mice compared to controls (repeated measures ANOVA, main factors 'treatment' ($p=0.149$), 'Light-Dark', 'day' $p<0.05$).