The rhythm of feeding

Effect of nutrients on metabolism and the molecular clock

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General discussion

Most organisms on earth are subject to a daily exposure of sunlight due to rotation of the earth. In order to anticipate the upcoming day – or night, most organisms have evolved to acquire an internal clock. In mammals, this internal clockwork is the suprachiasmatic nucleus (SCN), located within the anterior hypothalamus (Aschoff, 1984; Hastings *et al.*, 2003), although all cells have their own endogenous molecular clock. The daily variation in light and dark coincides with the presence of predators and the availability of food. From an evolutionary point of view, it is advantageous to adapt the clock to nutrient status, so that activity is synchronized to periods in which food is available and hazards are the least. Therefore, nutrient intake – or energy status, should be closely linked to the internal clockwork. Indeed, whereas the central clock is entrained to light, in mammals peripheral clocks which have no access to light, are entrained mainly by the feeding-fasting cycle (Damiola, 2000; Hirota & Fukada, 2004). Next to this exogenous entrainment, peripheral clocks are entrained by signals from the SCN (Buijs & Kalsbeek, 2001; Buijs *et al.*, 2003). In an optimal situation, SCN- and metabolic signals reaching the peripheral clocks are in synchrony. However, in the contemporary 24/7 society, feeding moments are distributed over a great part of the day, not always respecting the exogenous light-dark cycle, which may contribute to currently pandemic diseases such as obesity and type 2 diabetes. An important hypothesis, taking into account these chronobiological principles, is that aberrantly timed feeding, out of sync with the endogenous biological clock, contributes to this pandemic (Zarrinpar *et al.*, 2016). Yet, the exact mechanisms of how this desynchrony impairs the metabolic profile is unknown. From a physiological perspective, setting the phase of peripheral clocks by food allows metabolic organs to prepare for food metabolism at the correct time. However, the entraining signal from the SCN cannot be entirely overruled by feeding signals, as approximately 14% of cycling transcripts in the liver are under central circadian control (Kornmann *et al.*, 2007; Vollmers *et al.*, 2009), eventually leading to conflicting signals reaching the peripheral liver clock.

Many studies focus on the effect of high-energetic diets on metabolism and the molecular clock. Even more studies have been published on the effects of timing of food intake on the molecular clock mechanism [reviewed in (Oosterman *et al.*, 2015)]. However, there are still gaps in our understanding on how nutrients can affect the clock.

The first outstanding question is whether food-entrained signals from ingested nutrients arise from the amount of circulating nutrients, *i.e.* energy content, or from the composition of the circulating nutrients, *i.e.* diet composition. No comparative studies have been published to date.

The next outstanding question, with regard to studies on diet timing [e.g. a high-fat diet during the inactive period leads to increased body weight compared to high-fat diet in the active period in mice (Arble *et al.*, 2009)], is whether macronutrient composition *per se* or macronutrient timing is most important for the observed changes in energy homeostasis. Although it has been proposed that the combined effect of eating palatable food at an ''inappropriate'' time of day could be more detrimental to a healthy energy

balance than the sum of each single factor (Arble & Sandoval, 2013), this had not been studied before.

Therefore, we assessed the effects of sugar and fatty acids (macronutrients abundantly available in the human diet), on the daily rhythmicity of hepatic clock genes and whole-body metabolism *in vivo* in male Wistar rats, and on circadian rhythmicity of clock genes *in vitro* in immortalized hypothalamic cells.

Thus, the **aim** of this thesis was twofold:

1. To assess the (differential) effects of energy content and nutrient content on the molecular clock mechanism.
2. To assess the combined effects of consuming palatable food on an inappropriate time of day, on whole-body metabolism and molecular clock rhythms.

We **hypothesized** that:

1. Both fat and sugar affect the clock in a direct manner, independent of caloric content.
2. There is a complementary effect of diet timing and composition on whole-body metabolism and peripheral clock gene expression.

First, in **chapter 2** we conducted a study in which we assessed a time-dependent effect of diet composition on energy homeostasis. Animals were randomized to either a chow *ad libitum* diet, or a high-fat high-sugar diet (HFHS). The HFHS diet was provided either with all components *ad libitum* (AL), with *ad libitum* access to chow, tap water and a 30% sugar solution, but with access to saturated fat only during the light period (LF), or with *ad libitum* access to chow, tap water and saturated fat, but access to a 30% sugar solution only during the light period (LS).

The most important finding from this study was that LS animals gained significantly more body weight and showed a higher food efficiency and altered substrate oxidation pattern compared to LF animals, despite similar caloric intake, heat production and locomotor activity. The daily pattern of food intake was similar between those groups, suggesting that the increased body weight gain was not due to a disturbed feeding pattern alone, but also depended on the timing of ingestion of the different nutrients. In other words, macronutrient composition is important for energy homeostasis, independent of caloric content. Although the results have to be interpreted with caution due to the complex study design, we proved for the first time that timing of diet composition can differentially affect body weight, and we show that this is most likely due to a shifted oxidation pattern, which can predispose for obesity. The notion of decreased energy expenditure in light-fed animals is not novel (Reznick *et al.*, 2013), but the differential effects of diet composition on energy expenditure were not described before. Indeed, we suggest that drinking sugar during the light period is more disruptive for normal daily rhythms in substrate oxidation than eating fat intake during the light period, at least in Wistar rats. However, the nocturnal

food composition was also different between the LF and LS group. Therefore, we cannot exclude that these nocturnal differences may have contributed to our findings. Indeed, it was shown in mice that a high-fat diet resulted in alterations in the behavioral and metabolic circadian rhythms (Kohsaka *et al.*, 2007).

In order to assess the exact contribution of daily timing of fat and sugar consumption on energy balance and to assess the mechanism by which this occurs, we conducted a more elaborate study, which is presented in **chapter 3**. Animals were fed a chow or HFHS diet, either with *ad libitum* access to food, or with restricted access only during the light or dark period. The most important finding in this study was the interaction effect of diet timing and diet composition on hepatic fat accumulation, which was not observed for body weight or adiposity within the two diet groups. Triglyceride accumulation in the liver has clearly been linked to non-alcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma (Caldwell & Crespo, 2004; Poonawala et al., 2000; Wong et al., 2014), and is associated with insulin resistance and the metabolic syndrome in humans (Clark et al., 2002).

Whereas light-phase chow feeding resulted in significantly less liver fat compared to chow *ad lib* fed animals, HFHS light-fed animals showed the opposite, indicating that not timing *per se*, but the combination of timing and diet is important for the observed effect on hepatic fat accumulation. Possible cause for this is that the HFHS light-fed animals, as compared to chow light-fed animals, were unable to reverse their energy expenditure pattern, leading to a discrepancy in timing of energy intake and expenditure. A similar phenomenon was observed in high-fat high-sucrose diet-fed mice (Yasumoto *et al.*, 2016). Yet, the controversy between liver fat and whole-body fat remains difficult to explain, and the clinical relevance of increased hepatic steatosis, without a significant increase in general adiposity needs to be studied further. A possible explanation is that increased liver fat is associated with an increased rate of adipocyte lipolysis, independent of insulin action (Vatner *et al.*, 2015). The absence of increased hepatic fat accumulation in light-phase chow fed animals is in contrast to the work of Salgado-Delgado and colleagues, in which livers of light-phase chow-fed animals showed a significantly larger area of microvesicular fat deposits compared to *ad lib*- and dark-fed chow animals (Salgado-Delgado *et al.*, 2013). This discrepancy may be due to the length of the fasting period, which was 12 h in the Salgado-Delgado study versus 14 h in our study. Although this difference seems small, a correlation between the length of the fasting period and metabolic outcome parameters was described in mice (Chaix *et al.*, 2014).

A second important finding from this study was the observed shift in circadian expression profiles of hepatic clock genes between HFHS and chow-fed animals. A 3 h advance was found for the HFHS-light fed animals compared to chow light-fed animals, a difference not observed between HFHS dark- and chow dark-fed animals. This again suggests a complementary effect of diet timing and diet composition. It also suggests that the liver clock is more prone to (diet-induced) alterations when the regular feeding pattern is already out of phase with the exogenous light-dark cycle.

The amplitude of the expression profiles of *Glucokinase*, *Acc1* and *Fas*, involved in the lipogenesis pathway, was consistently more pronounced in the HFHS-fed compared to the chow groups, yet not different between light- and dark-fed animals. Hence, this does not explain the increased fat deposition in the liver in the HFHS light-fed group. In mice, it was found that most hepatic triglycerides of high-fat-fed mice were formed from the re-esterification of existing or ingested lipids, and did not arise from *de novo* lipogenesis (Duarte *et al.*, 2014), which may explain the apparent discrepancy between increased liver fat and absence of upregulation of genes involved in lipogenesis. A combined diet and timing-dependent effect was observed in *Ppar-γ* expression: HFHS-light fed animals expressed high levels of *Ppar-γ* during the light period. Upregulation of *Ppar-γ* upon a HFHS diet is in accordance to the literature (Inoue *et al.*, 2005), yet the timing-dependent effect is new. Perhaps the relative desynchronization between clock gene expression and metabolic gene expression in the HFHS-light fed group accounts for the increased hepatic fat accumulation, although the underlying mechanism remains unclear.

Summarizing the two *in vivo* studies, we learn that the combination of diet timing and composition is indeed important for body weight (**chapter 2**), hepatic fat accumulation (**chapter 3**), feeding efficiency (**chapter 2**), and substrate oxidation patterns (**chapter 2 and 3**).

Still, important questions remain:

1. What is the molecular mechanism behind the differential effects of light- and dark-phase HFHS feeding?
2. Do fat and sugar affect the clock in different ways, independent of energy content?
3. Does desynchrony between the central clock and the liver clock play a role in the observed alterations in metabolism?

Regarding the second point, it is noteworthy that we also included high-fat (HF) light- and HF dark-fed animals in the study (data not shown). No difference between HFHS-fed and HF-fed animals was observed with respect to caloric intake, locomotor activity and heat production (when comparing HFHS-L with HF-L and HFHS-D with HF-D). A major difference between HFHS and HF-fed animals was the RER, which was much lower in HF-fed animals compared to HFHS-fed animals, which can be explained by the fact that HF-fed animals consume, and hence, oxidize a greater amount of fatty acids. Furthermore, a diet composed of high-fat and high-sucrose was shown to induce more lipogenesis compared to a high-fat diet (Duarte *et al.*, 2014). Interestingly, we did not find a difference in hepatic fat accumulation between HFHS and HF light-fed animals. Noteworthy however, was the observation that there was no significant timing effect on hepatic fat accumulation in the HF-fed group (*i.e.* HF light-fed animals did not show significantly more liver fat than the HF dark-fed animals, as was observed in the HFHS-fed animals). This implicates that the timing-effect of a HFHS diet on hepatic steatosis is more pronounced than of a HF diet. However, a proper control group with sugar-fed animals is lacking thus preventing

a definite conclusion. Another remarkable finding was found with regard to clock gene expression, as there was a clear 6 h advance in clock gene expression in the HF light-fed group, compared to the chow light-fed group, a shift even more pronounced than observed in the HFHS light-fed group. The clinical relevance of this larger advance in clock gene expression needs to be studied further, as it did not lead, in this study, to an increase in hepatic fat accumulation or adiposity (data not shown).

To further determine the role of fatty acids and glucose on the molecular clock mechanism, and in particular in hypothalamic cells, we next performed *in vitro* experiments to assess the direct effects of these nutrients on the clock. First, we determined the effect on hypothalamic clock gene expression by palmitate, a saturated fatty acid abundantly available in human diet- and accounting for 25% of the total fatty acid content in the lard given to our rats. In **chapter 4** we describe the effects of palmitate on clock gene expression in the isolated hypothalamic murine cell line mHypoE-37, which endogenously expresses the core clock genes and has been well-characterized previously (Belsham *et al.*, 2004). The most important finding was the increased expression of *Bmal1*. The low-grade inflammatory response followed by high amounts of free fatty acids, is generally accepted as a resetting factor for the molecular clock (Pivovarova *et al.*, 2015), and could be causal for the observed effects on *Bmal1* expression. Therefore, we assessed the expression profiles of the inflammatory markers IκBα and IL-6, and of TLR4, because binding of free fatty acids to TLR4 leads to induction of pro-inflammatory pathways. The concentration of palmitate did not have significant effects on these pro-inflammatory markers, suggesting that another mechanism is responsible for the observed effects on the clock. This may also be a cell-type specific effect, as an inflammatory response was found in fibroblasts upon palmitate treatment (Kim *et al.*, 2016). In order to differentiate between a direct effect of palmitate and the energetic content of palmitate, we assessed the effect of palmitate on AMP-activated protein kinase (AMKP), an important nutrient sensor. Palmitate did not alter the phosphorylation of AMKP over the experimental time course, suggesting that the effects of palmitate on *Bmal1* are independent of AMPK. Next, cells were incubated with docosahexaenoic acid (DHA), a polyunsaturated fatty acid (PUFA). PUFAs have been described to be able to ameliorate the lipotoxic effects of saturated fatty acids via AMPK (Sakamoto *et al.*, 2017). We observed that DHA altered *Bmal1* expression, but in a different manner than that of palmitate, and independent of AMPK activity. Although AMPK is only one of the many nutrient sensors that may be involved in fatty-acid dependent effects on the clock, this observation does suggest that the observed effects are independent of nutrient status. Furthermore, the fact that a similar amount of DHA, which is also a fatty acid, affects *Bmal1* differentially from palmitate adds to the idea that not the upregulation of energy, but the composition of the fatty acid is important for the effects on the clock genes.

In **chapter 5**, we describe that glucose dose-dependently altered the expression profile of *Per2* in mHypoE-37 neuronal cells, whereas the expression profile of *Bmal1* was not affected. The nutrient sensors AMPK and GSK3β were assessed for their possible role in

this effect. GSK3β activity was not affected by different concentrations of glucose, whereas phosphorylation (*i.e.* activation) of AMPK was (non-significantly) repressed by a high dose of glucose. Chemically repressing AMPK resulted in both decreased phosphorylation of AMPK, as well as repression of *Per2* mRNA expression. This suggests an effect of glucose on the clock through energy sensing by AMPK, although as for now this is only correlational, and not causal, evidence.

Taken together, from the described *in vitro* studies, we learn that both palmitate and glucose can dose-dependently affect the rhythmic expression profile of core clock genes in murine, hypothalamic (non-SCN) cells. The use of this isolated hypothalamic cell line allowed for a thorough examination of the direct effects of nutrients on the molecular clock mechanism within this specific hypothalamic neuronal population. Although previous studies showed direct effects of glucose on the expression profile of clock genes [e.g. (Balsalobre *et al.*, 2000)], to our knowledge we were the first to show these rhythms in hypothalamic neurons. Firstly, this is important, as the hypothalamus is necessary for control of food intake and glucose homeostasis. Secondly, not all cell types respond similarly to nutrient stimuli. Taib *et al.* showed that glucose inhibits palmitate oxidation via AMPK in hypothalamic neuronal cell lines, primary hypothalamic astrocyte cultures, and MBH slices *ex vivo*, but not in cortical astrocytes and slice preparations, indicating differential effects amongst different cell types (Taib *et al.*, 2013).

The advantage of combining in vivo and *in vitro* models

Both *in vivo* and *in vitro* models have advantages and disadvantages. In terms of translatability, an *in vivo* model is of more clinical importance than an *in vitro* model. As adaptation to different food stimuli is a physiological response, it is important to look at the organism as a whole. However, to study the direct effects of specific nutrients on the molecular clock mechanism, using an *in vivo* model is almost impossible. For instance, the effects of sugar on the expression of circadian clock genes *in vivo* cannot be separated completely for other humoral factors, as increased glucose levels will be accompanied by alterations in hormones, including insulin, which itself may affect the clock (Chaves et al., 2014). Another complicating factor is that, due to the blood-brain barrier, it is still unknown what percentage, or which individual elements, of dietary components eventually reach the brain. It was found that extracellular glucose concentrations in the rat brain are approximately 20% of serum glucose levels and range from ~0.2 mM during systemic hypoglycemia to ~4.5 mM during systemic hyperglycemia (Silver & Erecinska, 1994), but how this translates to humans is still unknown. Therefore, it can be debated whether the glucose concentrations used in **chapter 5** are representative of what the brain normally senses. Other remarks concerning the cell studies and animal studies, are that in the *in vitro* studies, we used murine cells, whereas we used rats in the *in vivo* studies. Furthermore, using the *in vitro* model, we studied circadian patterns, whereas we studied daily patterns in the *in vivo* model. Also, it is unknown how the findings in the embryonic cell line can be translated to adult cells.

Clinical relevance

The studies presented in this thesis are part of a larger project with the ultimate goal to give recommendations, or design diet paradigms to strengthen the internal clock and improve metabolic health or, at the other end of the spectrum, prevent or reduce metabolic problems caused by chronodisruptive behaviors such as shift work. We did find that the timing and composition of the diet affects food efficiency and the amount of hepatic steatosis, presumably due to an imbalance between energy intake and expenditure. This was not just timing-dependent, but also composition-dependent. It is therefore tempting to speculate about the clinical relevance for, for example, shift workers. Extrapolating our *in vivo* findings to humans, we would suggest that, when someone is forced to eat during the normally inactive period because of shift work, it would be healthier to eat a diet rich in carbohydrates and protein (mimicking chow) instead of a high-fat, high-glucose diet. Yet, more research needs to be done, both in animal models and in human studies, to exactly delineate which factors are contributing most to the unhealthy metabolic effects of shifting the timing of food intake.

With regard to the translational aspect of our studies, an important question of course is whether evidence from animal studies can be translated to the human situation. For example, Rothshild *et al.* reviewed animal and human studies for the effects of time-restricted feeding (TRF) on body weight and markers of metabolic disease. Beneficial effects on plasma lipids and insulin sensitivity were found for both animals and humans, but a differential effect of TRF on body weight between animals and humans was found, *i.e.* TRF was associated with weight loss in animals, but this effect was not consistently found in humans, for unknown reasons (Rothschild *et al.*, 2014). Moreover, it should be taken into account that even within animal studies, species differences are found. Mice and rats are generally used as a model for the human situation, but within these species body weight, adiposity, and locomotor activity often are affected differentially (Opperhuizen *et al.*, 2015). Another complicating factor in the translation of animal studies to the human situation, is the difference in used diets. Even within animal studies, a broad variety in diets is used to mimic a human obesogenic, or palatable diet. Most studies use commercial pellet diets, with all different components in one pellet. This prevents choice of the different nutrients throughout the day and may lead to overconsumption of certain nutrients at times that the animal would normally not consume that specific component. Noteworthy, in diets used as control diets for a high-fat diet, the fat component is usually replaced by carbohydrates, which often also consist of sucrose. Therefore, these so-called control diets indeed contain less fat, but the sucrose content is higher, complicating the comparison [reviewed in (van den Heuvel *et al.*, 2011)]. Therefore, we believe that the used choice diets, as described in this thesis, are more representative of human situation than commercially available pellets. Furthermore, we believe that offering sugar as a liquid component, is highly representative for the human situation, as soft-drink intake is a great contributor to obesity (Malik *et al.*, 2006).

Irregular meal patterns due to shift work have been associated with the development of obesity and type 2 diabetes. However, not only extreme shifts in meal timing, but also the distribution of meal timing throughout the day is associated with more or less beneficial health outcomes. For example, a high-energy breakfast and low-energy dinner, *vs.* low-energy breakfast and high-energy dinner improved postprandial hyperglycemia throughout the entire day, as proved by a better glucose response to an identical meal (Jakubowicz *et al.*, 2015). Furthermore, the success of weight loss was associated with timing of food intake throughout the day (Garaulet *et al.*, 2013), indicating that also in humans timing of food intake may be an important contributor to metabolic health. Interestingly, ancient wisdom has already been suggesting this for decades: 'Breakfast like a king, lunch like a prince, dine like a pauper' (Adelle Davis [1904 – 1974]). In line with this, an association was found between individual chronotype and timing of energy and macronutrient intake. It was found that evening types had a postponed energy and macronutrient intake timing, with generally higher energy intake in the evening than in the morning, which may put them at higher risk for obesity, although this was not investigated (Maukonen *et al.*, 2017).

Not only the caloric timing, but also the distribution in time of different macronutrients is important. For instance, carbohydrates can be better metabolized during breakfast then during (late) dinner, as the body responds better to a glucose stimulus in the early morning, an effect even more pronounced in people with impaired glucose tolerance (Dos Santos *et al.*, 2006). This may be explained by the fact that the response to a meal of many metabolically relevant hormones, including insulin, cycle with a daily pattern (Van Cauter *et al.*, 1992). Furthermore, circadian misalignment in humans disturbs glucose metabolism and substrate oxidation. It has been shown that glucose tolerance is much lower in the biological evening versus the morning (Morris *et al.*, 2015). Thus, the circadian system affects glucose tolerance and thereby importantly affects 24 h glucose regulation which could contribute to the development of diabetes.

Although human studies show effects of altered meal timing on whole-body metabolism, with the suggestion that this is the result of desynchronization between peripheral clocks and the central clock, little research has addressed how temporal aspects of feeding can actually regulate the circadian system of humans. In a recent study by Wehrens *et al.*, healthy young men were subjected to either early or late meals. In a constant routine schedule following the meal protocol, it was found that late meals resulted in delayed plasma glucose rhythms, a decrease in average glucose concentration, and delay of *PER2* mRNA rhythms in adipose tissue (Wehrens *et al.*, 2017). This indicates that in humans molecular clocks may also be regulated by feeding time, and could account for the changes in plasma glucose levels (Wehrens *et al.*, 2017). The human clock was also found to be sensitive to changes in macronutrient availability. In healthy subjects, a switch from a high-carbohydrate/low fat diet to an isocaloric low-carbohydrate/high fat diet resulted in a phase delay in salivary cortisol levels, and altered gene expression of core clock genes and inflammatory genes in circulating monocytes (Pivovarova *et al.*, 2015). This suggests both an effect on the central clock (cortisol levels), as well as peripheral clocks (clock genes

in monocytes). One suggested mechanism is the low-grade inflammatory response caused by a high-fat diet, which can reset the circadian clock in peripheral tissues and the CNS (Pivovarova *et al.*, 2015).

In addition to alterations in metabolic hormones, many more metabolic processes in the body show a clear daily variation. For instance, gastrointestinal motility and gastric emptying occur in a circadian pattern [reviewed in (Moran-Ramos *et al.*, 2016)]. As the gastrointestinal tract is the largest endocrine organ, it is conceivable that disruption of its rhythmic coordination – e.g. by high-fat diet -, may influence the effects of altered diet timing (Moran-Ramos *et al.*, 2016). In mice, a high-fat, high-sugar diet combined with a shifted light-dark cycle resulted in gut microbiota imbalance (Voigt *et al.*, 2013), which might be causally related to metabolic syndrome (reviewed in (de Groot *et al.*, 2017)), especially since the intestinal microbiota also shows a daily rhythm itself (Thaiss *et al.*, 2014).

In a cross over laboratory setting, it was found that there is a circadian and behavioral pattern in substrate oxidation profiles in humans. The postprandial respiratory quotient (RQ) and carbohydrate oxidation were lower, whereas lipid oxidation was higher after dinner compared to after breakfast. Also, when behavioral influences were excluded, there is a circadian phase in oxidation pattern as well, with a lower RQ and lower carbohydrate oxidation in the biological evening *versus* the morning.

It was found that human skeletal muscle mitochondrial oxidative capacity in healthy human subjects shows a profound day-night rhythm (van Moorsel *et al.*, 2016). Therefore, it can be speculated that, also in humans, alterations in feeding times will cause a desynchronization between energy intake and energy oxidation, possibly adding to the unhealthy metabolic outcomes of circadian misalignment.

Obviously, these are lab-based studies, which are necessary to control for factors other than food intake. Although it is generally assumed that most people ingest three main meals per day -breakfast lunch, dinner-, the daily rhythm in eating patterns in humans in non-lab-based settings has rarely been investigated. Recently, the Panda group reported the results of daily food eating patterns of nearly 100 subjects during 21 days. Adults logged their food and beverage intake using a camera phone. The researchers analyzed all photos for timing of food intake and calories ingested. It was shown that, in contrast to the general belief that most food is ingested over 3 meals within ~12 h during the day, eating events were widespread throughout the day, with <30% of calories consumed before noon and >30% consumed in evening and late-night hours. Surprisingly, more than 50% of people spread their caloric intake events over 15 h or longer (Gupta *et al.*, 2017). In a study using the same mobile application, people were asked to reduce their food intake window to 10-12 h per day. By doing so, the participants lost a significant amount of body weight. The time-restricted feeding coincided with a reduction in caloric intake, contributing to the weight loss. However, better sleep and a smaller social jetlag were also recorded during the time-restriction, indicating that time-restricted feeding in

humans can have additive beneficial effects on health besides body weight loss alone (Gill & Panda, 2015).

In summary, feeding is an important modulator of the internal clock. Understanding the exact role of nutrients in clock functioning may allow for recommendations to use nutritional input more effectively in improving metabolic disorders arising from circadian misalignment (Ribas-Latre & Eckel-Mahan, 2016). Since timing of food intake is so important for human metabolic health, as shown by the studies described, regulation of meal timing may be used to synchronize peripheral rhythms in humans. This may be of particular interest for shift-workers or transmeridian travelers (Wehrens *et al.*, 2017). Noteworthy, in humans the circadian expression pattern of several clock genes was found to be advanced or delayed according to their chronotype (*i.e.* "early birds" *vs.* "night owls"). Taking the individual chronotype into account can be important for specific recommendations on food intake and timing (Ribas-Latre & Eckel-Mahan, 2016).

FUTURE STUDIES

Despite the interesting findings described in this thesis, more studies need to be performed to assess differential effects of nutrient timing on whole-body metabolism. Although we showed a nutrient-specific effect on feeding efficiency (**chapter 2**), we did not fully dissect the individual temporal contributions of sugar and fat on whole-body metabolism. Therefore, a study should be conducted including also a high-fat, and a high-sugar diet, either with *ad libitum* access to food or food restricted to only the light-or dark phase. As long as caloric intake between the different diet groups is similar, for instance, by using paired feeding, it would be possible to differentiate between the caloric effects of palatable diets, or the effects of the nutrient composition. Also, differential effects on clock and metabolic gene expression should then be assessed. We still hypothesize that differential effects between high-fat or high-sugar only *versus* high-fat high-sugar can be expected. Firstly, the effect of experimental diet-induced obesity on lipogenesis is, at least partially, dependent on the nutrient composition of the diet, as a high-fat, high-sucrose diet induced more lipogenesis than a high-fat diet (Duarte et al., 2014). Secondly, the composition of hypercaloric, palatable diets plays a role in the development of site-specific insulin sensitivity in rats (Diepenbroek *et al.*, 2017). Furthermore, it would be of interest to assess whether the molecular clock responds differentially to liquid sugar, as we used in our diets, compared to solid sugar, which is also highly abundant in human diet.

In line with this, in order to dissect the molecular pathways through which nutrients can affect the clock, more nutrient sensors need to be studied. Due to the techniques used, we only focused on a few nutrient sensors that we hypothesized to be important for the observed effects of glucose and palmitate on the molecular clock. However, more nutrient sensors could be involved (including, but not limited to GSK3β, PPARγ and OGT), as well as other pathways, for instance, inflammatory pathways in palmitate-mediated effects. Micro-array studies, in which entire pathways are investigated, would provide

more integrated information about the exact mechanism through which palmitate and sugar exert their effect on the molecular clock.

Another outstanding question is if, and how, nutrients can differentially affect the central clock *versus* peripheral clocks. It would be of interest to study whether nutrients themselves can differentially alter the central and peripheral clocks, and whether this leads to desynchrony in clock output rhythms and altered rhythms of downstream metabolic pathways. High-fat intake affects both the central and peripheral clocks. Conversely, feeding-dependent alterations in the rhythmic expression of PPARα only occur in peripheral organs and not in the central clock, although individual nuclei and specific neuropeptide-expressing neurons were not individually studied. If alterations in PPARα lead to alterations in the molecular clock, and subsequently in clock output genes, this might be a mechanism that promotes circadian desynchronization between the central and peripheral clocks.

Given the reciprocal relationship between the molecular circadian clock and energy homeostasis, these are important questions that directly affect metabolic homeostasis. Understanding the impact of specific macronutrients on the circadian clock will allow for guidance towards the composition and timing of meals optimal for physiological health, as well as putative therapeutic targets to regulate the molecular clock.

REFERENCES

Arble, D.M., Bass, J., Laposky, A.D., Vitaterna, M.H. & Turek, F.W. (2009) Circadian timing of food intake contributes to weight gain. *Obesity (Silver Spring)*, **17**, 2100-2102.

Arble, D.M. & Sandoval, D.A. (2013) CNS control of glucose metabolism: response to environmental challenges. *Front Neurosci*, **7**, 20.

Aschoff, J. (1984) Circadian Timing. *Ann N Y Acad Sci*, **423**, 442-468.

Balsalobre, A., Marcacci, L. & Schibler, U. (2000) Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol*, **10**, 1291-1294.

Belsham, D.D., Cai, F., Cui, H., Smukler, S.R., Salapatek, A.M. & Shkreta, L. (2004) Generation of a phenotypic array of hypothalamic neuronal cell models to study complex neuroendocrine disorders. *Endocrinology*, **145**, 393-400.

Buijs, R. & Kalsbeek, A. (2001) Hypothalamic integration of central and peripheral clocks. *Nat Rev Neurosci*, **2**, 521-526.

Buijs, R.M., la Fleur, S.E., Wortel, J., Van Heyningen, C., Zuiddam, L., Mettenleiter, T.C., Kalsbeek, A., Nagai, K. & Niijima, A. (2003) The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. *J Comp Neurol*, **464**, 36-48.

Caldwell, S.H. & Crespo, D.M. (2004) The spectrum expanded: cryptogenic cirrhosis and the natural history of non-alcoholic fatty liver disease. *J Hepatol*, **40**, 578-584.

Chaix, A., Zarrinpar, A., Miu, P. & Panda, S. (2014) Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges. *Cell Metab*, **20**, 991-1005.

Chaves, I., van der Horst, G.T., Schellevis, R., Nijman, R.M., Koerkamp, M.G., Holstege, F.C., Smidt, M.P. & Hoekman, M.F. (2014) Insulin-FOXO3 signaling modulates circadian rhythms via regulation of clock transcription. *Curr Biol*, **24**, 1248-1255.

Clark, J.M., Brancati, F.L. & Diehl, A.M. (2002) Nonalcoholic fatty liver disease. *Gastroenterology*, **122**, 1649-1657.

Damiola, F. (2000) Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes Dev*, **14**, 2950-2961.

de Groot, P.F., Frissen, M.N., de Clercq, N.C. & Nieuwdorp, M. (2017) Fecal microbiota transplantation in metabolic syndrome: History, present and future. *Gut Microbes*, **8**, 253-267.

Diepenbroek, C., Eggels, L., Ackermans, M.T., Fliers, E., Kalsbeek, A., Serlie, M.J. & la Fleur, S.E. (2017) Differential effects of hypercaloric choice diets on insulin sensitivity in rats. *J Endocrinol*, **232**, 49-57.

Duarte, J.A., Carvalho, F., Pearson, M., Horton, J.D., Browning, J.D., Jones, J.G. & Burgess, S.C. (2014) A high-fat diet suppresses de novo lipogenesis and desaturation but not elongation and triglyceride synthesis in mice. *J Lipid Res*, **55**, 2541-2553.

Garaulet, M., Gomez-Abellan, P., Alburquerque-Bejar, J.J., Lee, Y.C., Ordovas, J.M. & Scheer, F.A. (2013) Timing of food intake predicts weight loss effectiveness. *Int J Obes (Lond)*, **37**, 604-611.

Gill, S. & Panda, S. (2015) A Smartphone App Reveals Erratic Diurnal Eating Patterns in Humans that Can Be Modulated for Health Benefits. *Cell Metab*, **22**, 789-798.

Gupta, N.J., Kumar, V. & Panda, S. (2017) A camera-phone based study reveals erratic eating pattern and disrupted daily eating-fasting cycle among adults in India. *PLoS One*, **12**, e0172852.

Hastings, M.H., Reddy, A.B. & Maywood, E.S. (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci*, **4**, 649-661.

Hirota, T. & Fukada, Y. (2004) Resetting mechanism of central and peripheral circadian clocks in mammals. *Zool Sci*, **21**, 359-368.

Inoue, M., Ohtake, T., Motomura, W., Takahashi, N., Hosoki, Y., Miyoshi, S., Suzuki, Y., Saito, H., Kohgo, Y. & Okumura, T. (2005) Increased expression of PPARgamma in high fat diet-induced liver steatosis in mice. *Biochem Biophys Res Commun*, **336**, 215-222.

Jakubowicz, D., Wainstein, J., Ahren, B., Bar-Dayan, Y., Landau, Z., Rabinovitz, H.R. & Froy, O. (2015) High-energy breakfast with low-energy dinner decreases overall daily hyperglycaemia in type 2 diabetic patients: a randomised clinical trial. *Diabetologia*, **58**, 912-919.

Kim, S.M., Neuendorff, N., Chapkin, R.S. & Earnest, D.J. (2016) Role of Inflammatory Signaling in the Differential Effects of Saturated and Poly-unsaturated Fatty Acids on Peripheral Circadian Clocks. *EBioMedicine*, **7**, 100-111.

Kohsaka, A., Laposky, A.D., Ramsey, K.M., Estrada, C., Joshu, C., Kobayashi, Y., Turek, F.W. & Bass, J. (2007) High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab*, **6**, 414-421.

Kornmann, B., Schaad, O., Bujard, H., Takahashi, J.S. & Schibler, U. (2007) System-driven and oscillator-dependent circadian transcription in mice with a conditionally active liver clock. *PLoS Biol*, **5**, e34.

Malik, V.S., Schulze, M.B. & Hu, F.B. (2006) Intake of sugar-sweetened beverages and weight gain: a systematic review. *Am J Clin Nutr*, **84**, 274-288.

Maukonen, M., Kanerva, N., Partonen, T., Kronholm, E., Tapanainen, H., Kontto, J. & Mannisto, S. (2017) Chronotype differences in timing of energy and macronutrient intakes: A population-based study in adults. *Obesity (Silver Spring)*, **25**, 608-615.

Moran-Ramos, S., Baez-Ruiz, A., Buijs, R.M. & Escobar, C. (2016) When to eat? The influence of circadian rhythms on metabolic health: are animal studies providing the evidence? *Nutr Res Rev*, **29**, 180-193.

Oosterman, J.E., Kalsbeek, A., la Fleur, S.E. & Belsham, D.D. (2015) Impact of nutrients on circadian rhythmicity. *Am J Physiol Regul Integr Comp Physiol*, **308**, R337-R350.

Opperhuizen, A.L., van Kerkhof, L.W., Proper, K.I., Rodenburg, W. & Kalsbeek, A. (2015) Rodent models to study the metabolic effects of shiftwork in humans. *Front Pharmacol*, **6**, 50.

Pivovarova, O., Jurchott, K., Rudovich, N., Hornemann, S., Ye, L., Mockel, S., Murahovschi, V., Kessler, K., Seltmann, A.C., Maser-Gluth, C., Mazuch, J., Kruse, M., Busjahn, A., Kramer, A. & Pfeiffer, A.F. (2015) Changes of Dietary Fat and Carbohydrate Content Alter Central and Peripheral Clock in Humans. *J Clin Endocrinol Metab*, **100**, 2291-2302.

Poonawala, A., Nair, S.P. & Thuluvath, P.J. (2000) Prevalence of obesity and diabetes in patients with cryptogenic cirrhosis: a case-control study. *Hepatology*, **32**, 689-692.

Reznick, J., Preston, E., Wilks, D.L., Beale, S.M., Turner, N. & Cooney, G.J. (2013) Altered feeding differentially regulates circadian rhythms and energy metabolism in liver and muscle of rats. *Biochim Biophys Acta*, **1832**, 228-238.

Ribas-Latre, A. & Eckel-Mahan, K. (2016) Interdependence of nutrient metabolism and the circadian clock system: Importance for metabolic health. *Mol Metab*, **5**, 133-152.

Rothschild, J., Hoddy, K.K., Jambazian, P. & Varady, K.A. (2014) Time-restricted feeding and risk of metabolic disease: a review of human and animal studies. *Nutr Rev*, **72**, 308-318.

Sakamoto, A., Saotome, M., Hasan, P., Satoh, T., Ohtani, H., Urushida, T., Katoh, H., Satoh, H. & Hayashi, H. (2017) Eicosapentaenoic acid ameliorates palmitate-induced lipotoxicity via the AMP kinase/dynamin-related protein-1 signaling pathway in differentiated H9c2 myocytes. *Exp Cell Res*, **351**, 109-120.

Salgado-Delgado, R.C., Saderi, N., Basualdo Mdel, C., Guerrero-Vargas, N.N., Escobar, C. & Buijs, R.M. (2013) Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PLoS One*, **8**, e60052.

Silver, I.A. & Erecinska, M. (1994) Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. *J Neurosci*, **14**, 5068-5076.

Taib, B., Bouyakdan, K., Hryhorczuk, C., Rodaros, D., Fulton, S. & Alquier, T. (2013) Glucose regulates hypothalamic long-chain fatty acid metabolism via AMP-activated kinase (AMPK) in neurons and astrocytes. *J Biol Chem*, **288**, 37216-37229.

Thaiss, C.A., Zeevi, D., Levy, M., Zilberman-Schapira, G., Suez, J., Tengeler, A.C., Abramson, L., Katz, M.N., Korem, T., Zmora, N., Kuperman, Y., Biton, I., Gilad, S., Harmelin, A., Shapiro, H., Halpern, Z., Segal, E. & Elinav, E. (2014) Transkingdom control of microbiota diurnal oscillations promotes metabolic homeostasis. *Cell*, **159**, 514-529.

Van Cauter, E., Shapiro, E.T., Tillil, H. & Polonsky, K.S. (1992) Circadian modulation of glucose and insulin responses to meals: relationship to cortisol rhythm. *Am J Physiol*, **252**, E467-E475.

van den Heuvel, J.K., van Rozen, A.J., Adan, R.A. & la Fleur, S.E. (2011) An overview on how components of the melanocortin system respond to different high energy diets. *Eur J Pharmacol*, **660**, 207-212.

van Moorsel, D., Hansen, J., Havekes, B., Scheer, F.A., Jorgensen, J.A., Hoeks, J., Schrauwen-Hinderling, V.B., Duez, H., Lefebvre, P., Schaper, N.C., Hesselink, M.K., Staels, B. & Schrauwen, P. (2016) Demonstration of a day-night rhythm in human skeletal muscle oxidative capacity. *Mol Metab*, **5**, 635-645.

Vatner, D.F., Majumdar, S.K., Kumashiro, N., Petersen, M.C., Rahimi, Y., Gattu, A.K., Bears, M., Camporez, J.P., Cline, G.W., Jurczak, M.J., Samuel, V.T. & Shulman, G.I. (2015) Insulin-independent regulation of hepatic triglyceride synthesis by fatty acids. *Proc Natl Acad Sci U S A*, **112**, 1143-1148.

Voigt, R.M., Forsyth, C.B., Green, S.J., Mutlu, E., Engen, P., Vitaterna, M.H., Turek, F.W. & Keshavarzian, A. (2013) Circadian Disorganization Alters Intestinal Microbiota. *PLoS One*, **9**, e97500.

Vollmers, C., Gill, S., DiTacchio, L., Pulivarthy, S.R., Le, H.D. & Panda, S. (2009) Time of feeding and the intrinsic circadian clock drive rhythms in hepatic gene expression. *Proc Natl Acad Sci U S A*, **106**, 21453-21458.

Wehrens, S.M.T., Christou, S., Isherwood, C., Middleton, B., Gibbs, M.A., Archer, S.N., Skene, D.J. & Johnston, J.D. (2017) Meal Timing Regulates the Human Circadian System. *Curr Biol*, **27**, 1768-1775 e1763.

Wong, R.J., Cheung, R. & Ahmed, A. (2014) Nonalcoholic steatohepatitis is the most rapidly growing indication for liver transplantation in patients with hepatocellular carcinoma in the U.S. *Hepatology*, **59**, 2188-2195.

Yasumoto, Y., Hashimoto, C., Nakao, R., Yamazaki, H., Hiroyama, H., Nemoto, T., Yamamoto, S., Sakurai, M., Oike, H., Wada, N., Yoshida-Noro, C. & Oishi, K. (2016) Short-term feeding at the wrong time is sufficient to desynchronize peripheral clocks and induce obesity with hyperphagia, physical inactivity and metabolic disorders in mice. *Metabolism*, **65**, 714-727.

Zarrinpar, A., Chaix, A. & Panda, S. (2016) Daily Eating Patterns and Their Impact on Health and Disease. *Trends Endocrinol Metab*, **27**, 69-83.