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THESIS SUMMARY

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Daily rhythms in muscle mitochondria. Effects of time-restricted feeding and exercise

The incidence of type 2 diabetes mellitus (T2DM) is reaching pandemic proportions with an estimated 500 million diabetics worldwide. T2DM is characterized by hyperglycemia caused by a reduced insulin sensitivity. In early stages of the disease glucose levels can be kept within normal ranges through an increased release of insulin by the pancreas, but at later stages this release becomes insufficient due to failure of the pancreatic beta-cells and hyperglycemia develops. Comorbidities of T2DM include an increased risk for blindness as well as cardiovascular disease. The main risk factors for developing T2DM are excessive caloric intake as well as physical inactivity, but other behavioral risk factors such as performing shift-work have also been found to contribute to disease development. As our society increasingly relies on shift-work it is crucial to develop strategies that minimize these harmful effects.

It is currently unknown what the mechanisms are behind the increased risk of shift-workers for metabolic disease. There is a possible role for erratic eating patterns as shift-workers often eat during the night, which is the inactive period of humans. An often used animal model to study the effects of shift-work and erratic eating patterns is time-restricted feeding (TRF). In this model animals are limited in their opportunity to feed themselves by restricting food access to a specific time of day, usually either in the light (=inactive period for nocturnal rodents such as rats) or dark period (=active period for rats). Restricting food access to the inactive period is often associated with disease development, whilst restricting food access to the active period is associated with health improvements.

Throughout the first 2 parts of this thesis we consistently subjected rats to the same 3 feeding conditions: *ad libitum* (unrestricted access as the control condition), light-fed (TRF for 10h in the middle of the inactive period) and dark-fed (TRF for 10h in the middle of the active period). The only exception to this is **Chapter 4** in which we employed a 6-meals-a-day (6M) group, which could eat during 6 short time (10 – 12 minutes) windows evenly distributed throughout the day in order to eliminate the daily rhythm in feeding behavior.

In **part I** we first discussed important aspects of the experimental design and statistical analysis for research on biological rhythms. Importantly, we recommended to never duplicate time-series data before statistical analysis is done as this dramatically increases the false-positive rate. Furthermore, it is essential to control for multiple testing if several metabolites or gene expression profiles are measured from the same biological samples as is the case in "–omics" approaches as well as in our own experiments where we tested a wide variety of clock and metabolic genes using real time quantative PCR. The other chapters in part I focused on characterizing the effects of the

timing of food intake and diet composition on metabolism in general, with a specific emphasis on clock and metabolic gene expression in different peripheral tissues. The tissues that we focused on in this part all are metabolically important, with the liver being important for e.g. carbohydrate, protein, lipid and amino acid metabolism as it is responsible for glycogenesis, gluconeogenesis as well as lipogenesis. Skeletal muscles (SM), including the *Soleus* and *Gastrocnemius* muscles, that were investigated in the various chapters in this thesis are important for, amongst others, activity, shivering thermogenesis and glucose uptake. Brown adipose tissue (BAT) is important for body temperature regulation through non-shivering thermogenesis by burning the stored lipids of the body. In **Chapters 3.1**, **3.2** and **4** we found that both the rhythm in feeding behavior and diet composition differentially affect clock and metabolic gene expression in these metabolically important peripheral tissues. In **Chapters 3.1** and **3.2** we found that in light-fed animals the acrophase (=peak) in gene expression of several clock genes was shifted in BAT. Contrasting, in both muscle types studied, the *Soleus* and *Gastrocnemius*, rhythmic expression of clock genes was almost completely abolished for this group. In the light-fed group only Reverbα in the *Gastrocnemius* was still significantly rhythmic. The difference between these two muscles can likely be attributed to their different nature and functions: the *Soleus* is primarily a slow-twitching muscle consisting of oxidative fibers and plays a major role in maintaining posture and during endurance exercise. On contrary, the *Gastrocnemius* is a fast-twitching glycolytic fiber that plays a major role in high power output movements of the leg such as resistance exercise training. Although most metabolic genes were not rhythmically expressed in BAT in any of the groups, in SM some metabolic genes displayed altered rhythmic expression in the light-fed group, with for example the mitochondrial gene uncoupling protein 3 (Ucp3) showing a shift in acrophase of several hours in both SM types.

In addition, we found in **Chapter 3.1** that animals that were on a free-choice high-fat and high-sugar (fcHFHS) diet displayed more pronounced rhythms in clock gene expression in BAT, probably because of the increased lipid oxidation. In SM some of the clock genes that had lost rhythmic expression in the light fed group had their rhythmic expression rescued in the fcHFHS group that could only eat in the light period. Together these findings confirmed previous reports that feeding behavior and diet composition differently affect peripheral clock rhythms. Eliminating the daily rhythm in food intake using the ultradian 6M study protocol showed that the rhythmic expression of core clock genes in the tested peripheral tissues is not only driven by the daily rhythm in food intake (**Chapter 4**). In fact, most clock genes in liver, BAT and SM were unaffected by this absence of a daily rhythm in food intake. Clearly other factors, including daily rhythms in (muscle) activity, body temperature and hormone release, contribute in synchronizing peripheral clocks.

In the experiments presented in **Part II** we shifted away from the gene expression approach used in Part I and attempted to characterize several functional measures of glucose metabolism after disturbing or enhancing the rhythms of the biological clock through our TRF model. Using our light period TRF condition we found in **Chapter 6** that disturbing the clock leads to a reduced amplitude in the daily rhythm of mitochondrial respiration in SM as well as reduced overall levels. Conversely, strengthening the clock as during our dark period TRF condition slightly enhanced the natural

rhythm in mitochondrial respiration. Although the mechanisms behind these findings need further study, since we found no changes in the total number of mitochondria, we propose mitochondrial dynamics as potential mechanism behind these changes in respiration, i.e., the changes in mitochondrial activity through biogenesis, mitophagy, fission and fusion.
Using glucose tolerance tests (GTT) at 2 different time points along the 24h cycle in our 3 different feeding groups we found that disturbing or enhancing the biological clock through TRF did not result in altered glucose tolerance as compared to *ad libitum* feeding (**Chapter 7**). Surprisingly, when the GTT was performed in the experimental feeding period we found in both TRF groups lower insulin responses as compared to the *ad libitum* feeding group. It therefore seems that the negative effects of light period feeding are outweighed by the positive effects of a daily prolonged fasting period of 14h in the TRF groups. In line with this is the finding that the lowest insulin responses were found in the dark fed animals. However, when GTT's were performed during the experimental fasting period insulin responses were increased, especially in the light fed group. Taken together these findings indicate that the daily fasting period during TRF enhances insulin sensitivity, but only during the regular feeding period, and especially when the fasting period is in line with the natural circadian rhythm, i.e., fasting during the normal sleep period.

In **Chapter 8** we found that following 4 weeks of TRF several effects of this shift-work model lasted for at least 11 days after reverting back to *ad libitum* feeding. For example persisting changes in daytime lipid substrate usage and feeding and (locomotor) activity behavior were found, concurrently with remaining changes in clock gene expression in liver, BAT, *Soleus* and *Gastrocnemius* muscle. Similar to our TRF experiments in Part I these alterations in clock gene expression profiles were both tissue- and clock gene dependent. Importantly, these after-effects were found both in the groups that were light-fed and dark-fed for 4 weeks. These findings indicate that shift-workers that frequently switch between night-shifts and day-shifts are at an increased risk of having misaligned clocks. However, in the dark-fed group several positive after-effects of TRF were found including increased lipid substrate usage. This finding indicates that when applying TRF or other variants of intermittent fasting it is not necessary to apply this every single day in order to profit from their beneficial effects.

In **Part III** we confirmed that Zeitgebers other than feeding behavior can synchronize the muscle clock. We found that both removal of the adrenal gland and the timing of voluntary running wheel activity affected muscle clock gene expression. In **Chapter 9** we observed that hormones, or better absence of hormones (in this case adrenal hormones), also affect the muscle clock and metabolism. Removal of the adrenal glands resulted in reduced amplitude of Reverbα as well as a loss of daily rhythmicity for Per1 in *Soleus*. Reverbα is involved in lipid metabolism and our findings that also several genes involved in lipid metabolism were dysregulated are in line with this result. The finding of the loss of rhythm in Per1 was not surprising as it is well known that the Per1 gene contains a glucocorticoid response element. The loss of rhythm in Per1 that we found thus further strengthens the general idea that the SCN synchronizes the peripheral clocks through different pathways, amongst others also hormones such as the glucocorticoid hormone corticosterone.

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Using time-restricted running to manipulate activity behavior we found in **Chapter 10** that voluntary activity during the light period dampened the rhythms of the molecular clock in *Soleus*, but not *Gastrocnemius* muscle. Furthermore, animals that could only run during the light period had increased body fat as compared to animals that could run *ad libitum* or during the dark period only. This implies that running activity during the inactive period has negative effects on health as compared to activity during the active period. However, these light period running animals ran substantially less compared to the other two groups and this difference in body fat could thus also be explained by less total activity. Interestingly, when rats could only run during the dark period they had reduced daily glucose levels as compared to animals that had *ad libitum* access to their running wheel, without differences in total daily running activity. This would imply that strengthening the day/night difference in activity could be beneficial to counteract hyperglycemia without the need for increased activity, but these findings need further investigation.

Concluding, the studies presented in this thesis confirm the close relationship between the circadian timing system, timing in feeding behavior, diet composition and energy metabolism at the systemic (whole body), cellular and organellar level. More specifically, we conclude that the biological clock controls mitochondrial metabolism in skeletal muscle. In addition, disturbing or strengthening the biological clock using TRF affects both mitochondrial metabolism in skeletal muscle as well as systemic insulin sensitivity. However, we were unable to determine if disturbing mitochondrial metabolism directly impaired glucose tolerance and/or insulin sensitivity. Furthermore, our findings indicate that TRF is not always the best model to study the effects of disturbed biological rhythms on glucose and lipid metabolism. After all, the negative effects of eating at the wrong time-of-day are, at least partly, counteracted by the positive effects of daily prolonged fasting periods regardless of the time of day.

The diverse range of metabolic disturbances that we found in our animal model of shift-work supports the idea of internal desynchronization of peripheral clocks and thereby disturbed metabolism as the underlying course of the increased risk for metabolic disease in shift-workers. Future studies should therefore focus on the after-effects of disturbing the biological clock as a result of shift-work. More importantly, future studies should attempt to develop new shift-work strategies and lifestyle recommendations in order to minimize or even nullify the negative effects of shift-work.

On the other hand, strengthening the biological clock through fasting during the inactive period has several metabolic benefits. We found for example increased insulin sensitivity during the TRF regimen as well as improvements in fat metabolism that were still measurable 11 days after ending the TRF regimen. This last finding even implies that when using TRF as clinical therapy it does not necessarily needs to be enforced every single day. It thus would make it easier for patients to successfully comply with their TRF or similar fasting-related therapy.