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Paternal Circadian Arrhythmia Affects Offspring Health and Feeding Behavior

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TABLE OF CONTENTS

TABLE OF CONTENTS **2**

ABBREVIATIONS **4**

TABLE OF FIGURES **6**

SUMMARY **8**

ZUSAMMENFASSUNG **9**

INTRODUCTION **10**

TYPE 2 DIABETES 10
METABOLIC HOMEOSTASIS 12
Glucose metabolism *12*
Pathomechanisms of type 2 diabetes *17*
RISK FACTORS OF TYPE 2 DIABETES 19
Obesity & physical inactivity *19*
Genetic and epigenetic factors *20*
Circadian arrhythmia *21*
CIRCADIAN RHYTHM 22
Molecular and hormonal determinants of circadian rhythm *22*
Chronopharmacology *26*
Chrononutrition *27*
EPIGENETIC INHERITANCE 28
Definition of epigenetic inheritance *29*
Molecular mechanisms of epigenetic inheritance *30*
ALTERNATIVE MECHANISMS OF EPIGENETIC INHERITANCE 36
Seminal fluid and embryonic development *36*
Influence of glucocorticoid and the HPA axis on the metabolism *37*
DEVELOPMENTAL WINDOWS OF SUSCEPTIBILITY 37
Dutch Hunger Winter *38*
Överkalix study *39*
Inter-generational and transgenerational epigenetic inheritance *40*
SUMMARY 42

STUDY DESIGN, AIMS AND HYPOTHESIS **43**

MATERIALS **44**

BUFFERS, SOLUTIONS AND CONSUMABLES 44
CHEMICALS AND KITS 46
LABORATORY DEVICES AND EQUIPMENT 47
QPCR PRIMERS 48
HOUSEKEEPING GENES 48
PRIMARY ANTIBODIES 48
SECONDARY ANTIBODIES 49

METHODS **50**

ANIMAL HOUSING 50
F0 GENERATION SPECIFICS 50
PHENOTYPING AND METABOLIC METHODS 51

Bodyweight and visual inspection of mouse health ... *51*
Food intake measurements of the F1 generation ... *51*
Calorimetric cages ... *51*
ELISA ... *52*
Meso Scale Discovery (MSD) kits ... *52*
Blood glucose measurements ... *52*
Glucose tolerance tests ... *52*
TISSUE COLLECTION ... 53
Sperm and seminal fluid collections ... *53*
GENOTYPING OF GRHET MICE ... 54
IVF, OOCYTE ISOLATION AND EMBRYO TRANSFER ... 55
RNA SEQUENCING ... 55
Analysis of the first cohort ... *56*
Analysis of the second cohort ... *56*
STATISTICAL ANALYSIS ... 57

RESULTS ... 58

1) F0 CIRCADIAN ARRHYTHMIA FOR 30 DAYS ... 60
2) CHOW FED F1 OFFSPRING OF RF F0 ... 62
Metabolic phenotype of chow fed F1 offspring of RF F0 ... *62*
3) POTENTIAL VEHICLES OF TRANSMISSION FROM RF F0 TO RF F1 ... 67
Clock gene expression of RF F0 and RF F1 ... *67*
Corticosterone levels of RF F0 and RF F1 ... *69*
Gene expression of clock genes in germ cells of RF F0 ... *72*
Corticosterone concentration levels in the seminal fluid of RF F0 ... *73*
Metabolic phenotypes of F1 conceived via IVF ... *75*
4) EPIGENETIC, INTERGENERATIONAL AND DEVELOPMENTAL MECHANISMS ... 79
RNA sequencing of liver and hypothalamus samples of RF F1 ... *80*
Clustering of liver genes ... *82*
Clustering of hypothalamus genes ... *84*
5) F1 OFFSPRING OF FATHER'S THAT EXPERIENCED RESTRICTED AND THEN AD LIBITUM FEEDING ... 86
6) F2 GENERATION OF RF F0 ... 89
7) RF F1 FED A HIGH-FAT DIET ... 91
8) MECHANISTIC MODEL: F1 FROM FEMALE MICE WITH A HETEROZYGOUS GLUCOCORTICOID-RECEPTOR ... 96

DISCUSSION ... 100

PATERNAL CIRCADIAN ARRHYTHMIA AND ITS INFLUENCE ON THE F1 GENERATION ... 100
SEMINAL FLUID AS A MECHANISTIC TRANSPORTER ... 102
INVOLVEMENT OF THE BRAIN ... 104
EXPOSURE TIME OF F0 AND PENETRANCE OF CIRCARDIAN ARRHYTHMIA TO FURTHER GENERATIONS ... 105
LIMITING FACTORS AND FUTURE EXPERIMENTS ... 106
Mice and humans ... *106*
Paternal circadian arrhythmia and the impact on the offspring ... *106*
Differences between male and female offspring ... *107*
Influence of seminal fluid on placenta environment ... *107*
THE INFLUENCE OF CIRCADIAN ARRHYTHMIA ON MICE AND HUMANS ... 108
CONCLUSION AND RELEVANCE OF THE RESULTS FOR HUMANS ... 109

REFERENCES ... 111

ACKNOWLEDGMENT ... 127

PUBLICATIONS, TALKS AND POSTERS ... 129

ABBREVIATIONS

Abbreviation	Full Name
°C	Celsius
µL	Microliter
µM	Micromolar
AB	Antibody
ACTH	Adrenocorticotropic hormone
cDNA	Complementary DNA
CA	Circadian Arrhythmia
CpG	5‘-Cytosin-phosphate-Guanine-3‘
CRH	Corticotropin-releasing hormone
CTR	Control group
Dexa	Dexamethasone
dL	Deciliter
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
ELISA	Enzyme-linked imunnosorbent assay
E	Embryonic day
ET	Embryo transfer
F	Filial generation
FGF21	Fibroblast growth factor 21
GC	Glucocorticoid
GLP-1	Glucagon-like peptide-1
GR	Glucocorticoid receptor
GRhet	Heterozygous glucocorticoid receptor
GTT	Glucose tolerance test
GWAS	Genome-wide association studies
H2O	Water
HCG	Human chorionic gonadotropin
HDAC	Histone deacetylases
HDL	High density lipoprotein
HFD	High-fat diet
HPA	Hypothalamus pituitary adrenal
hCTR	Control group fed a high-fat diet
hRF	Restricted fed and high-fat diet
HTF	Human tubal fluid
Ip	Interperitoneal
IRS1	Insulin receptor substrate 1
ITT	Insulin tolerance test

IVF	In vitro fertilization
ivCTR	Control group concieved via in vitro fertilization
ivRF	Restricted fed group concieved via in vitro fertilization
kDa	Kilo Dalton
LDL	Low density lipoprotein
mCTR	Wild type mother
mg	Miligram
mGRhet	Mother with heterozygous glucocorticoid-receptor
mL	Mililiter
mM	Milimolar
miRNA	Micro RNA
ncRNA	Non-coding RNA
NGS	Next generation sequencing
NPY	Neuropeptide Y
NTC	No template control
oGTT	Oral glucose tolerance test
PI3K	Phosphoinositide 3-kinases
PMSG	Pregnant mare's serum gonadotropin
PTM	Post-translational modification
revCTR	Control group of the reversed restriction fed mice
revRF	Reversed restricted fed group
RNA	Ribonucleic acid
RF	Restricted feeding
RT	Room temperature
rpm	Rounds per minute
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SGLT2	Sodium-glucose linked transporter
SGP	Slow growth phase
Seq.	Sequencing
SNP	Single nucleotide polymorphism
STD	Standard
TMM	Trimmed middle of M-value
TRF	Time-restricted feeding
tRNA	Transfer RNA
wt	Wildtype
ZT	Zeitgeber

TABLE OF FIGURES

Figure 1: Number of people (20-79 years) with diabetes, 2019. 10
Figure 2: Number of people (20-79 years) with diabetes globally and by IDF Region 11
Figure 3: Total diabetes-related health expenditure (USD billion) by age group 12
Figure 4: Sucrose molecule 13
Figure 5: Insulin receptor signaling pathway and GLUT4 translocation 14
Figure 6: Distribution of glucose in the body 15
Figure 7: Distribution of glucose, fructose and sucrose in different fruits and vegetable 19
Figure 8: Circadian rhythm 24-hour scheme 23
Figure 9: Circadian clock core genes and feedback loop 24
Figure 10: The day/night pattern of cardiovascular events 26
Figure 11: From chromosomes to double helix 31
Figure 12: DNA methylation 32
Figure 13: Histone modifications 34
Figure 14: Inter- / Transgenerational inheritance 41
Figure 15: Setup of the experimental F0, multiple F1 and F2 cohort 59
Figure 16: Bodyweight, food intake and clock core gene expression levels of RF and CTR F0. 61
Figure 17: Food intake of RF and CTR F1 62
Figure 18: Food intake of RF and CTR F1 during the daytime at different age 63
Figure 19: Food intake of RF and CTR F1 during nighttime at different ages 64
Figure 20: Bodyweight of RF and CTR F1 at different ages 65
Figure 21: Energy expenditure of RF and CTR F1 over 48 hours 65
Figure 22: Blood glucose levels of RF and CTR F1 over 24 hours 66
Figure 23: Insulin levels of RF and CTR F1 over 24 hours 67
Figure 24: Heatmap of RNA-sequencing clock gene expression of RF and CTR F0 and F1 cohorts 69
Figure 25: Corticosterone levels in RF and CTR F0 70
Figure 26: Corticosterone levels in RF and CTR F1 at different Zeitgeber 71
Figure 27: Core clock gene expression levels in RF and CTR F0 germ cells 73
Figure 28: RF and CTR F0 seminal fluid corticosterone levels. 74
Figure 29: Cumulative food intake of ivRF and ivCTR F1 at different ages 76
Figure 30: Food intake of ivRF and ivCTR F1 77
Figure 31: Bodyweight of ivRF and ivCTR F1 at different ages 77
Figure 32: Corticosterone levels of ivRF and ivCTR F1 78
Figure 33: Blood glucose and insulin levels of ivRF and ivCTR F1 79

Figure 34: RNA-Seq data of oscillating liver genes of RF and CTR F1 80
Figure 35: RNA-Seq data of oscillating hypothalamus genes of RF and CTR F1 81
Figure 36: Heatmap visualization of RNA-Seq-based analysis of orexigenic and anorexigenic neuropeptide expression in the hypothalamus of RF and CTR.....82
Figure 37: Heatmap visualization of core clock genes in the liver of RF and CTR F1 83
Figure 38: Gene expression of selected core-clock genes in the liver of RF and CTR F1.....84
Figure 39: Heatmap visualization of core-clock genes in the hypothalamus of RF and CTR F1.....85
Figure 40: Gene expression of selected core-clock genes in hypothalamus of RF and CTR F1 86
Figure 41: Food intake of revRF and revCTR F1.....87
Figure 42: Bodyweight of revRF and revCTR F1 over 36 week.....88
Figure 43: Blood glucose of revRF and revCTR F1 at different Zeitgeber over a total of 48 hours 88
Figure 44: Food intake of RF and CTR F2.....89
Figure 45: Bodyweight of RF and CTR F2 over 48 weeks.....90
Figure 46: Blood glucose of RF and CTR F2 at different Zeitgeber 90
Figure 47: Food intake of hRF and hCTR F1.....92
Figure 48: Bodyweight of hRF and hCTR F1.....93
Figure 49: Fat mass gain of hRF and hCTR F1. 93
Figure 50: F1 Energy expenditure of hRF and hCTR F1 over 48 hours 94
Figure 51: Blood glucose and insulin levels of hRF and hCTR F1 over 24 hours.....95
Figure 52: Food intake of mGRhet and mCTR F1.....97
Figure 53: Bodyweight of mGRhet and mCTR F1 98
Figure 54: Blood glucose and corticosterone levels of mGRhet and mCTR F1 over 24 hours 98
Figure 55: Glucocorticoid receptor expression at different stages of embryonic development. 103

SUMMARY

This study is a first of a kind project to investigate circadian arrhythmia as an environmental stress factor and to examine how such an impact can lead to epigenetically transmitted phenotypes in the offspring.

We were successful in showing that circadian arrhythmia via restricted food intake (only 12 hours per day during the resting phase / no food intake for 12 hours during the active phase) in F0 males leads to significant metabolic and behavioral phenotypes in the unexposed F1 generation. We hereby observed that phenotypical alterations such as amount of food intake and corticosterone levels can be influenced by circadian arrhythmia of the fathers. This was especially interesting since the F1 offspring was completely unexposed to any environmental factors.

This study combines two emerging scientific fields: Firstly, Epigenetics, that has proven to deliver more explanations on inheritance phenotypes, which could not be explained by genetics. Secondly, research of disturbances of the circadian clock and the effects that are influenced by it. The importance of circadian clocks in humans, rodents and cells was cemented with awarding the Nobel Prize to Jeffrey C. Hall, Michael Robash and Michael W. Young in 2017 for their discoveries of the molecular mechanisms controlling the circadian rhythm.

To summarize, this study provides evidence that even short time environmental exposures such as circadian arrhythmia of the fathers can have tremendous metabolic and behavioral impact on the following generation. This is of utter importance, since nowadays more people than ever work at night or as shift workers and there is a growing incidences of early onset type 2 diabetes. Therefore this study can contribute to the explanation of how circadian arrhythmia and the rise of type 2 diabetes incidences are related and how the risk of developing type 2 diabetes can be passed on from the parents to their children.

ZUSAMMENFASSUNG

Diese Promotionsarbeit beschäftigt sich mit dem Zusammenhang von väterlichen Störungen der inneren Uhr im Körper und den daraus folgenden körperlichen Auswirkungen auf die Nachkommen. Wir haben hierbei herausgefunden, dass schon kurze Phasen von starken Umwelteinflüssen (wie der Störung der inneren Uhr) Einfluss auf die Kinder haben kann, auch wenn sie selbst niemals dem Umwelteinfluss ausgesetzt wurden. Dies sieht man im Mausmodell sowohl an einer erhöhter Futteraufnahme als auch an veränderten metabolischen Werten wie Glucose- und Cortisolkonzentrationen.

Dieses Doktorarbeits-Projekt verbindet zwei Wissenschaftsgebiete, denen in den letzten Jahren immer mehr Wichtigkeit zugesprochen wurde: Erstens die Epigenetik, durch die Vererbungsfaktoren erklärt werden konnten, für welche die Genetik allein bisher keine Erklärung liefern konnte. Zweitens, die Erforschung von Störungen der inneren Uhr und ihre Auswirkungen auf den kompletten Metabolismus und das Verhalten. Die Verleihung des Nobelpreises für Medizin im Jahre 2017 an Jeffrey C. Hall, Michael Robash und Michael W. Young zeigt, wie wichtig die Erkenntnis ist, dass molekulare Mechanismen die innere Uhr regulieren und zu unterschiedlichen Zeiten des Tages unterschiedlich stark gesteuert werden.

Zusammenfassend zeigt diese Doktorarbeit, dass schon kurzfristige starke Umweltfaktoren (wie eine gestörte innere Uhr) in Vätern, sich stark auf den Metabolismus und das Verhalten der Nachfahren auswirken können, auch wenn diese niemals diesem Umweltfaktor selbst ausgesetzt wurden. Wir sehen unsere Ergebnisse daher als wichtigen Beitrag um Risikofaktoren für Typ 2 Diabetes und metabolische Erkrankungen besser erklären zu können. Die Erforschung der Störungen der inneren Uhr und ihre Auswirkungen auf die Nachkommen ist in der heutigen Zeit, in der immer mehr Menschen in der Nacht oder als Schichtarbeiter arbeiten, besonders wichtig. Daher soll diese Veröffentlichung ein weiteres Puzzleteil darstellen, das dabei hilft zu verstehen, wieso in der heutigen Zeit immer mehr auch jüngere Menschen an Typ 2 Diabetes erkranken.

INTRODUCTION

TYPE 2 DIABETES

Type 2 diabetes (T2D) has become a pandemic over the past few years and currently around 500 million people are suffering from this disease [1](Figure 1). Epidemiological trends show that the number of people living with type 2 diabetes will continue to rise in the next few years (Figure 2). Once a disease of affluence of the west, type 2 diabetes has now spread to every country in the world and the greatest increases are expected to occur in Sub-Saharian Africa, the Middle East North Africa and South-East Asia (Figure 2). Increasingly sedentary lifestyles and transition to a westernized diet with high intake of refined carbohydrates are factors that have contributed to the increasing prevalence of type 2 diabetes in developing countries.

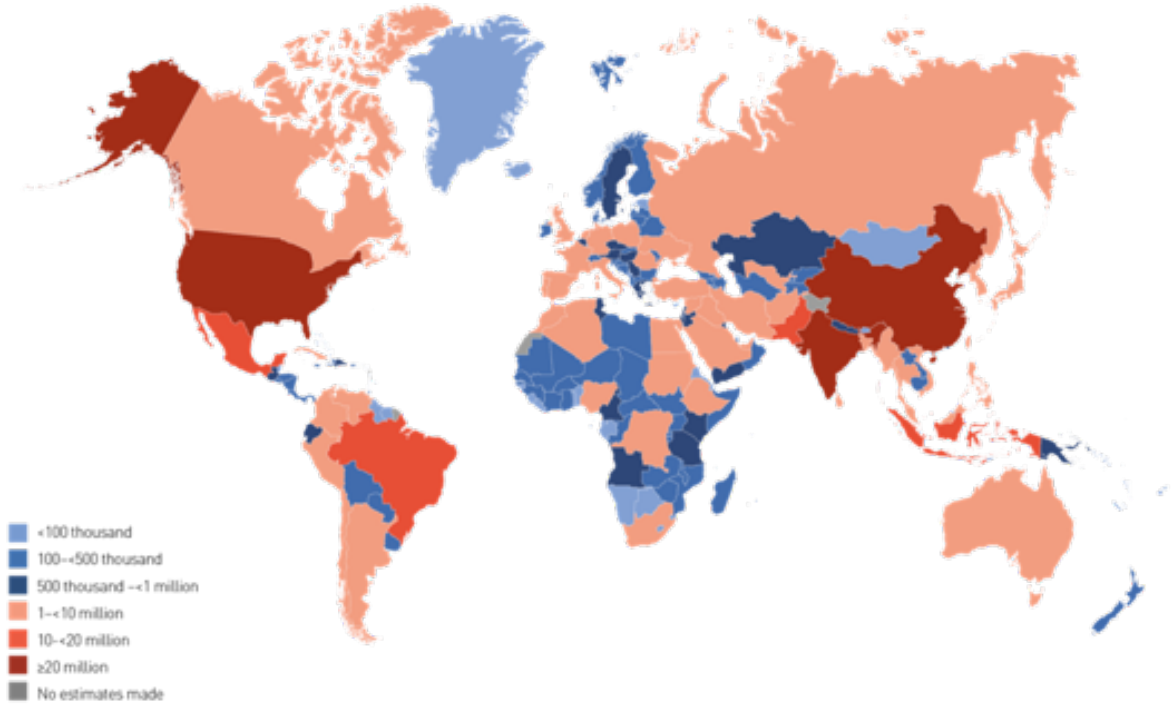

Figure 1: Number of people (20-79 years) with diabetes, 2019
The image shows the number of people with diabetes in a heatmap per country. The red color shows a higher incidence of diabetic people. The blue color shows a lower number of diabetic people [1].

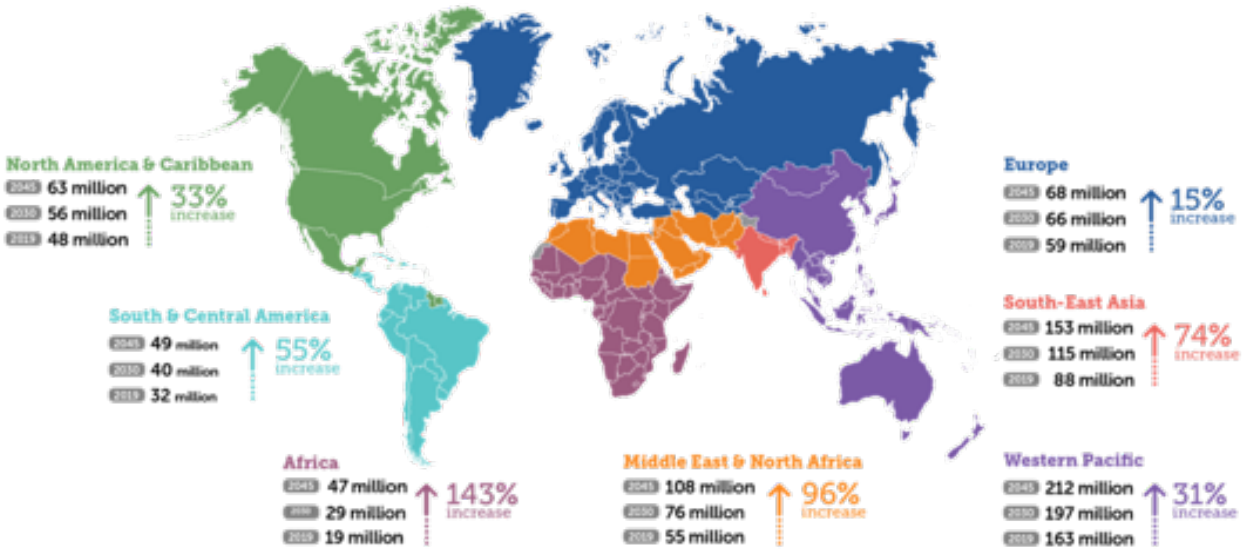

Figure 2: Number of people (20-79 years) with diabetes globally and by IDF Region
The image shows the increasing number of people suffering from diabetes with every 10 years. The forecast predicts even increasing numbers until the year 2045 [1].

Many people diagnosed with type 2 diabetes cannot achieve their target blood sugar levels with exercise and diet alone but need to take diabetes medications including insulinemic drugs [2] such as metformin [3-5], sulfonylureas [6], thiazolidinediones [5], DDP-4 inhibitors [6, 7], GLP-1 receptor agonists [7, 8] or SGLT2 inhibitors [9]. Type 2 diabetes is a chronic disease which increases the risk of developing co-morbidities [10] such as cardiovascular diseases, chronic inflammation and metabolic syndrome. Type 2 diabetes is, however, not only a big health and social problem [11], it is also a large burden for the health care system, leading to tremendous costs for patients as well as governments [12-14] (Figure 3). It is expected that as the incidence of type 2 diabetes is rising, the cost for the treatment of the disease and its complications will increase drastically and will impose a substantial economic burden on patients and healthcare systems [15].

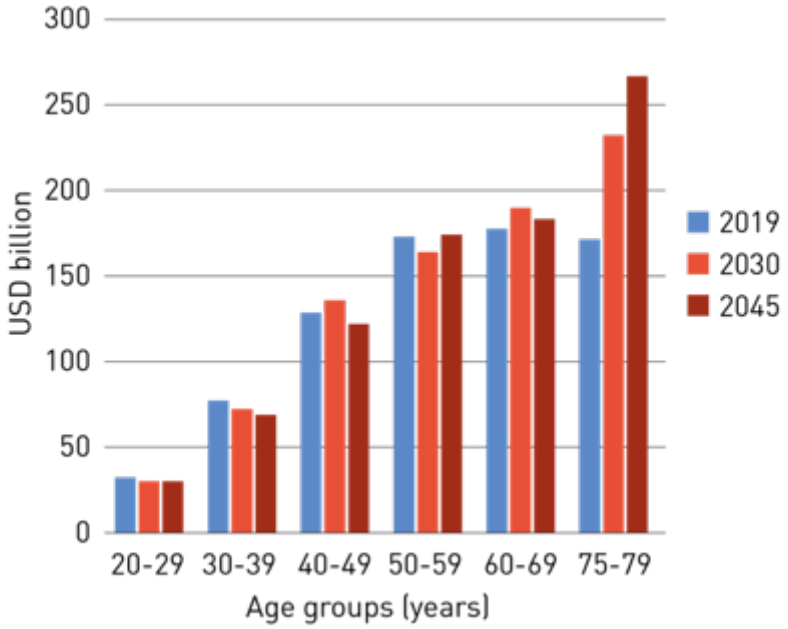

Figure 3: Total diabetes-related health expenditure (USD billion) by age group
Costs related to diabetes expenditures by age groups and for the years 2019, 2030 and 2045 [1].

METABOLIC HOMEOSTASIS

Glucose metabolism

In order to understand the pathomechanisms of type 2 diabetes, one has to understand how food is metabolized in our bodies. Food composition can be grossly subdivided into 3 macronutrients: carbohydrates, proteins and fats. Carbohydrates are, chemically speaking, chains of saccharides, often labelled as polysaccharides. To metabolize carbohydrates, polysaccharides are further digested by enzymes (such as amylase, maltase, sucrase and lactase) into oligo- or monosaccharides. The most abundant mono- and di-/oligosaccharides are glucose (monosaccharide), fructose (monosaccharide) and sucrose - a disaccharide consisting of one glucose bound to one fructose molecule (Figure 4).

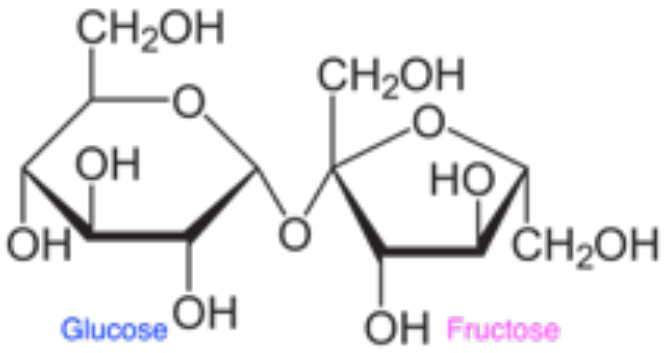

Figure 4: Sucrose molecule
A sucrose disaccharide molecule consist of a glucose and a fructose monosaccharide molecule [214].

Glucose homeostasis is therefore dependent on the interaction between insulin which lowers blood sugar levels and glucagon which increases blood sugar levels. For proper function the human body depends on a tight control of blood sugar levels [16]. Any defects, or diseases such as insulin resistance, in this system can lead to hyperglycemia (high blood sugar levels) or hypoglycemia (low blood sugar levels) that can lead to life-threatening dysfunctions. The roles of the pancreas, the liver and the muscles in the metabolism of glucose will be described in more detail in the following paragraphs in more detail.

Pancreas

In glucose homeostasis, glucose requires the hormone insulin in order to be taken up into the cells from the blood stream (Figure 5). The increase in blood sugar causes insulin to be released from the beta cells of the Langerhans islets in the pancreas. Insulin then binds to its transmembrane receptor (IR) and initiates an intracellular signaling cascade by autophosphorylation and recruitment of insulin receptor substrate 1 (IRS1).

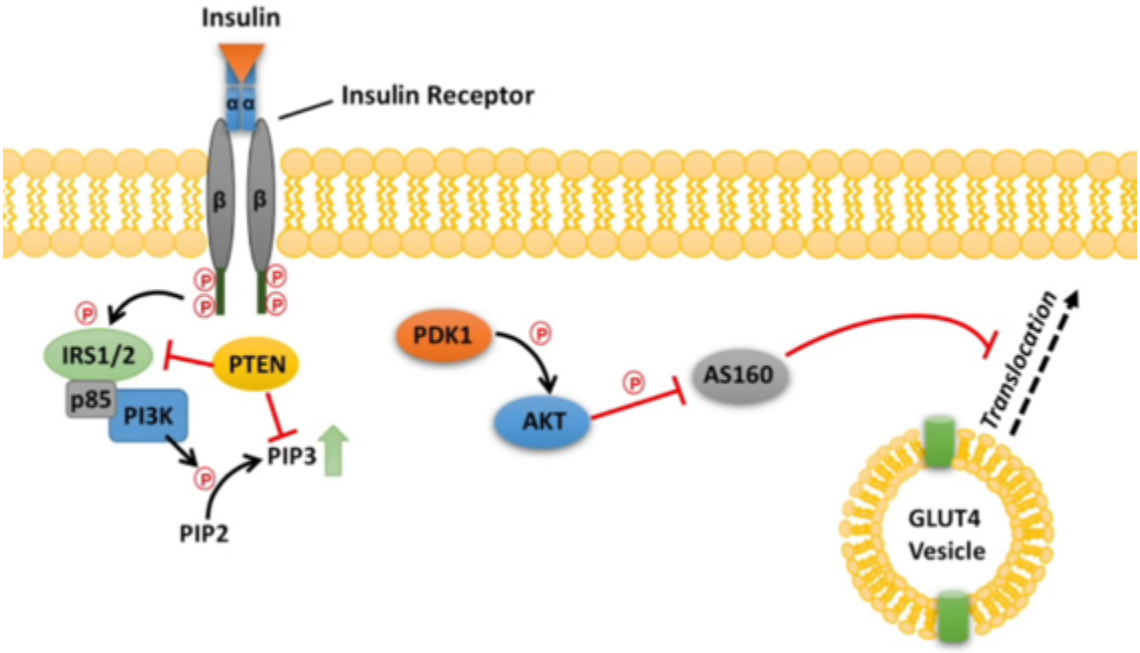

Figure 5: Insulin receptor signaling pathway and GLUT4 translocation
Scheme of the insulin receptor signaling pathway: Insulin binds to the insulin receptor and activates a cascade of PI3K, PIP2 and PIP3. This further activates PDK1 and phosphorylates AKT to inhibit AS160. As a result, GLUT4 translocates to the cell membrane to allow excess glucose uptake. Modified from [215].

Activated IRS1 binds to the enzyme Phosphoinositide-3Kinase (PI3K), which phosphorylates Phosphatidylinisitol 4,5-bisphosphate (PIP2) to Phosphatidylinisitol (3,4,5)-trisphosphate (PIP3). PIP3 is a necessary cofactor for the phosphoinositide- dependent kinase 1 (PDK1), which phosphorylates and activates Proteinkinase B (PKB – also known as AKT). The phosphorylation of PKB inhibits AS160 (a substrate of 160kDA – originally known as TBC1 domain family member 4- TBC1D4). Being inhibited, AS160 can no longer inhibit the translocation of the GLUT4 into the cell membrane. Through the initiation of this complex signaling cascade, insulin allows excess glucose in the post-prandial period to pass though the GLUT4 (gene: SLC2A4) transporter in skeletal muscle and adipose tissue.

Postprandial glucose is distributed in equal parts to the liver, insulin-sensitive tissues (muscles and fat) and noninsulin-sensitive tissues (central nervous system, red blood cells) (Figure 6). Apart from glucose uptake and utilization, insulin also controls glucose storage in the form of glycogen, by activating PKB/AKT. Insulin also activates glycogen synthase (GS) by inhibiting the Glycogen Synthases Kinase 3 (GSK3). Metabolic pathways are also induced

by the uptake of glucose into the cells. Through the activation of PKB/AKT, mTORC1 phosphorylates its downstream target p70s6K, thereby starting protein synthesis and other metabolic pathways.

In terms of fat metabolism, insulin inhibits lipolysis, the process in which triglycerides are hydrolyzed into glycerol and fatty acids. Taken together, insulin works as a strong anabolic hormone, that regulates a variety of metabolic mechanisms to store nutrients [17].

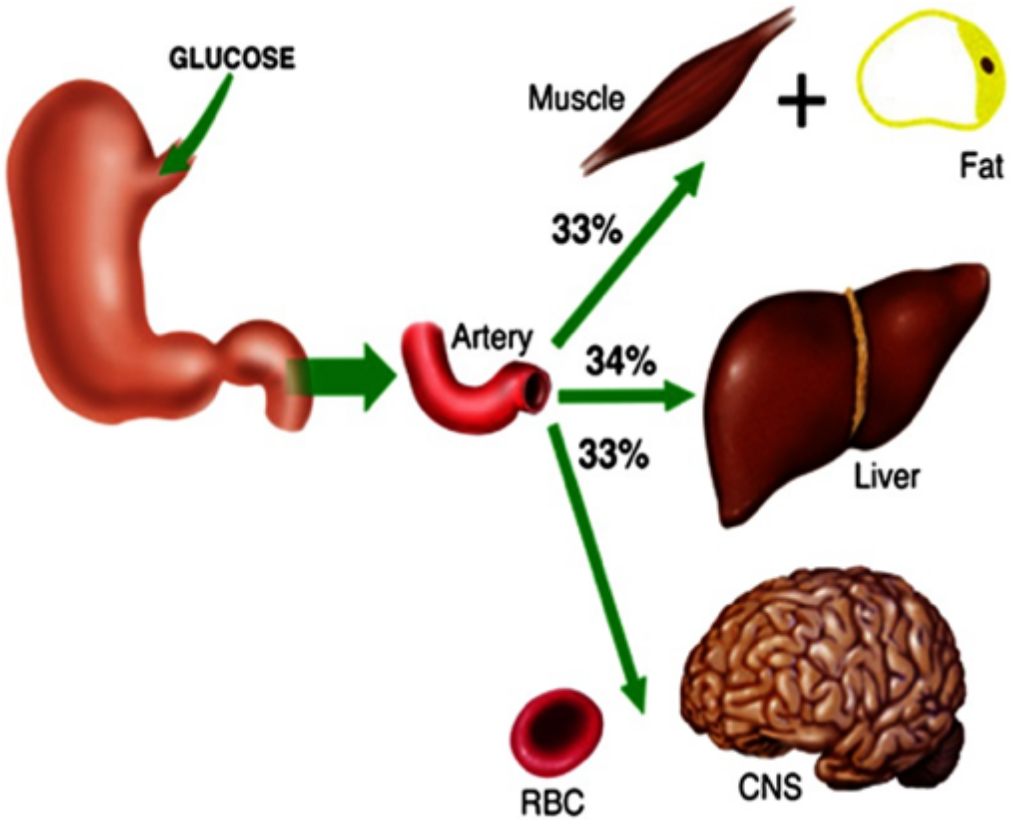

Figure 6: Distribution of glucose in the body
Glucose is distributed to muscles and fat tissue, the liver and red blood cells and the central nervous system. Glucose therefore fuels various metabolic pathway and is a crucial contributor in energy homeostasis. Modified from [18].

Liver

The liver performs a great number of vital functions in the human body and also plays a crucial role in glucose homeostasis. Its main functions include:

- Storage of glucose in the form of glycogen (~100g) [19]
- Release of glycogen in the form of glucose
- Transformation of amino acids, fatty acids and lactate into glucose via gluconeogenesis
- Amino acid synthesis & degradation
- Lipid metabolism: cholesterol synthesis & lipogenesis
- Production of bile
- Breakdown of insulin, hormones, bilirubin, toxic substances & drugs
- Storage of vitamins & minerals

By allowing for a constant flux between storage and release of glucose, the liver allows the body to buffer any changes in blood glucose levels between fasting and postprandial state [18].

Muscle

The largest reservoir of glucose are our skeletal muscles (~500g glycogen) [19]. Muscles lack the enzyme glucose-6-phosphatase, which is required to transform glucose-6-phosphate into glucose. Therefore glucose stored in the muscles in the form of glycogen cannot be released into the bloodstream and is solely used by the muscle cells as main energy substrate during exercise. Dependent on the human's physical properties and the intensity of the exercise, around 70% of the carbohydrate source is muscle glycogen [20].

Exercise training enhances insulin sensitivity in the muscles and furthermore increases expression of proteins GLUT4, hexokinases II and glycogen synthase which leads to an increase in glucose uptake into the muscles and therefore impacts whole body energy metabolism [21]. The body constantly uses glycogen for exercise and rebuilds glycogen storage in times of rest.

Central nervous system & red blood cells

The mammalian brain depends upon glucose as its main source of energy. The largest proportion of energy is consumed for neuronal activity, requiring continuous delivery of glucose from blood [22]. Thereby, the human brain consumes ~5,6mg glucose per 100g human brain tissue per minute [23, 24].

Pathomechanisms of type 2 diabetes

Type 2 diabetes is a chronic disease caused by a growing insulin resistance compensated (at least in the early phases of the disease) by an increase in insulin secretion. With time, insulin resistance results in an impairment in insulin secretion and overt hyperglycemia. This pathogenesis was demonstrated in a study that examined the incidence of type 2 diabetes among initially healthy subjects. Over the course of a median of 9.7 years of follow-up, 505 of the subjects were diagnosed with type 2 diabetes. In comparison to the people who remained healthy, the type 2 diabetics showed a marked decrease in insulin sensitivity during the five years prior to diagnosis. Three to four years before the diagnosis, the people who developed type 2 diabetes had an increase in insulin secretion and at diagnosis they had a decreased insulin secretion [25]. Type 2 diabetes is accompanied by different other health issues that are summed up as metabolic syndrome (hypertension, high serum low-density lipoprotein (LDL) cholesterol concentrations, and low serum high density lipoprotein (HDL) cholesterol). Insulin resistance and the resulting lack of insulin together with changes in lipid homeostasis, increase an individual's cardiovascular risk. People suffering from type 2 diabetes are two to three times more likely to get a heart attack or stroke than people who do not have diabetes.

Stage	Fasting blood glucose [mg/dl]
Normal	< 100
Pre-Diabetes	100-125
Type 2 Diabetes	>125

Table 1: Stages of type 2 diabetes and fasting blood glucose values. Fasting is defined as no caloric intake for 8 or more hours.

Eating food with a high glycaemic index and eating too much food raise the blood sugar and insulin levels and may increase the risk of developing pre-diabetes and later type 2 diabetes. Since all carbohydrate-based foods also contain glucose, they all require the release of insulin (Figure 7). Pre-diabetes is represented by reduced insulin sensitivity and elevated fasting blood glucose levels (e.g. glucose levels in the morning after night-fasting). While the blood glucose levels of a healthy individual are defined as <100 mg/dL, pre-diabetes is defined by blood glucose levels between 100-125 mg/dL. Interestingly, while indicative of early diabetes, pre-diabetes is still fully reversible by lifestyle interventions (diet/lifestyle/workouts), which prevent the onset of overt diabetes and the need for medications.

Carbohydrates, however, do not only consist of glucose but also to a large part of fructose or sucrose. Sucrose is a disaccharide consisting of a glucose and a fructose molecule. Fructose in the form of its chemically produced sucrose-analogue high fructose corn syrup (HFCS), is often used as a sweetening additive in the food technology and food processing industry due to its sweet taste. In 2009 the average American consumed about 35.7 pounds of high fructose corn syrup. Between 1970 and 1990 the consumption of high fructose corn syrup increased by 1000 % [26-28]. During this time the number of people with type 2 diabetes in the USA increased by 200%. While this is not the sole cause for the type 2 diabetes pandemic, it shows that not only the high consumption of glucose but also of fructose plays a role in the type 2 diabetes pandemic [29] [30].

Contrary to glucose, fructose is taken up by the insulin independent receptor GLUT5 (gene: SLC2A5) in the small intestine. The uptake of fructose is significantly slower than that of glucose.

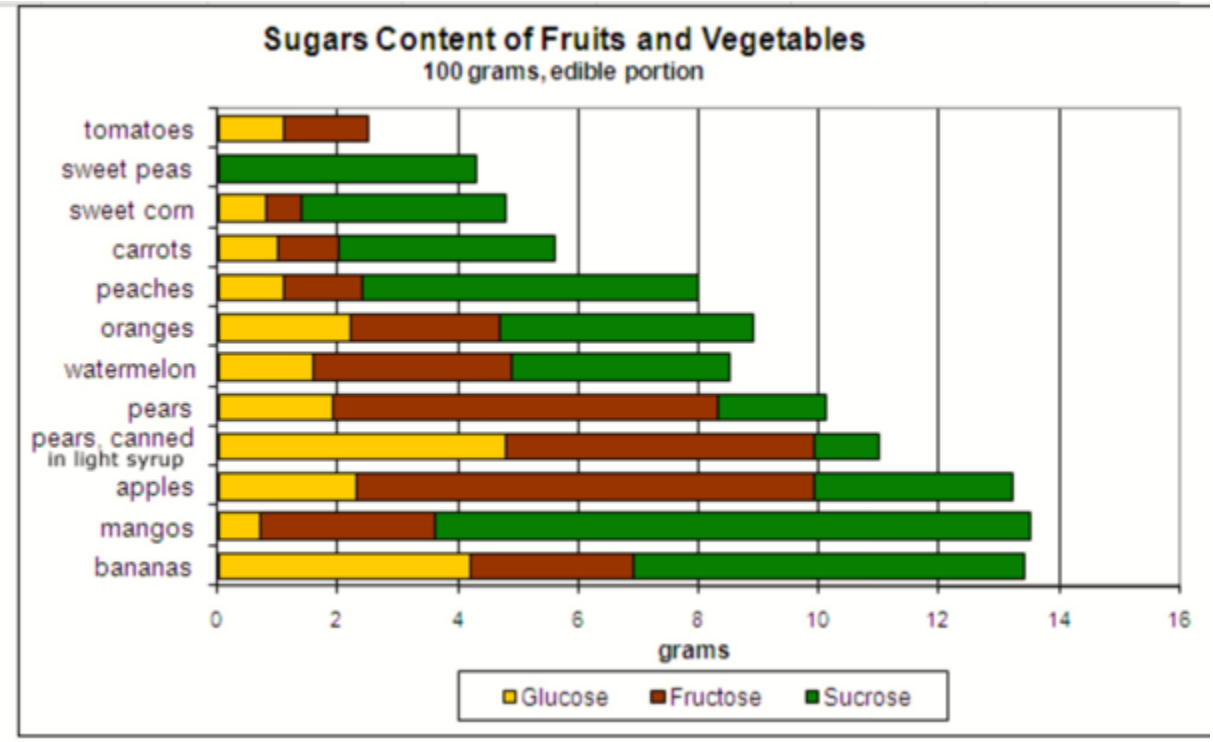

Figure 7: Distribution of glucose, fructose and sucrose in different fruits and vegetables
Glucose, fructose and sucrose are the main sugars in human nutrition. Fruits and vegetables contain these sugars in different distributions [216].

RISK FACTORS OF TYPE 2 DIABETES

Obesity & physical inactivity

The most prominent risk factor for type 2 diabetes is high body weight which leads to obesity [31, 32]. In the US, over 40% of the population older than 20 years are obese (BMI>30kg/m^2) and approximately 10% of the population has diabetes [33]. Although not every obese human will eventually develop type 2 diabetes and not everyone with type 2 diabetes is obese, obesity significantly increases the risk. Obesity causes insulin resistance and movement impairment. The precise mechanisms linking obesity and type 2 diabetes

still remain unclear, but recent studies have identified certain interactions such as proinflammatory cytokines, insulin resistance and deranged fatty acid metabolism that play a role in the pathogenesis of the disease. Already in 1991, Björntop et al. showed that not only the degree of obesity, but also the fat distribution impacts the type 2 diabetes risk. Increased upper body fat including visceral adiposity, was associated with a higher incidence of metabolic syndrome, type 2 diabetes, and cardiovascular diseases [34]. This theory was strengthened in 2009 by Cypess et al. who identified the importance of brown adipose tissue in humans and furthermore showed that adipose tissue can have different metabolic functions, based on its location [35]. Insulin resistance and impaired mitochondrial activity has been linked to both obesity and type 2 diabetes [36]. Therefore, Eckel et al. concluded that "mitochondrial dysfunction could be one important underlying defect linking obesity to diabetes, both by decreasing insulin sensitivity and by compromising beta cell function" [31].

Low physical activity is another major risk factor for developing type 2 diabetes. Studies have shown that both physical inactivity and obesity seem to be strongly and independently associated with diabetes and diabetes-related comorbidities [37]. We can conclude that physical activity prevents obesity and therefore reduces the type 2 diabetes risk. Also, studies have shown that physical activity directly impacts glucose metabolism and therefore insulin homeostasis [38]. To conclude, the scientific community agrees on the benefits of regular exercise and recommends avoiding long periods of physical inactivity to decrease the risk of developing type 2 diabetes.

Genetic and epigenetic factors

Studies have shown that certain genetic changes are linked to the occurrence of type 2 diabetes. Gene variations, known as single nucleotide polymorphisms (SNPs) discovered by genome-wide association studies (GWAs), could be linked to an increased risk for developing type 2 diabetes. Nevertheless, gene variations alone neither explain the rapidly increasing incidence of the disease nor the decreasing age of onset [39]. The lifetime risk of developing type 2 diabetes is 70% if both parents are affected. As the risk is not 100% if

both parents are affected, this indicates that also other factors play a role in the potential development of type 2 diabetes.

A growing body of literature has therefore investigated not only the genetic, but also the environmental and hence epigenetic factors that may play a role in the inheritance of diabetes risk susceptibility. It could be shown that several environmental challenges correlate to certain phenotypes not only in the F1 generation but even in the F2 or F3 generations. The following epigenetic factors have been investigated: exposure to different kinds of diet [40-42] [43, 44], chemicals/additives in drinking water [45, 46], restriction of the food intake times (e.g. feeding only during the resting time) [47-49], intermittent fasting / fasting mimicking diet [50, 51], changes in the light cycle [52, 53], exposure to different temperatures (cold, heat) and humidity [54, 55], exposure to psychological stress (changes in the mouse cage) [56], drugs [57, 58], and odors [59, 60].

The changes were not only visible if the F0 generation was exposed to the environmental factor over a longer period of time, but even if the F0 generation was only exposed over a short period of time [47]. The exact timing of the exposure such as during puberty and before or during pregnancy may also play an important role in the body's "epigenetic memory". Although not fully understood yet, the discovery of acquired epigenetic inheritance of disease risks is providing alternative potential explanations for the exponential increase in the incidence of type 2 diabetes. Epigenetic inheritance will be further discussed later in the corresponding chapter.

Circadian arrhythmia

Circadian arrhythmia is defined as a disruption of the circadian clock. Especially in combination with an unhealthy lifestyle, circadian arrhythmia has been shown to be another major risk factor for type 2 diabetes. The Nurses' Health Study conducted in 1976 and The Nurses' Health Study II conducted in 1989 were two of the largest and first studies demonstrating that circadian arrhythmia was a risk factor for the development of type 2 diabetes. 143410 nurses who were working nightshifts were followed over a period of 22-24 years. 10915 of the 143410 initially healthy nurses developed type 2 diabetes. For every

five years of working rotating night shifts, the nurses were 31% more likely to be diagnosed with type 2 diabetes. If the nurses also had an unhealthy lifestyle, for instance if they were smokers or overweight or even obese, their risk of developing type 2 diabetes was 2-3 times higher. The Nurses' Health Study was the first study to link circadian arrhythmia and unhealthy lifestyle with type 2 diabetes.

According to the Bureau of Labor Statistics almost 15 million Americans work full time on evening shift, night shift, rotating shifts, or other employer arranged irregular schedules [61] and the 30 occupations with the largest projected job growth between 2006 and 2016 were those with nonstandard work schedules. As this has become a central part of our modern society a growing amount of research is being conducted about the strong impact these working hours have on our natural diurnal biological clock and how this causes diseases such as type 2 diabetes.

CIRCADIAN RHYTHM

Molecular and hormonal determinants of circadian rhythm

The circadian clock is an adaptation to the rotation of the earth. It confers a 24-hour program on processes at all levels, including gene expression, hormonal secretion, and behaviors such as sleeping. The circadian clock is found in organisms from all phyla [62]. It allows organisms to coordinate their behavior with changes in their physical environment that are linked to daylight and nighttime [63]. It prevents, for instance nocturnal animals from venturing out of their caves during daytime and from being easily spotted and eaten by other animals. Thus, this synchronization ensures the survival of the species. The earliest research in this field was conducted by de Mairan in the 1700s [64, 65]. His observations showed that a plant, which moves its leaves up and down, will do so even if it is not exposed to sunlight. De Mairan's hypothesis was that these movements represented something more than a simple response to sunlight and were controlled by an internal clock [64]. His observations inspired the beginning of the study of biological circadian rhythms.

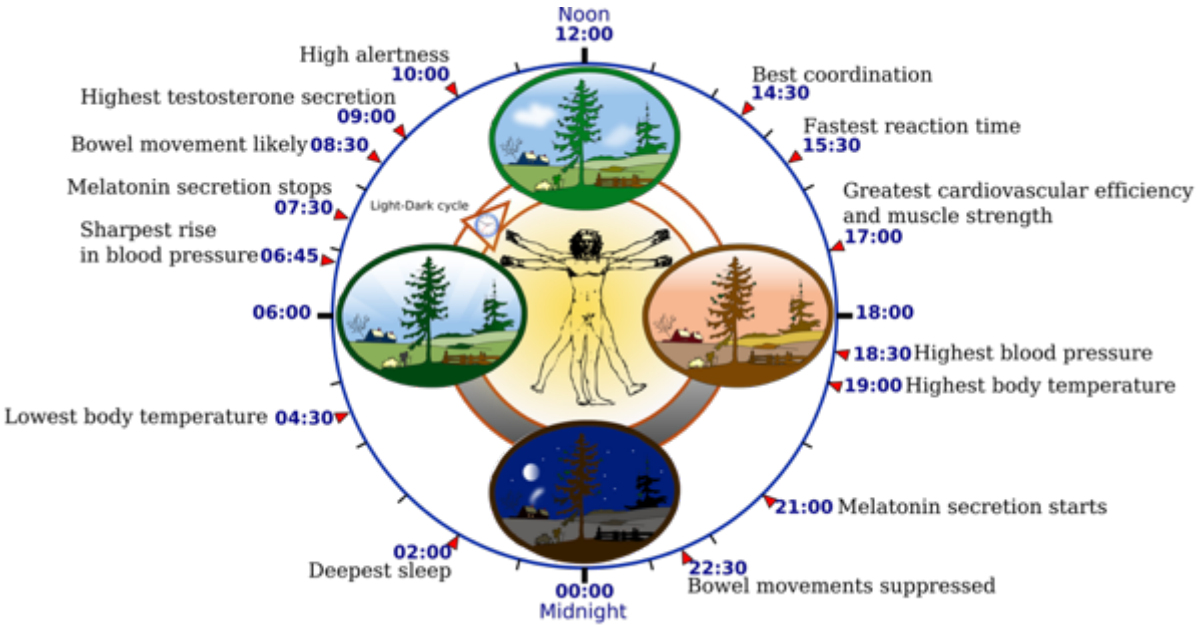

Figure 8: Circadian rhythm 24-hour scheme
The body acts differently at different times during a 24-hour day and shows significant differences in body temperature, hormone concentrations, blood pressure levels or behavioral traits such as alertness, coordination and reaction times [217].

The circadian clock has the ability to be synchronized to external time cues, such as the light-dark cycle. This is called entrainment. The circadian clock is deeply connected to human physiology. It regulates behavioral traits such as alertness and sleep, our immune system [66] and metabolism [67, 68] and therefore acts differently at different times during the 24-hour day (Figure 8). Manipulation of food intake has also been shown to impact metabolic functions [69, 70], including glucocorticoid levels [71, 72] and gut microbiota [73].

The intracellular molecular machinery ensuring circadian-dependent gene expression and function is highly complex. The four main transcription factors being: Clock (CLOCK), Bmal1 (ARNTL), Per (PER) and Cry (CRY). The proteins BMAL1 and CLOCK act as heterodimers to promote the expression of Per and Cry. The activation of these genes leads to the production of the proteins, PER and CRY. These protein in turn form a dimer that gradually inhibits the actions of BMAL1 and CLOCK – and with that the transcription of their own genes [74-76]. This whole process takes around 24 hours to complete, before it is repeated. Additionally, there is a second regulatory loop involving the nuclear receptor Rev-erb α and

ROR-α. Hereby they compete for the binding to RRE, which regulates repression / activation of Bmal1 [77] (Figure 9). Various intracellular processes are directly linked with these transcription factors and thus the synchronization of our cells to the 24-hour clock is ensured.

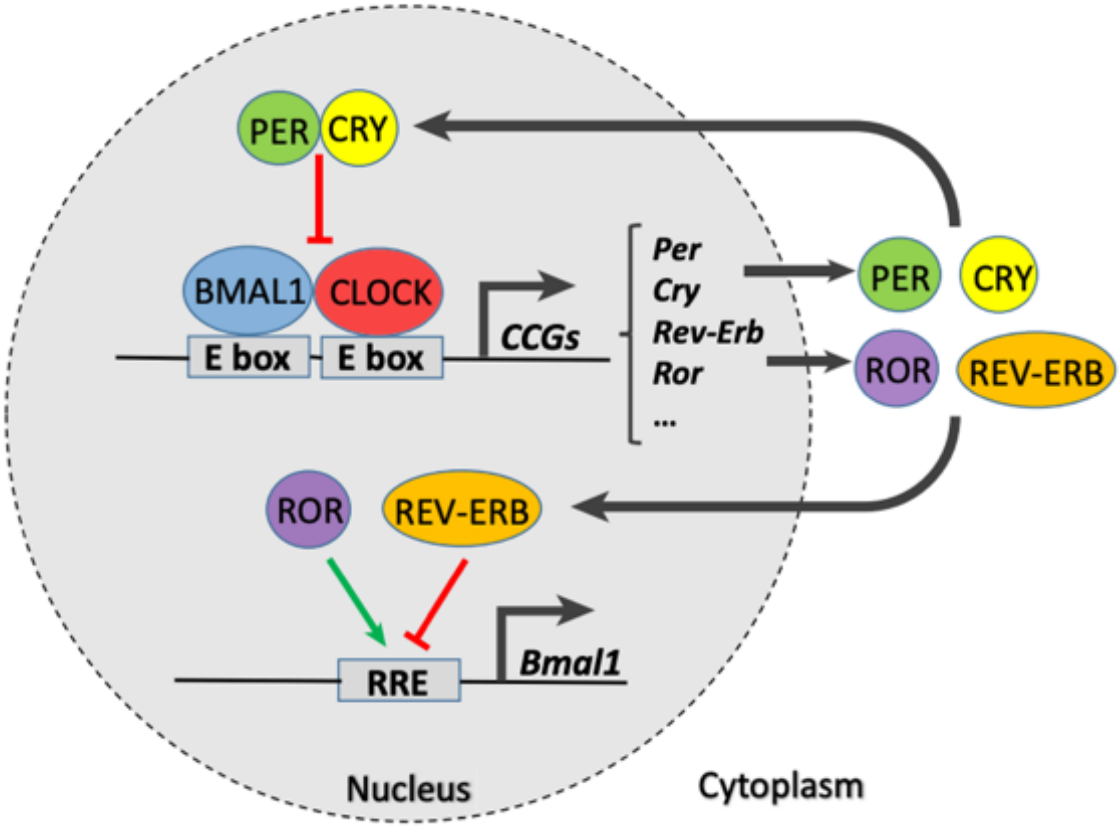

Figure 9: Circadian clock core genes and feedback loop
The circadian clock is mainly controlled by the core clock loop of BMAL & CLOCK and PER & CRY that oscillate around 24 hours. Nuclear receptors such as REV-ERB and ROR can also influence the response elements (RRE) and furthermore Bmal1 activity. Modified from [78].

The circadian rhythm is controlled by the suprachiasmatic nucleus (SCN) which is located on the anterior hypothalamus [79, 80]. Its neurons respond to changes in daylight and – through an intricate molecular system – entrain organismal circadian rhythm and body temperature rhythmicity [81]. In addition, there are internal clocks in organs (such as the liver and the heart) and tissues (including skeletal muscle) that are not entrained by the

dark/light cycle, and act independently of the master-clock in the suprachiasmatic nucleus [82-85]. Cellular NAD^+ level, for instance, shows circadian rhythm [86] and can affect the activity of posttranslational protein modification enzymes that use it as a co-substrate. This is how, for instance, several posttranslational modifications occur in the liver in response to cell-autonomous or extracellular signals that convey the feeding or fasting state. These posttranslational modifications include NAD^+ dependent acetylation and poly-ADP-ribosylation state of nonhistone proteins including some clock components. As many of these feeding-fasting–induced posttranslational changes also target clock components, they thereby also integrate the energy state with the molecular clock [87-89].

Desynchronization between the master-clock and internal clocks can happen if food intake is shifted to the night phase and the clock in the SCN is not aligned with the clock in the peripheral organs. In our 24/7 societies, a large portion of the workforce works during evening shifts or all night, which is counterintuitive to the biological processes in our body and eventually leads to circadian arrhythmia, stress and altered metabolic expressions. The above-mentioned Nurses' Health Study I and II have shown that circadian arrhythmia may lead to metabolic diseases such as impaired glucose homeostasis, obesity and higher likelihood of developing type 2 diabetes [70, 90]. Circadian clock dysfunction is also linked to cancer [91] and neurodegenerative diseases [92] such as familial advanced sleep phase syndrome where PER2 is mutated and leads to circadian arrhythmia.

The exact causative role of the dysfunction of the circadian clock still needs to be elucidated in more extensive studies. However, our knowledge of the circadian clock and its impact on cellular functions offers new therapy options. Since we know that cells are more susceptible to therapies at time when their target is primed and resistance mechanisms are ebbed, we can should use this knowledge to optimize the timing of pharmacotherapy and to enhance treatment efficiency [93].

Chronopharmacology

Although genome-wide studies have shown that the majority of drug target genes are controlled by the circadian timing system, chronobiology so far has only sparsely been implemented in the clinic. Studies by Thosar et al. could demonstrate that heart attacks and strokes occur more often in the morning hours [94] (Figure 10). Cancer therapy is the field in which the clinical application of chronopharmacology is greatest. The aim of the chronotherapeutic approach is to improve the tolerability and efficacy of the cancer therapy by applying it exactly when the tumor expresses proliferative targets relevant to cancer cell DNA synthesis or cancer cell division [95].

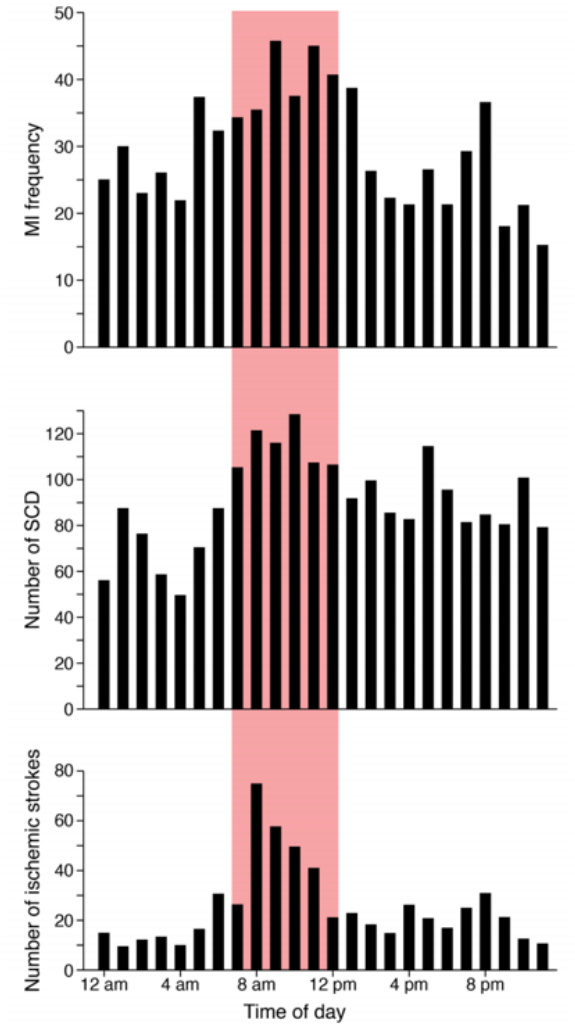

Figure 10: The day/night pattern of cardiovascular events
Epidemiological studies reveal that adverse cardiovascular events including myocardial infarction (MI), sudden cardiac death (SCD), and ischemic stroke increase in frequencies in the morning hours compared with other times of the day and night [94].

Chrononutrition

Not only chronopharmacology but also chrononutrition offers new clinical possibilities. Knowledge about how circadian arrhythmia affects our metabolism and thereby causes type 2 diabetes and metabolic diseases can be used to positively influence our eating habits and thereby prevent and even possibly reverse metabolic diseases. The importance of time-restricted feeding, where diurnal variants in oral glucose tolerance favored the morning meal, was earliest shown by Jarrett et al [96]. A comparison of the blood sugar levels showed that while the mean blood sugar levels of the oral glucose tolerance test in the afternoon and evening tests were similar for the tested patients, they were both significantly higher than those in the morning test. Since Jarrett's work in this field in the 1970's, we have gained further insight into how circadian rhythm affects a multitude of digestive processes including gastric pH and hormone secretion. The nascent field of chrononutrition therefore offers a paradigm shift: the timing of the diet that is consumed is equally, if not more important than the quality of the diet.

Without changing the amount of caloric intake or the diet composition, a change in the timing of the diet has been shown to have beneficial effects on the body [49, 70, 97]. This means, that there is a metabolic difference between ad-libitum food intake and eating the same amount of calories in a restricted time frame that is aligned with our circadian clock i.e. 8-10 hours during day time. Hatori et al. showed that mice that are fed a high-fat diet and had time-restricted access to food (12 hours during their active phase) not only have an improved oscillation of the circadian clock and their target gene expression but are also protected against obesity, hyperinsulinemia, hepatic steatosis, and inflammation. Time restricted feeding was found to improve nutrient utilization and catabolic and anabolic pathways [48].

The same results have been proven to equally hold true in human studies. Stekovic et al. showed in a randomized-control study that four weeks of restricted alternate day fasting improves various markers of general health [98]. Stekovic expanded Hatori's idea of time-restricted feeding. He not only investigated if food consumption in alignment with our natural clock (strict 12 hours) is beneficial in humans but also if times of complete fasting

(36-hours of fasting following food intake) are beneficial to our health. The study showed improved cardiovascular markers, reduced fat mass (particularly trunk fat), an improved fat-to-lean ratio, and increased β-hydroxybutyrate, even on non-fasting days. The mechanisms behind these results are not yet fully understood. However, they may be explained by a reduction in oxidative stress and thus a reduction in oxidative damage [99], as well as by the general improvement of metabolic functions via reduction of bodyweight, improved glucose tolerance and reduction in circulating insulin [100].

The safety and efficacy of caloric restriction and fasting diets are still debated, and more research is required to fully understand the exact processes behind these results. However, it is clear that our expanding knowledge about the circadian clock and the temporal orchestration of cell biology offers new possibilities to promote health and prevent diseases. Chrononutrition may offer a nonpharmacological strategy to reduce bodyweight long-term and thus to lower the risk of developing type 2 diabetes.

EPIGENETIC INHERITANCE

Circadian arrhythmia has been shown to adversely affect the health of the current generation. However, there are studies indicating that this effect may not be limited to the present generation but may even affect future generations. The offspring of rats, for example, that were exposed to chronic phase shifts during gestation had increased adiposity and hyperleptinemia when they were 3 months old. At 12 months, the female offspring of these rats displayed poor glucose tolerance and increased insulin secretion in response to an intraperitoneal glucose tolerance test [101]. These results highlight the need for a thorough analysis of how circadian arrhythmia exposure in utero affects the health of the adult offspring in humans. We also need to research how circadian arrhythmia and its effects are passed on to the next generation. A possible mechanism may be epigenetic inheritance. It has been demonstrated that the transgenerational plasticity of the plant circadian clock does not involve the alteration of clock gene DNA sequences, but instead manifests as reversible changes in the chromatin structure that determines the expression

of the core oscillator genes. Chromatin reshaping depends on epigenetic factors, such as histone post-translational modifications/replacements, which create a flexible loop of gene regulation. In the next chapter, epigenetic inheritance and its different mechanisms will be discussed.

Definition of epigenetic inheritance

According to Charles Darwin complex organisms have evolved from less complex ancestors as a consequence of accumulating spontaneous genetic mutations. These genetic mutations that occur under environmental pressure and are transmitted across generations result in completely different organisms and have survival advantages. This theory is best known as “natural selection”. Flies, for instance, with a mutation that resulted in their growing wings, survive and are so able to pass on their genetic mutations for wings onto their offspring. Flies that do not have this mutation are more easily caught by prey so they do not live long enough to pass on their genetic material [102].

Although Darwin’s theory of evolution is the most famous, many pre-darwinian naturalists had also conceptualized evolution. The best-known naturalist is Jean-Baptiste Lamarck. He anticipated Darwin in his theory of natural selection by pointing at the environment – independently from occurring genetic mutations – as the major driving force of evolution. Lamarck found out that when the environment changes, living organisms have to change their behavior (and their bodies) to survive. The giraffe's neck for instance must grow to enable the animal to eat the highest leaves. These acquired characteristics are passed on to the next generations to improve their adaptation and survival [103].

Being the first to postulate the transmission of non-genetic (or acquired) information across generations, Lamarck’s theory was strongly rejected by the community of Mendelian geneticists and modern molecular biologists until more than a decade ago, when it was rekindled by the discovery of acquired epigenetic inheritance. This change came when the British biologist C. Waddington discovered that exposure to ether (environmental change) caused the development of a second thorax in the developing embryos of Drosophila. He defined epigenetics as “the branch of biology, which studies the causal interactions

between genes and their products which bring the phenotype into being" [104]. Based on our current molecular understandings, Waddington's definition of epigenetics has been changed into "the group of molecular processes on DNA, which regulate genome activity independently from the DNA sequence". These changes are passed on to the next generation through the germline via environmentally modified epigenetic tags on the genome [103]. This is possible because epigenetic processes are mitotically stable.

Molecular mechanisms of epigenetic inheritance

Epigenetic inheritance was originally discovered in plants and lower organisms such as *C. elegans*. The mechanisms of epigenetic inheritance in these organisms is well understood. In mammals, the role of epigenetic inheritance has also been recognized. Experimental evidence suggests that parental health is important in defining offspring health and disease susceptibility. However, the underlying molecular mechanisms involved in mammalian epigenetic inheritance were grossly unknown until less than a decade ago. In mammals, epigenetic inheritance entails the intergenerational transfer of epigenetic information from gametes to the developing embryo. Upon fertilization and in the early stages of embryonic development (e.g. when the primordial germ cells are formed) the inherited epigenome is heavily reprogrammed. However, not all of the inherited genome is reprogrammed. The escaping loci constitute potential signals for epigenetic inheritance.

Chromosomes consist of chromatin fibers that are built from the DNA double-helix spun around histone proteins (Figure 11). To date, three mechanisms have been characterized as potential signals for epigenetic, intergenerational inheritance: DNA methylation (and in particular genomic imprinting), histone post-translational modifications and small non-coding RNAs.

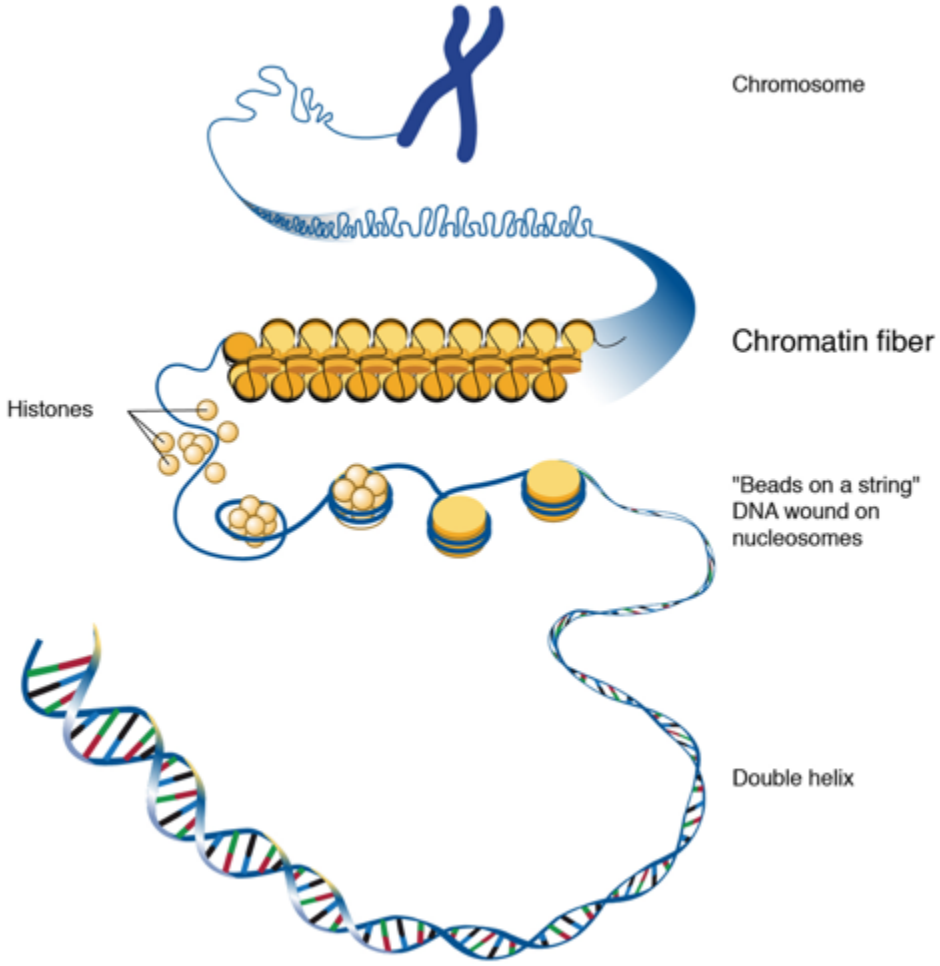

Figure 11: From chromosomes to double helix
Structure of a chromosome and the included microstructures. Chromatin fibers consist of the DNA double helix wound around histone proteins. Modified from [218].

DNA Methylation

DNA methylation describes the processes by which a methyl group is added (Figure 12) to DNA base pairs via three different DNA-Methyltransferases (DNMT1, DNMT3a and DNMT3b). In this process, the DNA is not altered but only modified. This leads to a suppression in the transcription and expression of the modified gene.

Only two of the four nucleotides can be methylated: cytosine (C) and adenine (A). Methylation of cytosine leads to the formation of 5-methylcytosine. This methylation primarily occurs at CpG sides (‘5-Cytosine-phosphate-Guanine-3’). Around 75% of the

mammalian CpG sites are methylated [105]. Cytosine can also be methylated at the 4' position, which results in N4-Methylcytosine. However, this form of methylation is mostly found in prokaryotes and not in eukaryotes. Adenine can be methylated at the 6' position to form N6-Methyladenine and is more frequently found in prokaryotes than in eukaryotes.

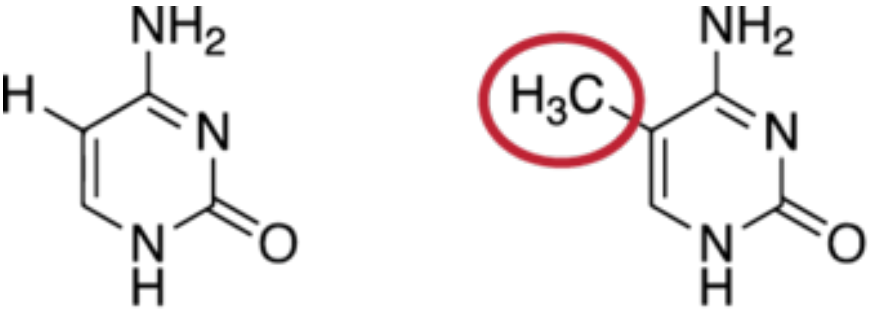

Figure 12: DNA methylation
Structure of cytosine and methylated cytosine as an example of DNA methylation. The methyl group (-CH3) is added at the 5 position of the pyrimidine ring and thus alters its function [219].

DNA methylation prevents gene activation by altering chromatin compaction and by reducing the affinity between the DNA and protein complexes. As a result, transcription and gene expression are up- or down-regulated. One of the most famous silencing events that involves DNA methylation is genomic imprinting [106]. In this process a subset of genes are expressed mainly from one allele, resulting in only one single parental allele being expressed [107, 108]. Imprinting is characteristic of mammals and affects around ~125 genes in humans and 100 in mice in a parent-of-origin specific manner. Imprinted genes are expressed during early development and are important for the formation and the function of the placenta [109], as well as for proper offspring development and health. Problems during the establishment of imprinting lead to severe human diseases, such as Prader-Willi, Angelman syndrome or male infertility [110].

Histone modifications

A nucleosome is the core component of chromatin and consists of four histone protein dimers (2x H2A, H2B, H3, H4) and 146 base pairs of DNA wrapped around them (Figure 13).

H1 acts as a linker histone and is crucial for the formation and packaging of the nucleosome. Similar to DNA methylation, posttranslational histone modification does not affect DNA nucleotide sequence, but can modify its availability to the transcriptional machinery [111]. The different types of histone modification are acylation, methylation, phosphorylation and ubiquitination. These different types of modification each impact the chromatin structure and genome function in unique ways. For example, histone acetylation usually leads to higher gene expression, while histone methylation has either a permissive or repressive function on transcription – depending on the location of the targeted amino acid [112].

Histone acetylation is regulated by either histone acetyltransferases (HATs) or histone deacetylases (HDACs). HATs are responsible for transferring an acetyl group from acetyl CoA to an amino group of the target residues on the histone tails. This process makes chromatin less compact and thus more accessible to transcription factors. By contrast, HDACs remove the acetyl group from the histone tail, which results in a repression of gene expression [113]. Recently, the role of histone post-translational modifications (PTM) in epigenetic inheritance in lower organisms and in mammals has been studied. It was demonstrated that germline patterns of histone PTMs regulate early embryonic development and the assembly of embryonic heterochromatin [114-116], thus potentially influencing embryonic development and adult phenotypes.

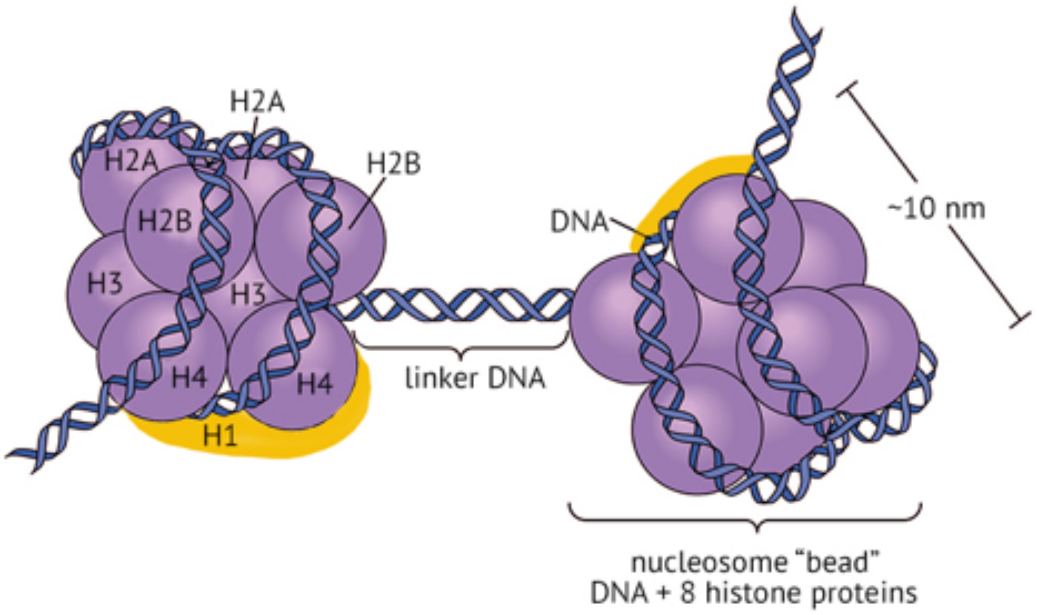

Figure 13: Histone modifications
Picture of a nucleosome consisting of the DNA double helix wound around histone proteins. Alterations or modifications of histones impacts the chromatin structure and functions [220].

In mature spermatozoa, the vast majority of histone-containing nucleosomes are replaced by protamines to allow sperm DNA to fit the small and hydrodynamic shape of the nucleus. Few loci (~1% in the mouse and 10% in humans) retain the nucleosomal structures and therefore represent the only potential sites that are directly inherited from one generation to the next. Only environmentally induced epimutations in these loci that retain their nucleosomal structure are passed on to the next generation.

In mouse spermatozoa, retained nucleosomes are located at non-methylated CpG islands [117] and contain histones that are mostly modified on the lysine 4 and/or 27 of the histone H3 (H3K4me or H3K27me) [118]. Genetic studies in mice have demonstrated that alteration of either H3K4me or H3K27me leads to intergenerational phenotypes. In particular, perturbation of H3K4me during spermatogenesis by overexpression of the K4-specific demethylase LSD1 (Lysine Specific Demethylase 1) alters the development in wild-type offspring across several generations [119].

Small RNAs

Small RNAs such as microRNAs (miRNAs), non-coding RNAs (ncRNAs) or transfer RNAs (tRNAs) have been discovered as potential transmitters of epigenetic information [40, 120]. Small RNAs were also found in spermatozoa, which could potentially mediate intergenerational transmission of paternally acquired phenotypes, such as mental stress and metabolic disorders [121]. Therefore, small non-coding RNAs, maternally or paternally provided, are suspected to play an important role in the pre-implantation development and the inheritance of acquired phenotypes [122].

Zygotic injection of "challenged" RNAs, indeed reproduces parentally acquired phenotypes, thus indicating small RNAs as important carriers of acquired epigenetic information [121]. Several environmental challenges, such as psychological stress [123-126] diet and exercise [127-129] can alter the abundance and composition of RNAs carried by the germ cells. Both microRNAs and tRNA fragments (tRFs) have been shown to mediate epigenetic inheritance of acquired phenotypes [40, 120, 123, 130]. Although the exact molecular mechanisms are still largely unknown, evidence indicates that inherited small RNAs control the expression of retroelements and their "target" genes [130] during early development. More recently, post-transcriptional RNA modifications, and in particular methylation of tRNA fragments, have been demonstrated to be necessary for paternal inheritance of diet-induced metabolic phenotypes [121]. While tRNA fragments seem to be predominant determinants of paternal intergenerational effects, genetic studies in mice have shown miRNAs to be important mediators of both paternal and maternal transmission. For example, conditional deletion of Dicer1 in male and female germlines impairs early embryonic development leading to highly penetrant embryonic lethality [106, 131, 132].

These and several other pieces of experimental evidence suggest that small-RNAs play a crucial role in epigenetic inheritance of parentally acquired phenotypes.

ALTERNATIVE MECHANISMS OF EPIGENETIC INHERITANCE

The above mentioned mechanisms previously explain the majority of the epigenetic phenotypes and may also explain the link between metabolic syndrome symptoms and type 2 diabetes in shift workers and their offspring. However, not all epigenetic inheritance mechanisms have been identified since the inheritance of some phenotypes has yet to be explained. It is therefore believed that other mechanisms, described below, may also contribute to the inheritance of deranged metabolic phenotypes via circadian arrhythmia. These mechanisms will be described below.

Seminal fluid and embryonic development

Paternal inheritance, for instance, may not only be transferred by DNA and epigenetic alterations in the spermatozoa but also via seminal fluid [133]. Seminal fluid consists of heterogeneous organic and non-organic substances such as hormones, amino acids, sugars, citric acid, exosomes, small RNAs. As seminal fluid stays in the reproductive tract for the first five days after fertilization, it may impact the oocyte and all embryonic stages up to the blastocyst [134, 135]. In his paper, Bromfield examined if ablating the plasma fraction of seminal fluid by surgical excision of the seminal vesicle gland had any consequences for the offspring. He could show that conception was substantially impaired and that when pregnancy did occur, placental hypertrophy was evident in late gestation. The embryotrophic factors Lif, Csf2, Il6, and Egf are down-regulated and the apoptosis-inducing factor Trail was up-regulated, which resulted in altered growth trajectories in the offspring, most profoundly in males. The offspring of these males exhibited obesity, distorted metabolic hormones, reduced glucose tolerance, and hypertension [136]. These findings show that seminal fluid has a lasting effect on the reproductive tract and thus on the environment of the embryo. Since during in-vitro fertilization (IVF) only the spermatozoa and not the seminal fluid is transferred to the oocyte, this method allows to distinguish between phenotypes in the F1 generation that are solely caused by epigenetic changes of the spermatozoa and phenotypes that are caused by the seminal fluid.

Influence of glucocorticoid and the HPA axis on the metabolism

Cortisol levels are also highly impacted by the circadian clock, since our cortisol level naturally peaks after the "waking up"-phase to anticipate activity and feeding. Light has also been shown to activate the adrenal gland and to lead to the release of glucocorticoid [137]. This is exemplified by the fact that night shift workers, who are exposed to circadian arrhythmia, have a higher stress level, and therefore higher levels of basal cortisol are released by the hypothalamus pituitary adrenal (HPA)-axis [138, 139]. Data shows that shift workers do not have a peak in cortisol levels in the morning, as they are missing the natural "waking up" phase [140]. Chronically elevated levels of cortisol can not only impact the body's metabolism but also oocyte development and thus reproductive functions [141]. Since the embryos of pregnant shift workers are exposed to higher cortisol levels, their development may be impaired [142]. Studies, for instance, show that women with elevated cortisol levels have a higher risk of having a preterm birth [143].

DEVELOPMENTAL WINDOWS OF SUSCEPTIBILITY

Not every period of life is equally sensitive to environmental challenges for the establishment of environmentally induced heritable epigenetic alterations. Therefore, the timing of when the parents are exposed to shift work may influence their offspring's chance of developing type 2 diabetes. Human observational studies on the Dutch Hunger Winter of 1944/1945 and the Överkalix study as well as the growing number of studies in mammalian animal models have identified several critical windows of susceptibility. These studies provide clear evidence that the preconceptional, the early developmental, the gestational and the early life periods are sensitive periods for changes in the environment. Among those - and in keeping with the definition of epigenetic inheritance given above - only the preconceptional and, to some extent, the early developmental periods can lead to parentally acquired epigenetically inherited phenotypes. Acquired parental information embedded into oocyte and/or sperm epigenomes is to a certain extent transferred to the offspring at conception.

Studies using in vitro fertilization (IVF) provide clear evidence that gametes are sufficient to intergenerationally transfer phenotypes acquired through exposures to different dietary [40, 41, 130], traumatic [120] and temperature [54] challenges. For example, maternal and paternal exposure to a chronic high fat diet (HFD) challenge, causes mice to become overly obese and metabolically compromised. This increases their offspring's susceptibility to diet-induced obesity and metabolic syndrome via oocyte and sperm-embedded information, in a gender and parent-of-origin specific fashion [41]. A similar approach has been used in a more recent mouse study to demonstrate that alteration of DNA methylation in sperm induced by preconceptional cold exposure improves offspring basal energy expenditure and metabolic homeostasis and protects the offspring from diet-induced obesity and metabolic syndrome [54].

Dutch Hunger Winter

The Dutch famine of 1944–45, known in the Netherlands as the Hongerwinter (hunger winter) of 1944/1945 is a prominent example of how changes in the parental diet during a critical time period can affect the F1 and even following generations via epigenetic inheritance. Occupied by German soldiers, parts of the Netherlands were cut off from food supply chains for more than five months. As a result, the caloric intake of more than 4 million Dutch decreased from around 1000kcal/day in November 1944 to only 500kcal/day in April 1945. This chronic severe caloric restriction led to a high number of deaths and diseases. Women who were at the mid to late gestation stage during this fasting exposure gave birth to offspring with significantly lower birth weight. By contrast, women who were at the early gestation stage did not see changes in the birth weight of their babies, compared to control children [144]. However, these babies showed a higher risk of suffering from obesity as a long-term consequence [145].

The data shows that the timing of the exposure to environmental changes impacts the phenotype of future generations. Research among the grandchildren of the affected women showed altered epigenetic DNA methylation patterns, although the grandchildren had not been directly affected by the restriction in calories. These altered DNA methylation

patterns were associated with risk factors for developing coronary heart diseases, impaired glucose tolerance and obesity [145] [146]. As previously postulated, exposure during critical development timepoints such as early pregnancy can lead to impactful changes in the epigenome and therefore to alterations of the whole-body metabolic physiology of future generations.

Överkalix study

The Överkalix study, one of the largest epigenetic studies in humans, has demonstrated that the time before puberty is also sensitive to environmental challenges which may impact the development and health of future generations. Överkalix is a small, self-sufficient town in Sweden. A detailed record of the town's food availability was kept and three cohorts born in 1890, 1905 and 1920 were followed until death or max. until 1995 [147]. The study allowed a to draw a correlation between the amount of food available during the slow growth phase period before puberty (SGP) and phenotypic traits such as bodyweight (BMI), body height, mortality risks and the appearance of diseases (including cardiovascular diseases and diabetes) in the exposed people, their children and even grandchildren.

For instance, Kaati et al. showed that if the father ($p=0.05$) and - with less statistical robustness - the paternal grandmother ($p=0.11$) faced food scarcity during their SGP, the subject was protected against cardiovascular diseases. If the paternal grandfather lived through a famine during his SGP, it tended to protect the proband from diabetes ($p=0.09$). If the paternal grandfathers, on the other hand, had an excess of food during their SGP, their grandchildren had a 4x over-risk for death of type 2 diabetes according to the point estimate ($p=0.01$) [148]. The outcome of the study shows that the SGP period before puberty, constitutes a sensitive window of susceptibility to environmental factors that can have a large impact on the future metabolic health of the exposed persons and even the next two to three generations. These findings are interesting because they suggest that exposure to environmental challenges can have greater or lesser effects, depending on the timing of the exposure.

The offspring's phenotype is also affected by the gender of the parent who was exposed to environmental changes. The following distinctions need to be made:

- Was the mother exposed? (maternal inheritance)
- Was the father exposed? (paternal inheritance)
- Or were both parents exposed? (parental inheritance)

Certain environmental exposures in the F0 may affect only male offspring, others may affect only female offspring and in other cases both sexes may be affected but the intensity of the phenotypical manifestation may be different.

An example of paternal inheritance is the mouse study published in Cell Metabolism by Wu et al. It showed that a rise in glucocorticoids due to stress caused extra methyl groups to be added to the Sfmbt2 gene in the sperm. This affected how the associated microRNA (the intronic microRNA-466b-3p) was expressed. The epigenetic change was also detected in the offspring's livers. The intronic microRNA-466b-3p was silenced due to the inherited epigenetic change and so the enzymatic function of PEPCK was impaired, resulting in elevated blood sugar levels in the offspring [126]. The reason for sex-dependent phenotypes could either be imprinting genes, hormonal differences between male and females or some yet unknown mechanisms [149].

Inter-generational and transgenerational epigenetic inheritance

Transmission of acquired phenotypes can be inter- or trans-generational depending on the number of generations presenting the phenotype with no direct exposure to the triggering environmental challenge. In the case of paternal transmission, transgenerational epigenetic inheritance is defined as the persistence of the inherited phenotypes at least to the second unexposed generation (F2)(Figure 14). This definition remains valid for maternal transmission, when the exposure to the environmental challenge is pre-conceptional (i.e. outside of pregnancy) with no mother-child physical interaction. Alternatively, one additional generation has to be included when exposure to the triggering environmental

challenge happens during pregnancy. In this case the physical interaction between the mother and the child leads to indirect exposure of the primordial germ cells to the environmental challenge and makes the child a new F0 [150].

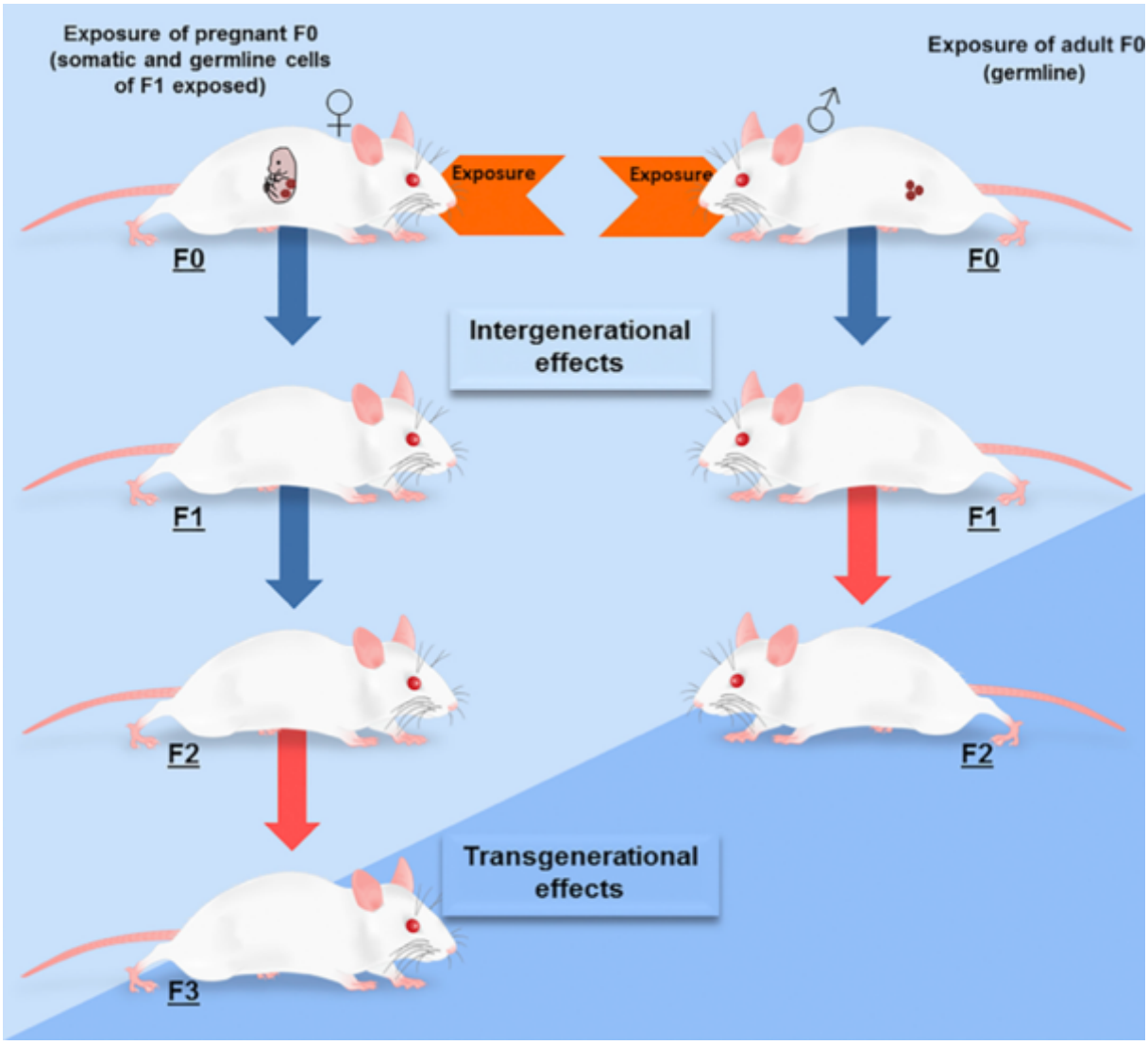

Figure 14: Inter- / Transgenerational inheritance
Classification of intergenerational and transgenerational effects depending on exposed females or males. Due to the F0 being in the womb of the exposed female the first generation does not count as unexposed. Contrary to that the first-generation offspring of exposed males counts as unexposed. The first unexposed generation can be investigated for intergenerational effects. After 2 unexposed generations transgenerational effects can be investigated. Modified from [151].

SUMMARY

In summary, type 2 diabetes is a pandemic with a rising prevalence. One of the reasons for the growing prevalence may be the increasing number of shift workers. Studies, including the Nurses' Health Study I & II, have shown that circadian arrhythmia may cause type 2 diabetes and metabolic syndrome. However, little is known about the effect of circadian arrhythmia on future generations. Human studies such as the Överkalix study have shown that the caloric intake of the parents determines the metabolic phenotype of future generations. It may therefore be that parental circadian arrhythmia causes type 2 diabetes and metabolic syndrome in the offspring. However, up to date no study has been conducted to investigate the effect of circadian arrhythmia on future generations. The timing of the paternal exposure to the shift work may also influence the development and health of the offspring and decide if the offspring will develop type 2 diabetes or not. The mechanisms that could be involved are not only epigenetic pathways but also higher cortisol levels or seminal fluid, influencing the embryo's development and health.

STUDY DESIGN, AIMS AND HYPOTHESIS

The main aim of this PhD project was to show that changes in metabolic function caused by circadian arrhythmia in the F0 generation can be inherited by the F1 and possibly also the F2 generation. Based on the publications of Mukherji et al. in 2015, mice suffering from circadian arrhythmia develop crucial metabolic phenotypes (such as impaired glucose levels, altered glucose tolerance test results and impaired food intake) after only 30 days of circadian arrhythmia [47, 152]. We were therefore interested in finding out if a short period of circadian arrhythmia is powerful enough to impact the metabolic phenotype of future generations. As most of the previously published data on this topic focus on maternal factors, we specifically focused our research on paternal circadian arrhythmia. We also aimed to investigate the underlying mechanisms of inter- and transgenerational epigenetic inheritance of acquired phenotypes via environmental exposure.

The results of this study are relevant since shift work, i.e. any work that is done outside of normal daytime working hours, has become an important part of many industries worldwide. The performance of shift work has been found to be associated with a relatively high health risk. People working during the night suffer from severely higher risk of developing type 2 diabetes, which can be a contributor to the recent strong increase in the type 2 diabetes pandemic [153]. Our results suggest that children of fathers working night shift may also have a higher risk of type 2 diabetes, which would explain the growing incidence numbers. To investigate the changes in metabolic function caused by circadian arrhythmia, we raised the following key questions:

- Are 30 days of exposure to circadian arrhythmia a sufficient exposure period to cause epigenetically transmitted phenotypes in future generations?
- If yes, how defined and how penetrant are the phenotypes in the F1 generation?
- Is this phenotype also visible in the F2 generation?
- What are the underlying molecular mechanisms for the epigenetic transmission of the phenotype?
- What implications do our findings have for humans?

MATERIALS

BUFFERS, SOLUTIONS AND CONSUMABLES

Solutions	Composition
Blocking Buffer	Tween 1x PBS 1x
Digestion Solution 1 (Germ cell extraction from testes samples)	DMEM 4mg/ml Collagenase IV 20ug/ml DNAse I
Digestion Solution 2 (Germ cell extraction from testes samples)	DMEM 4mg/ml Collagenase IV 20ug/ml DNAse I 0,66mg/dl Hyaluronidase
DNA Extraction Lysis Buffer	10 mM NaOH 2,5 mM EDTA dH_2O
PBS Tween	1 % PBS 1 % Tween

Chemicals	Company	Catalogue No.
100 bp DNA Ladder	Invitrogen	15628019
Agarose	Sigma Aldrich	A9539
Bolt LDS	Life Technologies	BT00061
Bolt Sample Reducing Agent	Life Technologies	B0004
Bolt Transfer Buffer (20x)	Life Technologies	Bt00061
Bradford Reagent	Sigma Aldrich	B6916
BSA	Sigma Aldrich	A4503

Calcium Chloride	Sigma Aldrich	449709
Collagenase	Sigma Aldrich	C5138
DNA Loading Dye (6x)	Thermo Fisher	R0611
DNase I	Sigma Aldrich	DN25
EDTA	PanReacAppliChem	A3145,0500
Ethanol	Merck	100983
Fast Sybr Green Master Mix	Thermo Fisher Scientific	4385612
Formaldehyde Solution	Sigma Aldrich	F-8775
GelRed Nucleic Acid Gel Stain	Biotium	41003
Glucose	Sigma Aldrich	G8644
Glycogen	Thermo Fischer	R0561
HCG – Ovogest 300	MSD	340100
Hepes	Gibco	15630
HTF - Human Tubal Fluid	Irvine scientific	9922
Hyaluronidase IV	Sigma Aldrich	H4272
Ketamine	Heinrich Fromme	-
MagicMark XP Western Protein Standard	Thermo Fischer	LC5602
Magnesium Chloride Solution	Sigma Aldrich	M1028
Methanol	Merck	113351
NaCl 0,9 %	BBraun	L4263
PBS	Gibco	10010023
PCR MasterMix	Thermo Fisher	K0171
PMSG 500	Bioveta	665624A
Potassium Chloride	Sigma Aldrich	P9333
Proteinase K	Invitrogen	EO0492
Purelink RNAse A	Invitrogen	12091021
Pursept A Xpress	Schülke	-
RNA Later	Invitrogen	AM7020

Seeblue Pre-stained Protein Standard	Invitrogen	LC5925
Sodium Bicarbonate	Sigma Aldrich	S5761
Sodium Chloride	Sigma Aldrich	31434
Sodium Hydroxide (NaOH)	Sigma Aldrich	655104
Sodium Pyruvate	Sigma Aldrich	P2256
Sucrose	Sigma Aldrich	S0389
Tris-HCl	Sigma Aldrich	T2319
Trizol	Thermo Fisher	15596018
Tween-20	Panreac Applichem	A4974

CHEMICALS AND KITS

Kit	**Company**	**Catalogue No.**
Agilent RNA 6000 Pico Kit	Agilent	5067-1513
Agilent RNA 6000 Nano Kit	Agilent	5067-1511
Bioanalyzer High Sensitivity DNA Analysis	Agilent	5067-4627
ELISA Corticosterone Kit	Abcam	108821
ELISA FGF21	Abcam	212160
ELISA Leptin	Abcam	100718
i7 Index Plate for QuantSeq/SENSE for Illumina	Lexogen	044
LS Columns	Miltenyi Biotec	130-042-401
M Tubes	Miltenyi Biotec	130-093-236
MSD Insulin	MSD	K152BZC
MSD Cytokines	MSD	K15059D
MSD Mouse Metabolic Kit (Leptin/Insulin)	MSD	K15124C

Quantseq 3’mRNA-Seq mRNA Library Prep Kit FWD for Illumina	Lexogen	015
RNeasy® Mini Kit	Qiagen	74104
RNeasy® Plus Micro Kit	Qiagen	74004

LABORATORY DEVICES AND EQUIPMENT

Laboratory Equipment	**Company**
2100 Bioanalyzer Instrument	Agilent
Bio Safety Cabinet	
Centrifuge	Thermo Fisher
Freezer 4°C	Liebherr
Freezer -20°C	Liebherr
Freezer -80°C	Eppendorf
Genotyping and Westernblot Imaging System	Invitrogen iBright FL1000
Glassware	Schott
Glucometer Roche Akku Check Aviva	Roche
Glucometer Roche Akku Check Aviva Test stripes	Roche
Multipipette	Eppendorf
Nanodrop ND-1000	NanoDrop Technologies
qPCR Machine (96/384 plates)	Applied Biosystems QuantStudio 6 Flex
Scale 200g	0Haus
Shaker	HLC
Timer	Roth
Vortex	Neolab
Waterbath	Julabo

QPCR PRIMERS

Name	Forward Sequence	Reverse Sequence
Clock	CACAGGCCAGCACATGATAC	GTGACTGTAGCACTCTGGGT
Bmal1	CAATGAGCCAGACAACGAGG	TACGCCAAAATAGCTGTCGC
Cry1	TACAGCAGCCACAAACAACC	GAAGCTGAGTCATGATGGCG
Per1	TCTACCTCTCCTCTGCCAGT	CCACCTCTACTGCCCTGAAA
Per2	TTTCTGCCGTGTCAGTGTTG	CTCAGGAGGGATTCTAGGCG
Rev-Erb-a	TGGCATGGTGCTACTGTGTAAGG	ATATTCTGTTGGATGCTCCGGCG

HOUSEKEEPING GENES

Name	Forward Sequence	Reverse Sequence
Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA
36B4	AGATGCAGCAGATCCGCAT	GTTCTTGCCCATCAGCACC
GAPDH	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGGTAGGAACA
Rplp0	TGCCACACTCCATCATCAAT	CGAAGAGACCGAATCCCATA

PRIMARY ANTIBODIES

Antibody	Company	Catalog No.
Glucocorticoid Receptor	Thermo Fisher	MA1-510
OXPHOS	Abcam	ab110413
β-Actin	Santa Cruz	sc-130656
GAPDH	Cell Signaling Technology	2118

SECONDARY ANTIBODIES

Antibody	Company	Catalog No.
HRP linked – Anti-mouse	Cell Signaling Technology	7076
HRP linked – Anti-rabbit	Cell Signaling Technology	7074

METHODS

ANIMAL HOUSING

C57BL6/J mice (males and females) were purchased from Charles River Laboratories Germany (Sandhofer Weg 7, 97633 Sulzfeld) and housed in ventilated cages at constant temperature (22°C +/- 1C°) with moderate humidity (50% +/- 5%). The mice had unlimited access to water and, unless stated otherwise in the experiments, were fed ad libitum on a 12h light/dark cycle from 6:00 a.m. to 6:00 p.m. All housing protocols and animal experiments were performed according to European Union directive 2010/63/EU and were approved by the responsible authorities of the government of Upper Bavaria (Tierversuchsantrag No.: ROB-55.2-2532.Vet_02-17-33). All efforts were made to minimize suffering by considerate housing and husbandry. All phenotyping procedures were examined for potential refinements. Animal welfare was assessed routinely for all mice involved.

F0 GENERATION SPECIFICS

Our restricted fed (RF) F0 generation mice cohort consisted of 6-week-old male mice that were ordered from Charles River Laboratories Germany. They were accustomed to the mouse facility for one week and then put on a 30 days restricted feeding schedule with fasting over night for 12 hours from 6:00 p.m. to 6:00 a.m. and access to chow diet for 12 hours from 6:00 a.m. to 6:00 p.m. After 30 days, they were subsequently mated with unexposed wildtype females of the same age. At the time of mating, both males and females, were fed ad libitum with normal chow diet. The offspring of these matings was used as the experimental F1 generation. The control (CTR) F0 mice cohort consisted of 6-week-old male mice that were purchased from Charles River Laboratories Germany. They were accustomed to the mouse facility for one week and then given ad libitum chow diet for 30 days. After 30 days, they were mated with wildtype females of the same age. The offspring of these matings was used as control cohort for the F1 generation.

PHENOTYPING AND METABOLIC METHODS

Bodyweight and visual inspection of mouse health

For every mouse cohort, bodyweight was measured every 2 weeks by single mouse measurement on a precisionscale with 2 decimal places. Additionally, every single mouse was visually inspected for potential wounds or ill-behavior. If necessary, mice were treated in agreement with the animal caretakers or veterinarians and excluded from the study.

Food intake measurements of the F1 generation

The F1 generation mice were weaned after 3 weeks of age and kept in cages to a total of 4 littermates. Drinking water and chow diet were supplied ad libitum for their entire life. Food intake was measured manually every 2 months for 2 weeks by calculating the amount of food that was put in the cage minus the weight of the food pellets that were left in the cage at the end of the day. Food intake was calculated by dividing the net-amount of food by the number of mice per cage.

Calorimetric cages

Before sacrificing the F1 mice at 10 months of age, the entire cohort was kept in single caged calorimetric cages for 48 hours and phenotyped for the following metabolic parameters: food intake, water intake, activity, energy expenditure, as well as gas analysis of CO_2 and O_2. This testing was performed in the Metabolic Screening Facility of the German Mouse Clinic at the Helmholtz Zentrum Munich, with access to 32 metabolic calorimetric cages. The results were either analyzed by using the raw data in Microsoft Excel or by arranging the data in Graphpad Prism 7. In addition, the TSExplorer program of Dr. Jan Rozman and Dr. Martin Kistler at the German Mouse Clinic at the Helmholtz Zentrum Munich was used for the visualization and statistical analysis of the data.

ELISA

Mice at the age of 16 weeks were tailcut and blood (approx. 50 uL) was collected in 1.5 mL Eppendorf tubes that were immediately put on ice. The blood was directly centrifuged at 4°C and 10,000g for 10 minutes and the liquid supernatant was saved as blood plasma and either directly used for ELISA testing or stored at -80°C for later analysis. Depending on the specifics of the kit, a blood plasma volume of 3-20 uL (either undiluted or diluted with working media) was used for the assays. A variety of different ELISA kits was used, including Corticosterone, Leptin, FGF21 and ACTH (see Materials section) to detect their concentration in blood plasma and seminal fluid samples at different Zeitgeber of the day.

Meso Scale Discovery (MSD) kits

Meso Scale Discovery (MSD) kits were used to detect the concentration of insulin and cytokines (IL-10, IL-1 β, IL-2, IL-4, IL-5, IL-6, KC/GRO, TNF - α) in blood plasma and seminal fluid.

Blood glucose measurements

After tailcut, a small droplet of blood was measured using a Roche Akku Check glucometer. Blood sugar data was collected in the F1 generation at exactly 16 weeks of age, while collecting blood samples for ELISA testing. The blood glucose was measured in mg/dL.

Glucose tolerance tests

Blood glucose levels of the F1 RF cohort were measured by means of a glucose tolerance test (GTT) at 9:00 a.m. after 16 hours of fasting. Glucose was injected intraperitoneally with a calculated amount of 1.5g glucose per kg of bodyweight. We measured the blood glucose levels at timepoints 0, 15, 30, 60 and 120 minutes using a Roche Akku Check glucometer. Blood glucose was measured in mg/dL.

TISSUE COLLECTION

For tissue collection, 10-month-old mice were sacrificed in the ad libitum-fed state at different times of the day to achieve results that show cycling of certain parameters. In order to obtain results for 4 time points per day, the unfasted mice were sacrificed at Zeitgeber 0, 6, 12 and 18. These time points are equivalent to 6:00, 12:00, 18:00 and 24:00. Blood was collected by either puncturing the heart or the vena cava using a syringe and needle. The blood was collected in EDTA monovettes (Sarstedt) and immediately centrifuged in a 1.5mL Eppendorf tube to obtain the blood plasma (10000g, 4°C, 10 minutes). Samples from the following organs were collected: the liver sample was snap-frozen in liquid N_2 and transferred to a -80°C freezer, the adrenal gland and hypothalamus samples were collected in RNAlater and either stored or directly used for further processes. The snap-frozen sample as well as the samples in RNAlater could directly be transferred into TRIzol Reagent (Thermo Fisher) for RNA extraction. This method allowed to obtain high quality RNA.

Sperm and seminal fluid collections

Additionally to the tissue, sperm and seminal fluid were collected for further analysis. For this the cauda epididymis was dissected on both sides but only the caudal tissue was extracted. The tissue was put in a 2 mL Eppendorf tube containing sperm motility medium and then cut into small pieces using precision scissors. After 20 minutes of incubation at 37°C, the sperm cloud was separated from the diluted seminal fluid. This was done by centrifuging the mixture at room temperature for 1 minute at 1000g at room temperature and then by incubating it at 37°C for 10 minutes. This gave a buffy coat of sperm on top of the seminal fluid. The sperm and seminal fluid were separated and placed into new Eppendorf tubes. The diluted seminal fluid was subsequently used for ELISA or MSD analysis.

GENOTYPING OF GRHET MICE

Mice that are genetically heterozygous for the glucocorticoid receptor (GRhet mice) were kindly provided by Prof. Dr. Stephan Herzig at the Institute for Diabetes and Cancer at the Helmholtz Center Munich. Samples of ear clips were transferred to 0.2 mL 8-strip PCR tubes (Eppendorf) and dissolved in cell lysis buffer for 16 hours. We determined the genotype of the GRhet mice using the following oligonucleotide sequences:

Name	**Sequence**
GRflox1	5' – GGC ATG CAC ATT ACG GCC TTC T – 3'
GRflox4	5' – GTG TAG CAG CCA GCT TAC AGG A – 3'

For the PCR reaction the following protocol was used:

Reagent	**Volume**
2x Mastermix (Thermo Fisher)	10 uL
Primer GRflox1	0.5 uL
Primer GRflox4	0.5 uL
dH_2O	7.5 uL
Total	**18.5 uL**
	+ 15 uL DNA
Total	**20 uL**

Genotyping samples were prepared in 0.2 mL 8-strip PCR tubes (Eppendorf) and put in a PCR cycler using the following program:

Degrees	**Time**
95° (repeated 35 times)	30 seconds
55° (repeated 35 times)	30 seconds
72° (repeated 35 times)	1 minute
72°	10 minutes
4°	Indefinitely

The resulting DNA samples, together with a positive (GRhet positive) and a negative wildtype control, were loaded onto a 1% agarose gel with Gelred (Biotium) and run for 40 minutes at 120 V to separate them according to their size. The gel was then put into an imaging system (iBright FL1000 - Invitrogen) and the genotypes were analyzed visually according to the size of the DNA fragments (measured in base pairs, bp):

Band	Basepairs
Flox Mutant Band	275 bp
Wildtype Band	225 bp

IVF, OOCYTE ISOLATION AND EMBRYO TRANSFER

In vitro fertilization and oocyte isolation were conducted by the group of Dr. Susan Marshall at the German Mouse Clinic, Helmholtz Center Munich, following standardized procedures of the INFRAFRONTIER consortium [41, 154]. Native single unexposed females were super-ovulated with 7.5 U of Pregnant Mare Serum Gonadotropin (PMSG) and 7.5 U of human Chorionic Gonadotropin (hCG) before being sacrificed for oocyte collection. The oocytes were transferred into Human Tubal Fluid (HTF) at 37°C and 5% CO_2. Reaching the embryonic morula stage, the embryonic development was visually checked before embryonal transfer was performed to foster mothers. The IVF protocol of the German Mouse Clinic was followed [155]. After giving birth, the F1 offspring was metabolically phenotyped for the same parameters as the naturally mating RF F1 generation.

RNA SEQUENCING

RNA was collected from liver, adrenal gland, hypothalamus, placenta and morula embryos (50 morula per experimental group). The TRIzol reagent (Thermo Fischer) procedural guideline protocol was used for the liver, adrenal gland, hypothalamus and placenta samples and the RNeasy mini kit (Quiagen) protocol was used for the morula samples. As a

result isolated high-quality total RNA was obtained. The concentrations and quality of the RNA were controlled using Nanodrop and subsequently the Agilent Bioanalyzer system. Only RNA samples with RNA Integrity Number (RIN) values > 7 were used for the construction of libraries. Library construction and sequencing were performed in two different ways, depending on the samples:

Analysis of the first cohort

The RNA of the control and RF F0 and F1 mice liver samples at different Zeitgeber time points were outsourced and performed by IGA Technology Services (Udine, Italy). The libraries were constructed using the Nextera Library Prep Kit (Illumina) according to the manufacturer's instructions and sequenced on an Illumina HiSeq 2500 at 75bp single-ended, with a minimum output of 50 million reads per sample. Read mapping and differential expression analysis were performed using the A.I.R (Artificial Intelligence RNAseq) software from Sequentia Biotech.

Analysis of the second cohort

The second cohort of samples consisted of RF and control F1 RNA from:

- the liver at Zeitgeber 0, 6, 12, 18
- the adrenal gland at Zeitgeber 0, 6, 12, 18
- the hypothalamus at Zeitgeber 0, 6, 12, 18
- the placenta
- the embryo's fetal liver samples

The samples were sequenced at the Genomics Unit of the Center for Genomic Regulation in Barcelona on an Illumina HiSeq 2500 at 50bp single-ended reads, with a minimum output of 50 million reads per sample.

Data analysis for differentially expressed genes was either performed using edgeR or DESeq2 analysis methods. DEseq uses a geometric normalization strategy, whereas edgeR is a weighted mean of log ratios-based method. Both normalize data initially via the

calculation of size / normalization factors [156, 157]. The DEseq normalization method is included in the DESeq Bioconductor package (version 1.6.0) [158] and “is based on the hypothesis that most genes are not differentially expressed. A DESeq scaling factor for a given lane is computed as the median of the ratio, for each gene, of its read count over its geometric mean across all lanes“ [159]. The trimmed mean of M-values (TMM) edgeR normalization method is implemented in the edgeR Bioconductor package (version 2.4.0) [160, 161]. It is „also based on the hypothesis that most genes are not differentially expressed. For each test sample, TMM is computed as the weighted mean of log ratios between this test and the reference, after exclusion of the most expressed genes and the genes with the largest log ratios“ [159]. Heatmap and principal component analysis (PCA) analyses were performed with the web-application ClustVis using default parameters [162].

STATISTICAL ANALYSIS

All figures and statistical analyses were generated using GraphPad Prism 6 and/or 7 (San Diego, CA). Data was tested for gaussian distribution via D’Agostino-Pearson normality test. Statistical significance was tested by Student’s t-test or analysis of variance (ANOVA), when appropriate [163]. All data were expressed as mean ± SEM unless otherwise specified and a two-tailed p-value < 0.05 with multiple comparison correction (Bonferroni method) was used to indicate statistical significance (*<0.05, **<0.01, ***<0.001) [163].

RESULTS

The suprachiasmatic nucleus (SCN), located in the hypothalamus, senses day/light periods via the retina, while the liver and peripheral organs sense rest and active phases mainly via the timepoints of food intake. Normally, the SCN and the liver are synchronized to periods of food intake during the "active light phase" and to periods of no or significantly less food intake during the "resting dark phase". In previous studies it was shown that misalignment of the circadian rhythm via an impaired feeding schedule may lead to phenotypes comparable to metabolic-syndrome [47, 68]. It could be demonstrated that food intake of the F0 generation that was restricted to the resting phase leads to a complete desynchronization of this homeostasis, highlighted by an inverted expression of core clock genes in the liver of the F0 generation [47, 152]. In our experiments we could confirm these findings (Figure 16 D-G).

However, no data, has yet been published on the effect of these changes in the F0 generation on future generations. Therefore, we focused our research on how circadian arrhythmia in F0 males can affect F1 offspring. To test this, we restricted the food intake of the F0 males to their resting phase, during which they normally rest and their food intake is minimal. We restricted the feeding schedule for 30 days (Figure 15). Hereafter, these mice will be referred to as RF F0. After 30 days, we mated the RF F0 males to naïve unexposed females. We then investigated how the F1 offspring of the RF F0 males differed from the F1 of control mice (CTR F1) in regard to food intake, body weight, blood glucose and hormone levels at different timepoints within a 24-hour period. We focused on four different F1 generations (Figure 15):

1. **Group 1:** F1 cohort with a chow diet and ad libitum food access
2. **Group 2**: F1 cohort with a 60% high fat diet and ad libitum food access starting at the age of 6 weeks
3. **Group 3**: F1 cohort created via IVF and not via natural mating of RF F0 male mice with wild type female mice

4. **Group 4**: F1 cohort where the RF F0 male mice after four weeks of circadian arrhythmia where put back to an ad libitum feeding schedule

In all of these cohorts we matched the experimental mouse cohort with the same number of isogenic and age matched control mice (of unexposed parents) to guarantee comparable results under the exact same environmental conditions.

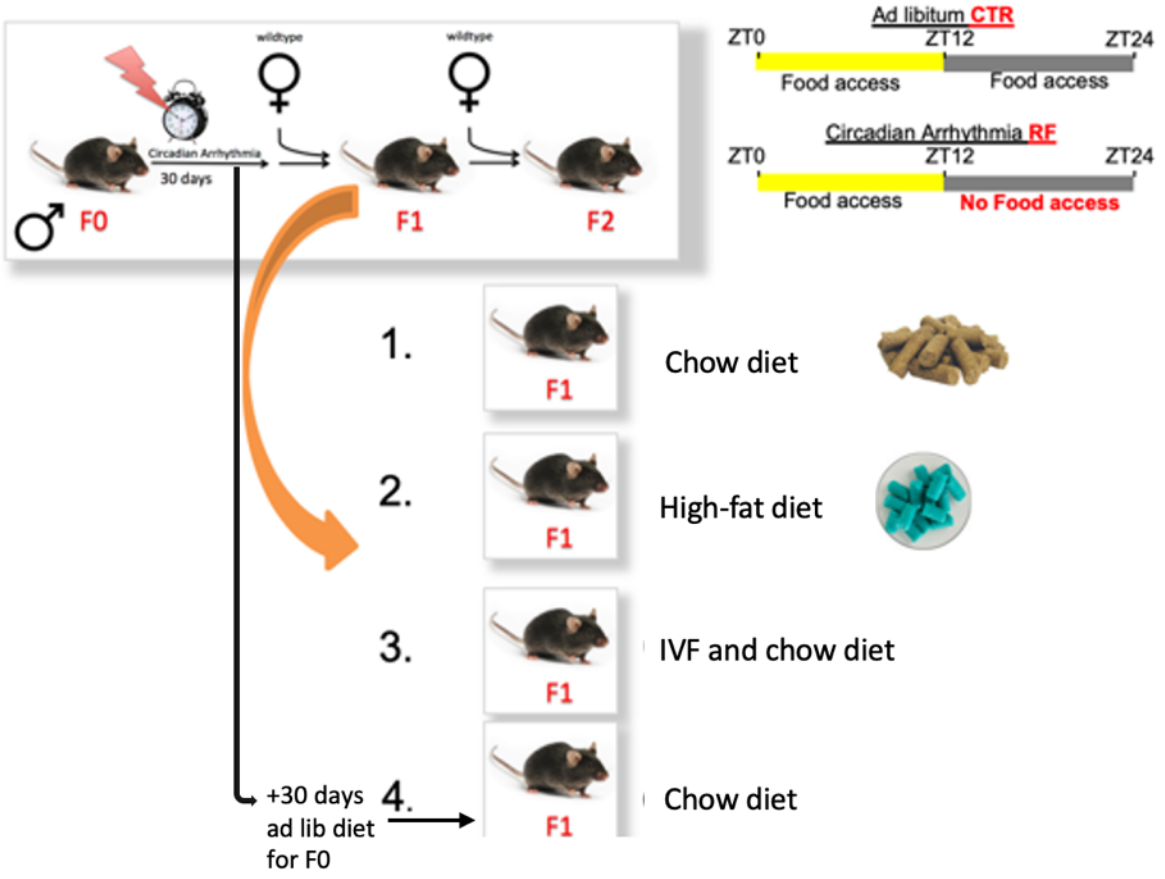

Figure 15: Setup of the experimental F0, multiple F1 and F2 cohort
Mouse cohorts after keeping the F0 male mice on a restricted feeding schedule for 30 days (chow food access during the resting phase and no food access during the active phase): 1. F1 generation with 24 hours access to a chow diet 2. F1 generation with 24-hours access to a high-fat diet 3. F1 generation created via IVF with 24 hours acess to a chow diet 4. Following the 30 days of restricted feeding for the F0, they had ad libitum acccess to a chow diet for 30 days and then were mated with wildtype mice to create the fourth F1 generation. This F1 generation had 24-hour access to a chow diet.

1) F0 CIRCADIAN ARRHYTHMIA FOR 30 DAYS

During the 30 days that the RF F0 was exposed to circadian arrhythmia we measured their weekly body weight and their daily food intake. A short period of adaptation could be seen in the first two days. In this time period, the mice adapted to the new environmental challenge and changed their behavior. The RF F0 began eating their food in the light periods, when they should normally would be resting.

As Figure 16B shows, the RF F0 mice almost ate the same amount in 12 hours as the CTR F0 ate in 24 hours (average of 3.2 gram of total food intake for RF F0 vs. average of 3.5 grams for the CTR F0). There was no significant change in body weight between the RF and CTR F0 (Figure 16 A). However, insulin levels of the RF F0 were completely inverted (Figure 16 H). To prove if the changes in behaviour were really linked to changes in the circadian rhythm of the RF F0 mice, we measured qPCR gene expression levels of the core clock genes Bmal1, Per2, Cry and the nuclear receptor Rev-erb α in the RF F0 mice. Gene expression levels where measured at different Zeitgeber 0, 4, 8, 12 and 24 and normalized to the housekeeping gene 36b4. Thereby we were able to confirm the previously published scientific results by the Chambon group [47, 152]. After 30 days of restricted feeding, the expression levels of the core clock genes were completely inversed ($p < 0.001$) (Figure 16 D-G). Our experiments confirm that exposure to an altered feeding schedule alone is powerful enough as an environmental stimulus to induce circadian arrhythmia and will lead to a complete inversion of core clock gene expression.

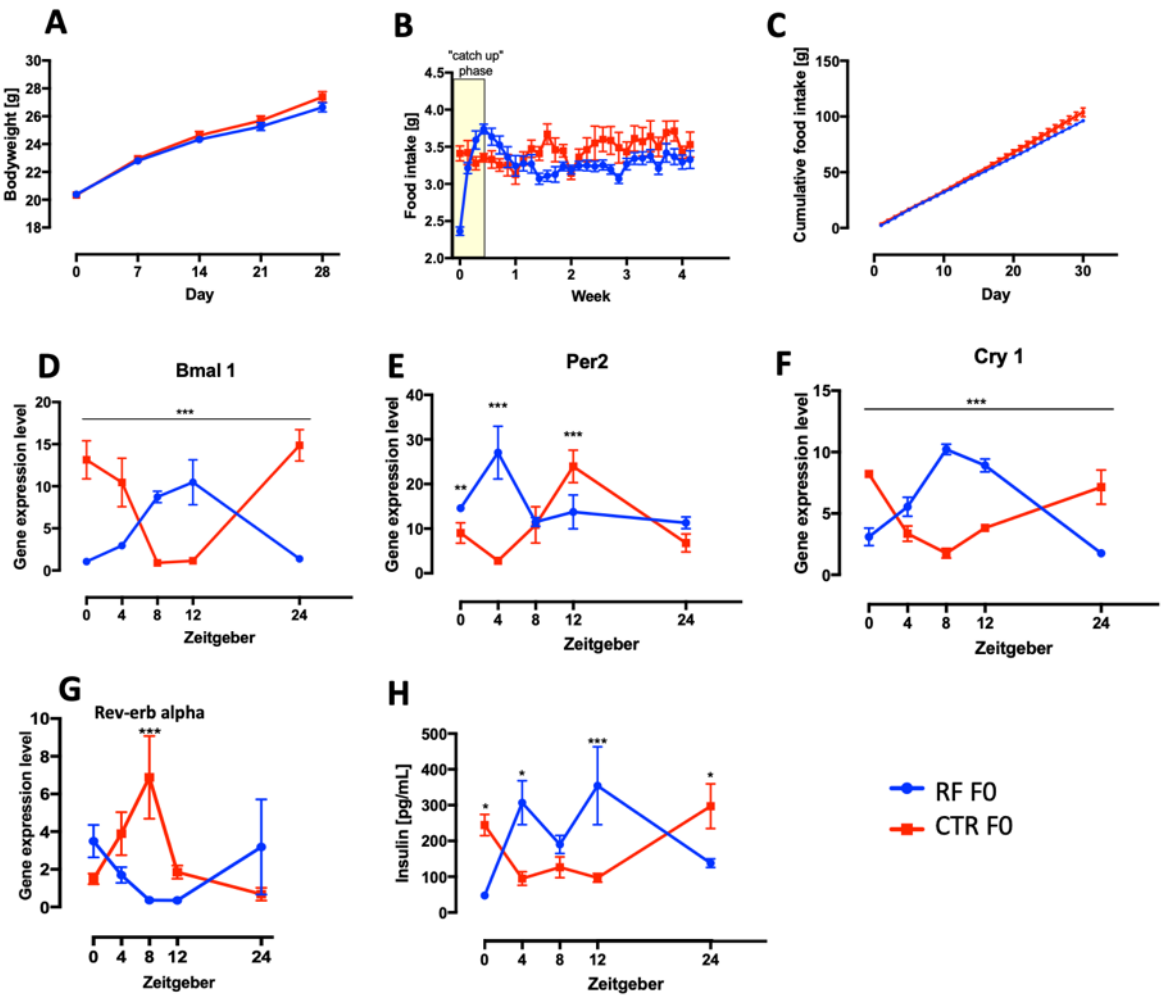

Figure 16: Bodyweight, food intake and clock core gene expression levels of RF and CTR F0
Average bodyweight of RF and CTR F0 over 30 days (A), Total food intake of RF and CTR F0 over 4 weeks (B), Cumulative total food intake of RF and CTR F0 over 30 days (C), qPCR gene expression levels of core circadian clock genes normalized to 36b4 from RF and CTR F0's liver samples at Zeitgeber 0, 4, 8, 12, 24 Bmal1 (D), Per2 (E), Cry 1 (F) and the nuclear receptor Rev-erb α (G). Insulin levels of RF and CTR F0 over 24 hours (H). n=20 for A-C, n=4/ZT for D-H. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001).

As described above, half of the mice cohort exposed to the circadian arrhythmia were mated with isogenic naïve females to generate a RF F1 cohort. The CTR F0 were also mated with isogenic naïve females, thereby generating a CTR F1. After checking for the female plug to confirm pregnancy [164], the F0 males were immediately removed from the mating cages to prevent any contact between F0 fathers and their F1 offspring. The other half of the F0 mice cohort were sacrificed for liver and hypothalamus samples. The results provided by these mice will be presented later in the text (Section 4: RNA-sequencing).

2) CHOW FED F1 OFFSPRING OF RF F0

Metabolic phenotype of chow fed F1 offspring of RF F0

No data has yet been published on how the described changes in the RF F0 generation affect future generations. Therefore, we focused on how the circadian arrhythmia in the RF F0 males affects their F1 offspring (RF F1). We investigated if the food intake patterns that we had induced in the RF F0 also lead to changes in food intake behavior of the RF F1. As seen in Figure 17, during daytime (resting phase) the food intake of the RF F1 is significantly higher than that of the CTR F1 (RF: 2.5g vs. CTR 1.8g $p<0.05$). The food intake during nighttime of RF F1 is identical with that of CTR F1. This means that in addition to eating the normal amount of food during the night, the RF F1, similar to their RF F0 fathers, also eat more during the day. This behavior results in a higher food intake over 24 hours among the RF F1 than among the CTR F1.

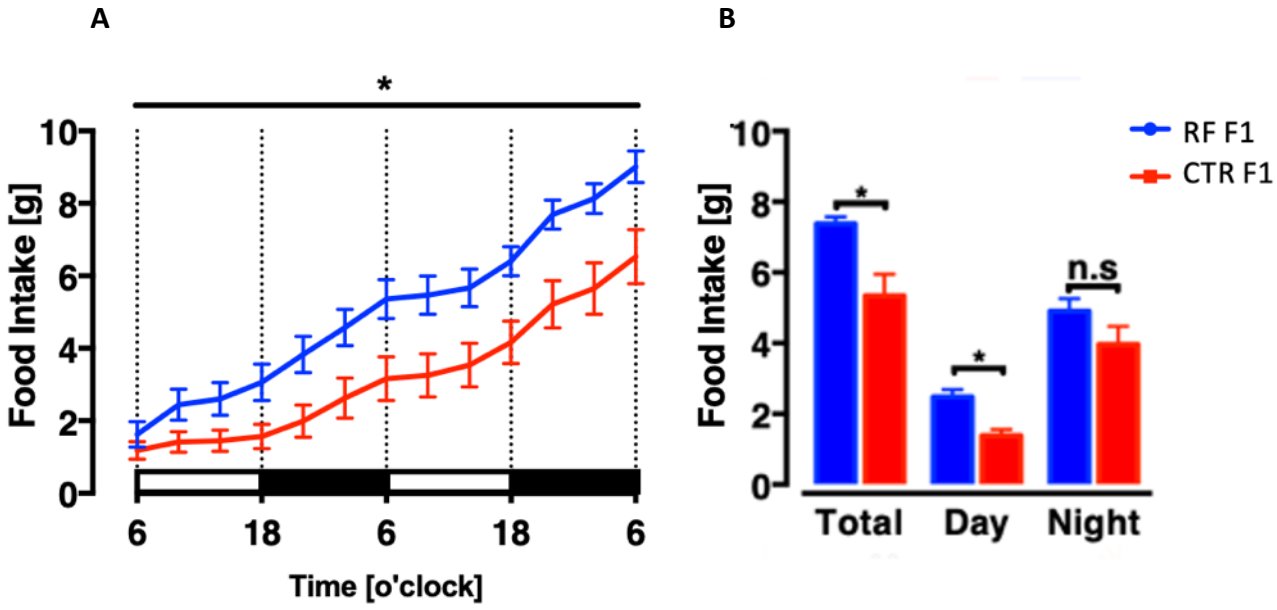

Figure 17: Food intake of RF and CTR F1
Cumulative food intake of RF and CTR F1 over 48 hours (A). Average total and average daily/nightly food intake of RF and CTR F1 measured over 24 hours in calorimetric cages (B) n=10. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001, n.s. = non-significant).

We examined if this food intake behavior is already seen at a young age or if this food intake pattern changes over the lifespan of the RF F1. As Figure 18 shows, the discrepancy between food intake of the RF and CTR F1 becomes more distinct as the mice become older. At the age of 4 months, the difference in average cumulative food intake between the RF and CTR F1 is 1.22 g ($p<0,001$), after 6 months it is 1.41 g ($p<0,001$) and after 10 months it is 1.62 g ($p<0,001$). There was no difference in food intake during the nighttime (Figure 19).

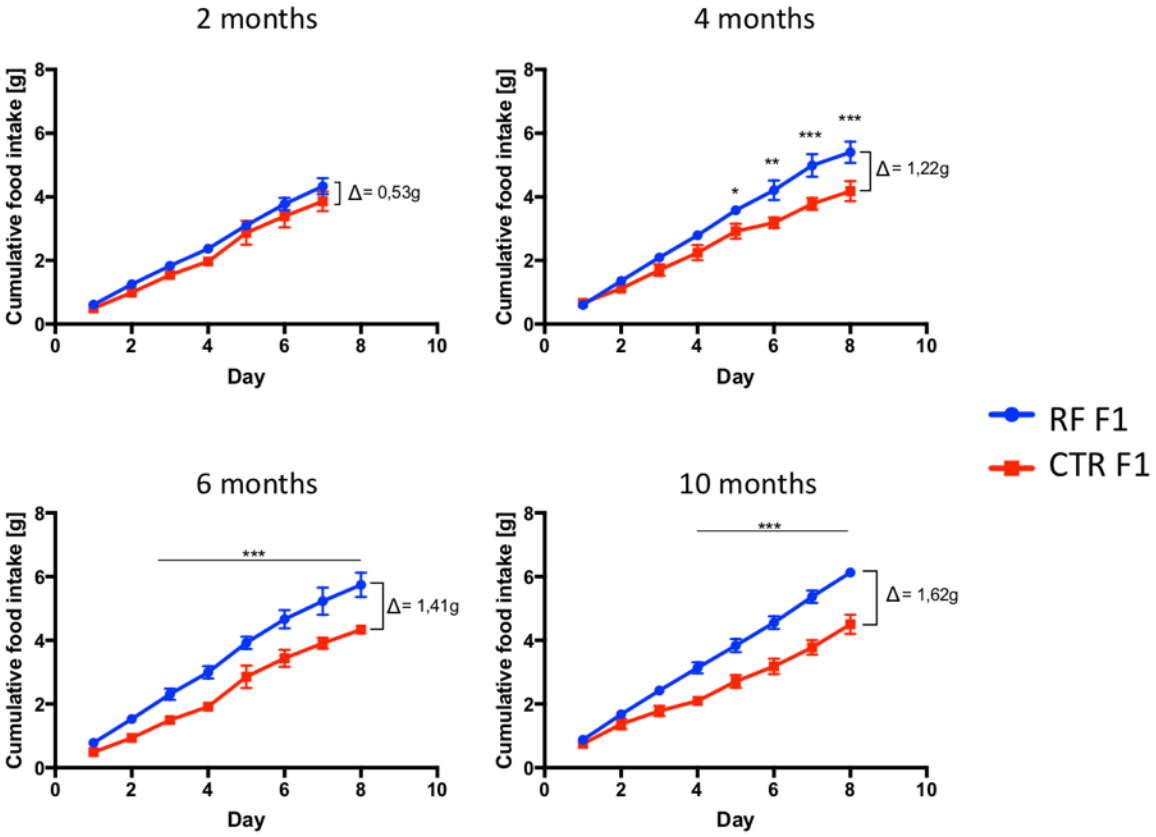

Figure 18: Food intake of RF and CTR F1 during the daytime at different ages
Cumulative food intake during the daytime of RF and CTR F1 at 2, 4, 6 and 10 months of age. n=6-7. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

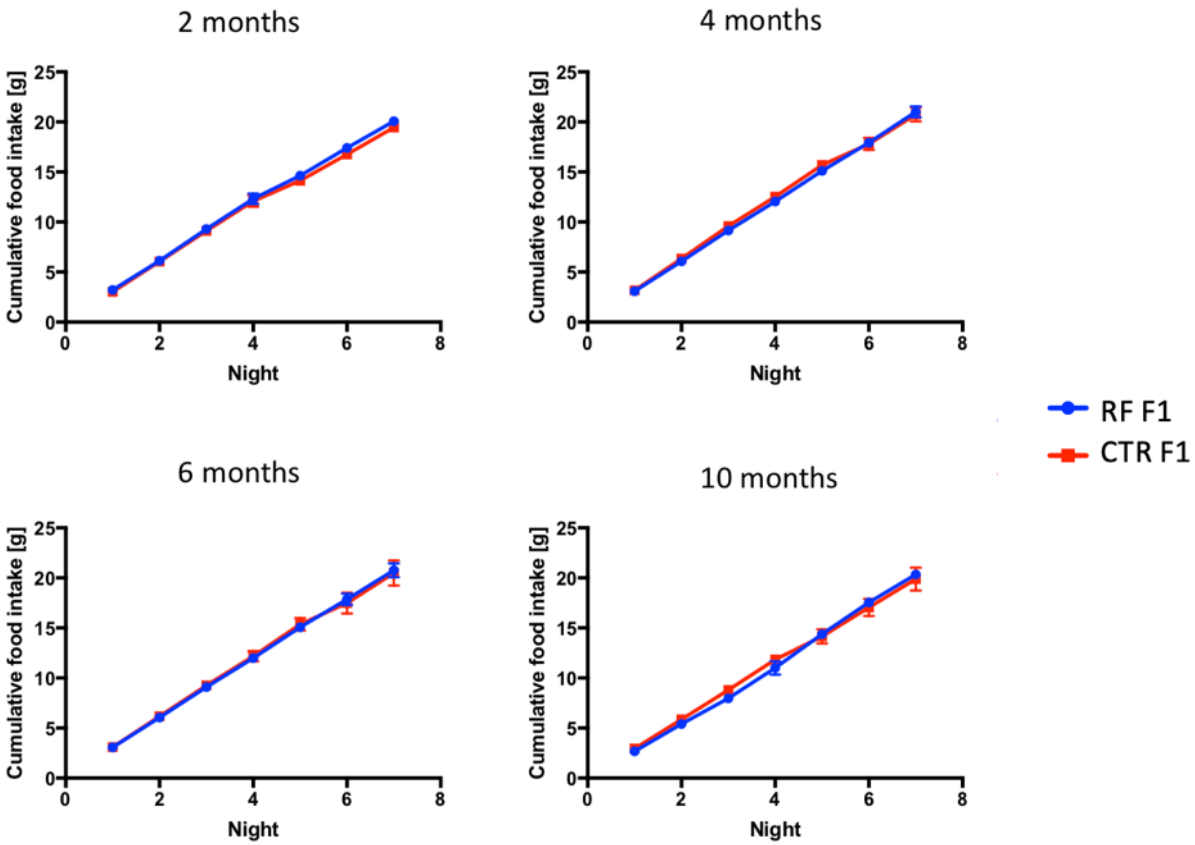

Figure 19: Food intake of RF and CTR F1 during nighttime at different ages
Cumulative food intake during the nighttime of RF and CTR F1 at 2, 4, 6 and 10 months of age. n=6. Error bars display SEM values.

The bodyweight of RF and CTR F1 are not significantly different (Figure 20). This raises the question where the surplus energy from the increased food and thus increased caloric intake is going. Results from indirect calorimetry measurements hereby showed slightly increased levels of energy expenditure, particularly during the day phase among the RF F1 (Figure 21). One can assume that this increase in energy expenditure comes from the RF F1 mice being active and looking for food.

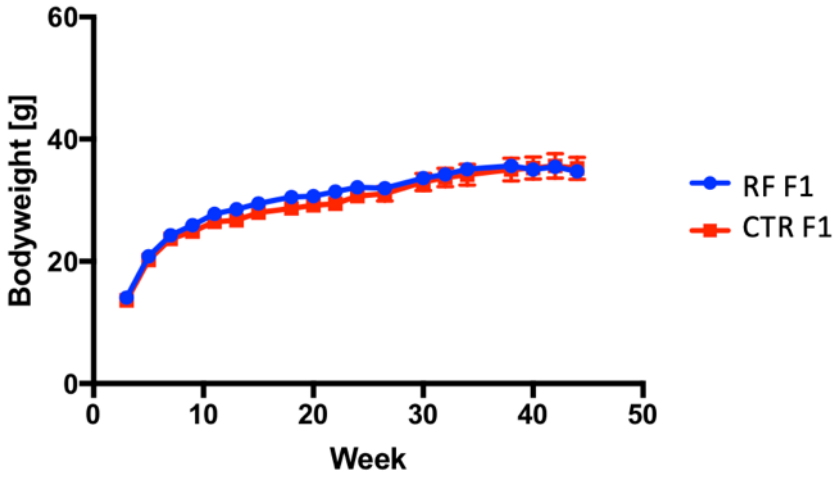

Figure 20: Bodyweight of RF and CTR F1 at different ages
Body weight of RF and CTR F1 measured over 44 weeks. n=10. Error bars display SEM values.

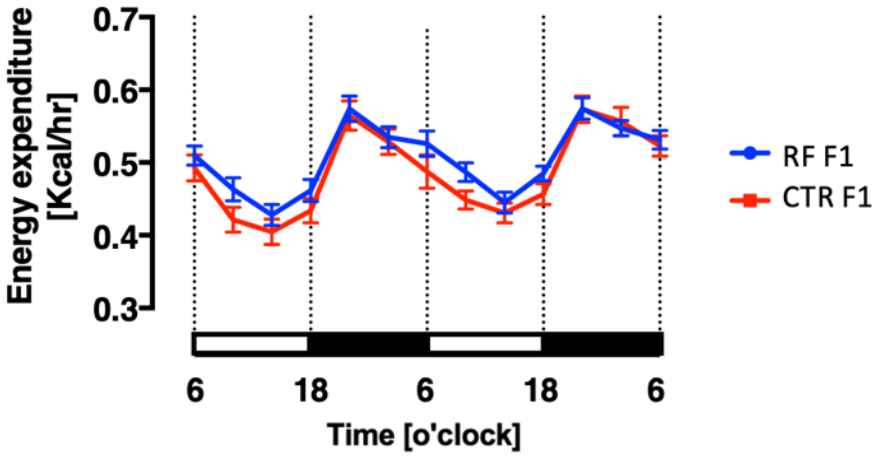

Figure 21: Energy expenditure of RF and CTR F1 over 48 hours
Energy expenditure of RF and CTR F1 over 48 hours in calorimetric cages. The time from 6 - 18 o'clock represents the light/resting phase and the time from 18 - 6 o'clock represents the dark/active phase. n=10. Error bars display SEM values.

Our data show that the disruption of the circadian clock results in the mice eating significantly more during the day phase. They, however, also burn more energy.

RF and CTR F1 also differ in their blood glucose levels, as seen in Figure 22. RF F1 mice in general have a higher blood glucose level throughout the 24 hours than CTR F1 mice. This is exemplified by the fact that the lowest blood glucose level of RF F1 mice (175.6 mg/dl) is almost equal to the highest blood glucose of the CTR F1 mice (175.5 mg/dl) at Zeitgeber 6. Already at the beginning of the active phase (Zeitgeber 12) the blood glucose level of the

RF F1 (181 mg/dl) mice is significantly higher ($p<0.001$) than that of the CTR F1 (152 mg/dl) mice. This difference in blood glucose is most prominent during the active phase.

The elevation in the blood glucose of RF F1 mice may be caused by a higher food intake over 24 hours. The RF F1 mice have higher glucose levels than the CTR F1 (RF average: 183mg/dl vs. CTR average: 164 mg/dl difference). The highly significant difference in blood glucose between the RF and CTR F1 during the active phase, during which both mouse groups have their highest food intake, may also be caused by the RF F1 transitioning from the resting phase to the active phase with a blood glucose level that is already high.

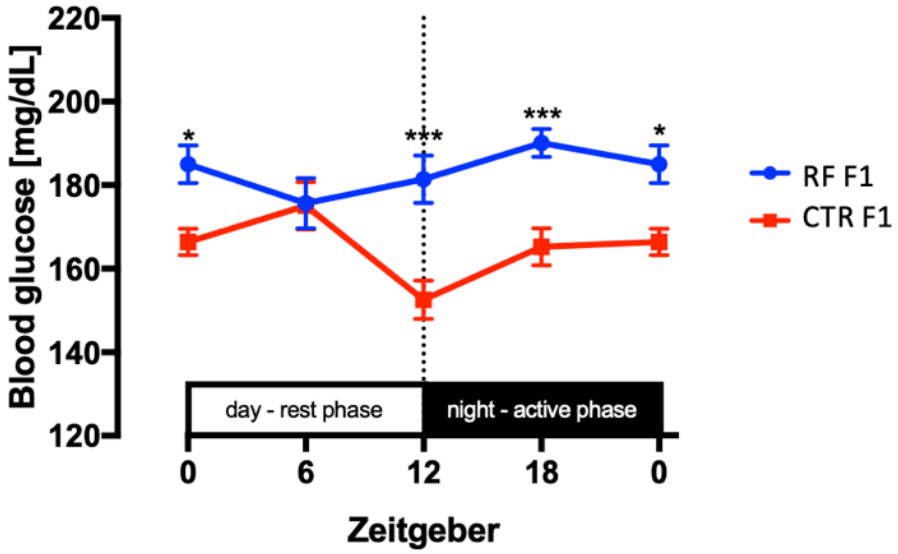

Figure 22: Blood glucose levels of RF and CTR F1 over 24 hours
RF and CTR F1 blood glucose levels at different Zeitgeber ZT0, 6, 12 and 18. n=9 / ZT. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

In addition to the blood glucose levels, we also checked the insulin levels at the different Zeitgeber. According to our data, there are no significant differences in insulin levels between the CTR and RF F1 (Figure 23).

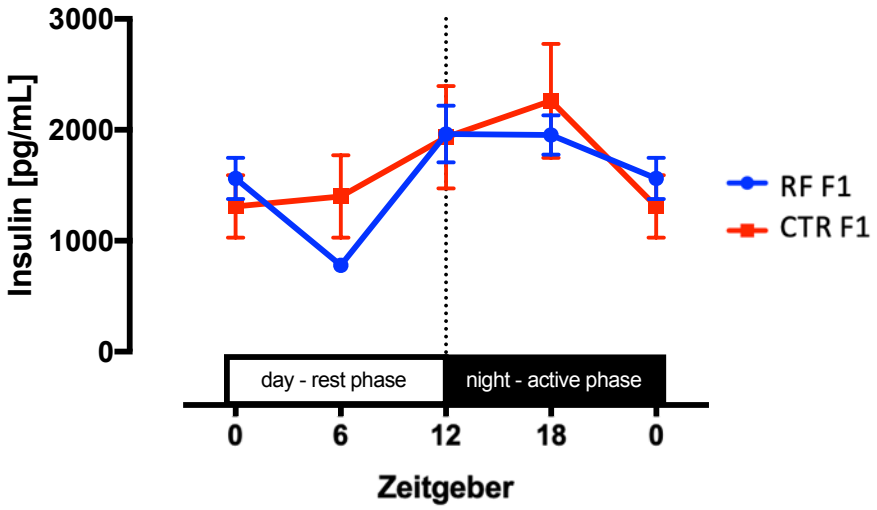

Figure 23: Insulin levels of RF and CTR F1 over 24 hours
RF and CTR F1 insulin levels at Zeitgeber 0 ,6, 12 and 18. n=9 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

3) POTENTIAL VEHICLES OF TRANSMISSION FROM RF F0 TO RF F1

Clock gene expression of RF F0 and RF F1

The qPCR of the RF F0 liver shows that the core clock genes in organs such as the liver are highly correlated to the timing of food intake and not to the rhythm of light. Even though the SCN works as a master controller of circadian clock oscillation throughout the body by sensing light and thus day and night time, peripheral clocks in organs and cells can be massively influenced by other environmental clues, especially via food intake [53, 84, 165]. We created a heatmap (Figure 24) where the expression levels of the different clock genes were compared between RF and CTR in F0 and F1. This method allowed us to visualize the expression levels of the clock genes in 16-week-old RF F0 (Figure 24 RF 1, 2, 3) and CTR F0 mice (Figure 24 CTR 1, 2, 3) at Zeitgeber 0. In the RF F0 expression levels of Bmal1, Clock and Cry1 vary between 0 and 1. The expression of Bmal1, Clock and Cry1 in the RF F0 generation, in comparison, varies between -1 and -2. This means that in the RF F0 mice the expression of these genes is half or even one quarter of the expression compared to CTR F0. For the other clock genes (RorA, Per1, Per2, Rev-erb α, Cry2, Dbp, Rev-erb β) that are

inversely expressed, the expression patterns of RF F0 and CTR F0 are completely inversed. In RF F0 therefore, the expression levels of these genes vary between 0 and 2. In comparison, in the CTR F0 mice the gene expression of these genes varies between 0 and -2.

We were interested in analyzing if these core clock genes were altered in the RF F1 and resulted in the mice being stimulated to eat more during the resting phase than their fathers, even though the RF F1 had never being exposed to circadian arrhythmia. We waited for the RF F1 to be at the comparable age of 16 weeks and then measured the gene expression at Zeitgeber 0 (6:00 a.m.) in their liver samples. As one can see in Figure 24, the expression pattern of the clock genes in the RF F1 and the CTR F1 (RF 4, 5, 6 and CTR 4, 5, 6) do not differ, contrary to the expression pattern of the clock genes in the RF F0 mice. Expression level of Bmal1, Clock and Cry1 vary between 0 and 2 in in both RF and CTR F1. The expression levels in both RF and CTR F1 of Per1, Per2, Rev-erb α, Cry2, Dbp and Rev-erb β, on the other hand, vary between 0 and -2. This shows that while in the RF F0 mice the expression of the clock genes was undeniably influenced by the change in food availability, this gene expression alteration was not inherited by the RF F1. The altered expression of the clock genes is the driving mechanism for the phenotype seen in the RF F0 mice.

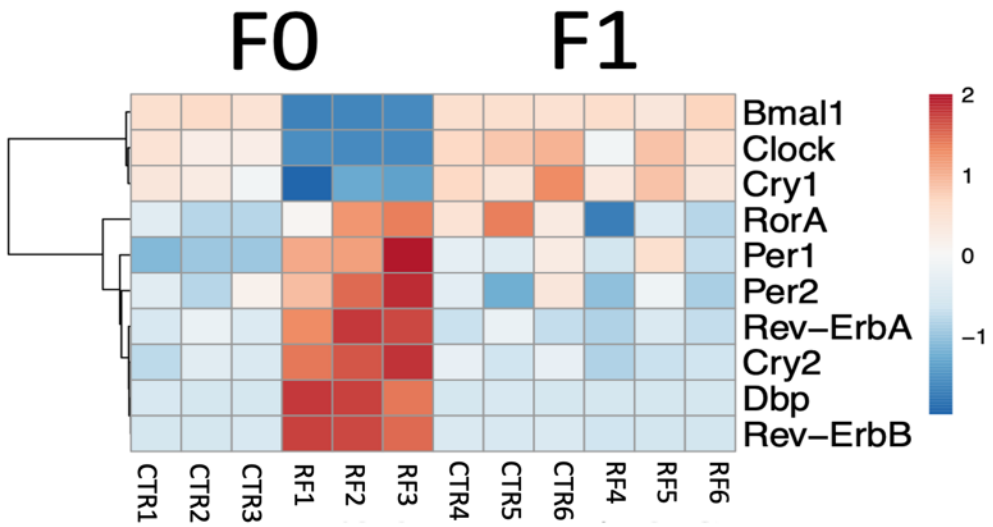

Figure 24: Heatmap of RNA-sequencing clock gene expression of RF and CTR F0 and F1 cohorts Heatmap representation of the expression levels of significant core clock genes of RF and CTR F0 and F1 liver samples at ZT0. Liver samples were taken when the RF and CTR F0 and F1 were 16 weeks old. High gene expression levels are symbolized via dark red boxes and low gene expression are symbolized by dark blue boxes. The F0 cohort consisted of the control mice CTR1, CTR2, CTR3 and the restricted fed mice RF1, RF2, RF3. The F1 cohort consisted of the control mice CTR4, CTR5, CTR 6 and the restricted fed mice consisted of RF4, RF5, RF6.

From consideration of our data the following conclusions can be drawn:

1) The changes of clock core gene expression are more likely to be caused by direct environmental factors rather than by any inherited factors.

2) The phenotypic alterations in the RF F1 must be caused by other driving factors than altered expression of core clock genes. These factors will be discussed in the following chapters.

Corticosterone levels of RF F0 and RF F1

While cortisol in humans is the primary glucocorticoid, in many species, including mice, corticosterone is the main glucocorticoid. The name glucocorticoids already indicates, that they are tightly connected to glucose metabolism. Both cortisol and corticosterone are also involved in stress responses of the body, which again are connected to many metabolic processes [166]. We decided to measure the corticosterone level of the RF F0 and RF F1 for the following reasons:

1. Corticosterone levels are a well-known readout for potential circadian arrhythmia, since corticosterone levels are highly connected to the circadian clock and fluctuate throughout the 24 hour day. In mice, corticosterone levels peak before nighttime, when mice are anticipating food. In humans, cortisol levels are generally higher in the morning directly after waking up [167].
2. Circadian arrhythmia and the manipulation of natural sleep cycles are severe stress factors [168]. Measuring the corticosterone levels throughout the day could provide us with information to what extent circadian arrhythmia and manipulation of sleep cycles impact not only glucocorticoid levels but also subsequent glucose levels and metabolic processes.

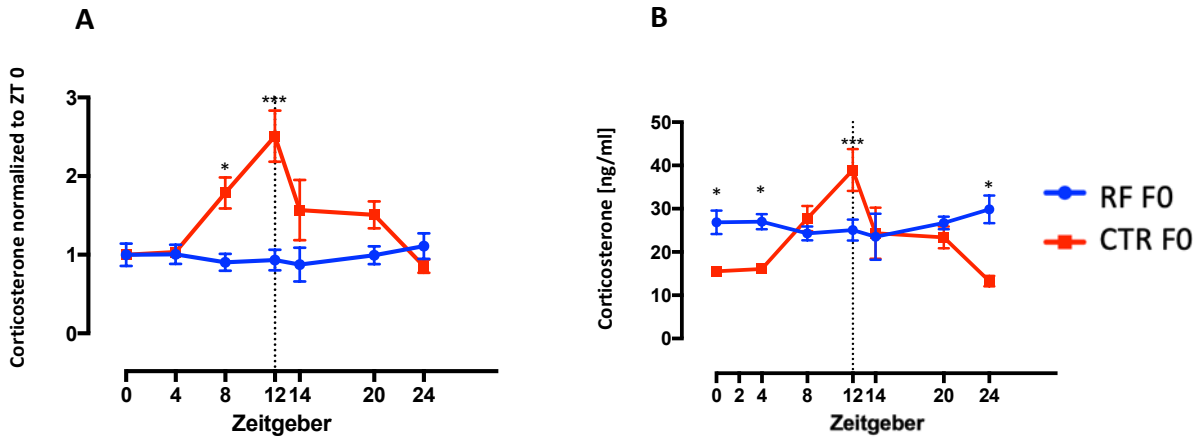

Figure 25: Corticosterone levels in RF and CTR F0
ELISA CTR and RF F0 corticosterone concentrations normalized to control at Zeitgeber 0 (A). Corticosterone concentration in the blood plasma of RF and CTR F0 at Zeitgeber 0, 2, 4, 8, 12, 14, 20, 24. n=4 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

In Figure 25 where the corticosterone levels were normalized to the starting values at Zeitgeber 0, we saw the peak that anticipates feeding and activity in the CTR F0, with a peak glucocorticoid concentration of around 40 ng/ml at ZT 12. The corticosterone expression levels of the RF F0 on the other hand were dampened and fluctuated between 25 and 30 ng/ml, without peaking ($p<0.001$) (Figure 25). At Zeitgeber 0 and 4, RF F0 corticosterone levels were almost twice as high as that of the CTR F0 group (ZT0: RF F0 26.8 ng/ml vs. CTR F0 15.5 ng/ml, ZT4: RF F0 26.9 ng/ml vs. CTR F0 16.0 ng/ml). The basal corticosterone levels

during the resting phase were therefore elevated in the RF F0. The data showed that by changing the eating patterns of the RF F0 mice, more stress hormones were produced and the difference in hormone levels between day (resting period) and night (active phase) became smaller.

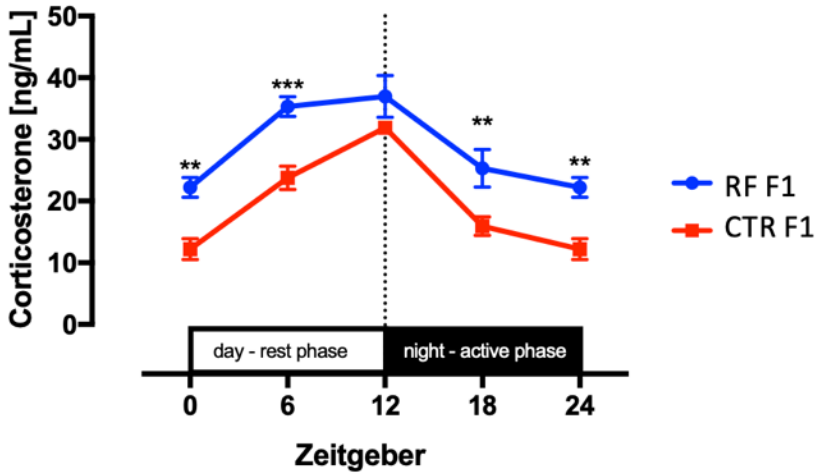

Figure 26: Corticosterone levels in RF and CTR F1 at different Zeitgeber
RF and CTR F1 blood plasma corticosterone levels measured via ELISA at Zeitgeber 0, 6, 12, 18, 24. n=9 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using a two-tailed Student's t-test (*<0.05, **<0.01, ***<0.001).

Furthermore, we measured the corticosterone levels in plasma samples of the consequent RF and CTR F1 mice. Like the RF F0 generation, the RF F1 had statistically significantly higher corticosterone levels than the CTR F1 at all Zeitgeber points (ZT0, ZT 18 and ZT24 $p<0,01$ and ZT6 $p<0,01$). We saw a dampening of the expected corticosterone peak in the RF F1 (Figure 26). Between ZT6 and ZT12 in the CTR F1 there was an increase of approximately 8 ng/ml corticosterone (ZT6: 23.7 ng/ml, ZT12: 31.8 ng/ml). In the RF F1 the elevation in corticosterone level between ZT6 and ZT12 was only 1,6 ng/ml (ZT6: 35.3 ng/ml, ZT12: 36.9 ng/ml). Even though the phenotype was not as pronounced in the RF F1 generation as in the RF F0 mice, the RF F1 cohort showed a significant phenotype in corticosterone levels. Circadian arrhythmia in the RF F0, induced by an altered feeding schedule, entails crucial changes in corticosterone homeostasis. These changes in the RF F0 generation can also

impact the corticosterone levels of the next generation (F1), even if it has never been exposed to circadian arrhythmia. Due to the strong link between corticosterone and glucose homeostasis, discussed in the introduction, the data suggest that the alteration in corticosterone levels passed from the RF F0 to the RF F1 may cause the food intake phenotypes previously discussed. To further explore the underlying mechanism, potential vehicles of transmission were explored:

1) Transmission from RF F0 to RF F1 via differentially expressed clock core genes in germ cells
2) Alterations in the level of corticosterone in the seminal fluid

Gene expression of clock genes in germ cells of RF F0

Only the sperm cells and the seminal fluid are transferred in the reproductive process from the RF F0 male to the wild type female. Via qPCR we investigated the expression of the core clock genes Bmal1, Per2 and Cry1 in the sperm germ cells of the RF F0. An epigenetic change in the DNA of the RF F0 mice could be a potential mechanism of inheritance. We therefore isolated the sperm of the RF F0 mice. We focused on the expression of the clock core genes since in the qPCR of the liver cells, described earlier in the text (Figure 16), gene expression patterns of RF F0 and CTR F0 mice had been completely inverted. In the sperm cells, however, we saw no difference between the RF F0 and the CTR F0 mice regarding the expression patterns of these genes at the different Zeitgeber 0, 4, 8, 12 and 24. We therefore concluded that, unlike in the liver cells, gene expression of the clock core genes in the sperm germ cells is not directly altered via exposure to a circadian arrhythmia environment (Figure 27). Our data thus exclude the transmission from RF F0 to RF F1 via differentially expressed clock core genes in germ cells as mechanism resulting in increased food intake in the RF F1 generation.

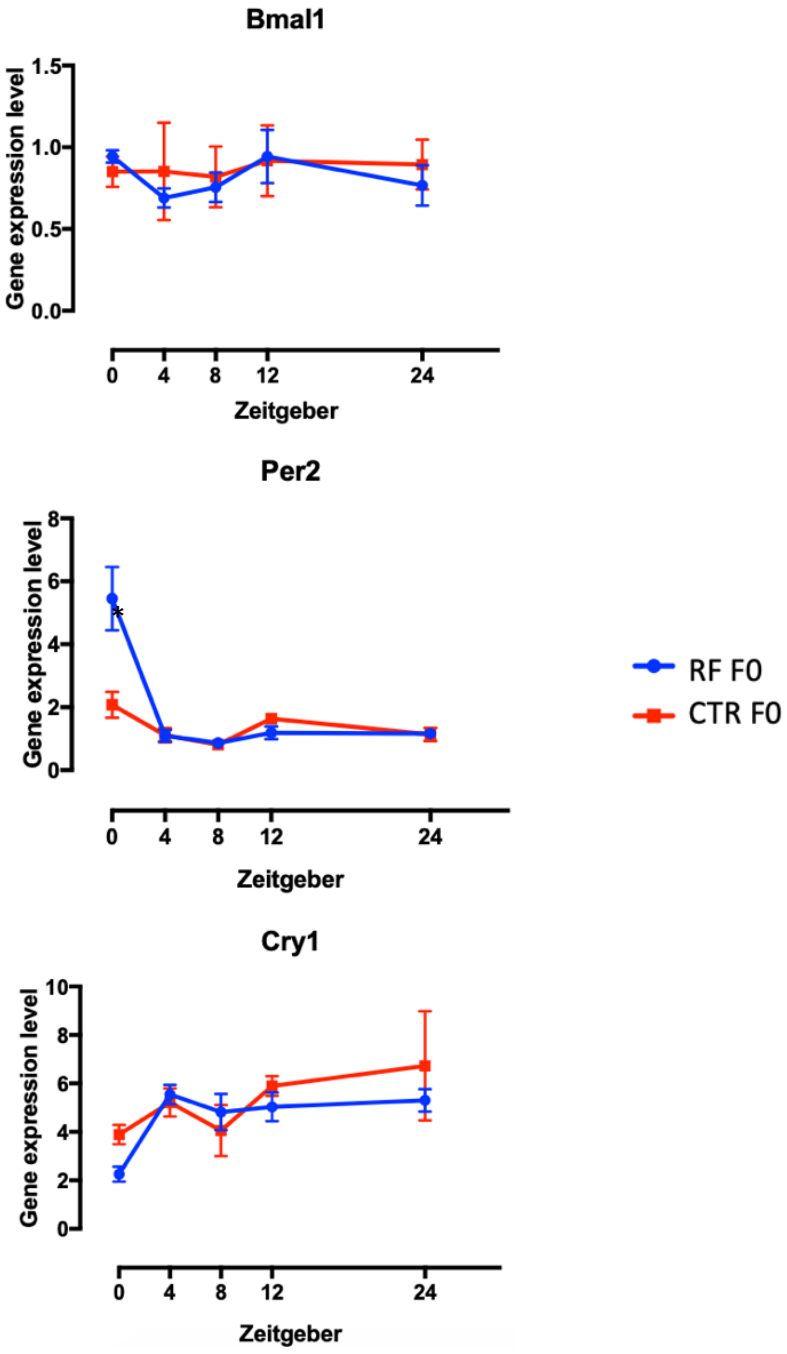

Figure 27: Core clock gene expression levels in RF and CTR F0 germ cells
Core clock gene expression of Bmal1, Per2 and Cry in germ cells of RF and CTR F0 (normalized to 36b4) at Zeitgeber 0, 4, 8, 12, 24 (measured via qPCR). n=4. Error bars display SEM values. Data was considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001).

Corticosterone concentration levels in the seminal fluid of RF F0

Since seminal fluid also plays a crucial role in reproduction [169-171] and influences pregnancy outcome and offspring development [172], we wanted to find out if the

corticosterone levels in the seminal fluid are altered by circadian arrhythmia. When we tested seminal fluid samples in the F0 we noticed the same pattern we had seen regarding the corticosterone levels in the blood plasma (Figure 25). In seminal fluid the normal peak was weakened in the RF F0 mice and there was hardly any fluctuation in corticosterone levels at the different timepoints (Figure 28 CTR F1: ZT4 7.6ng/ml, ZT12: 34.9 ng/ml – range: 27.3 ng/ml. RF F1: ZT4 4.1 ng/ml, ZT8: 18.5 ng/ml – range: 14.4 ng/ml).

The corticosterone level of CTR F0 seminal fluid was significantly elevated in comparison to that of the RF F0 (ZT8: RF 18.5 ng/ml vs. CTR 30.9 ng/ml $p<0.05$ and ZT12: RF 12.9 ng/ml vs. CTR 34.9 ng/ml $p<0.001$).

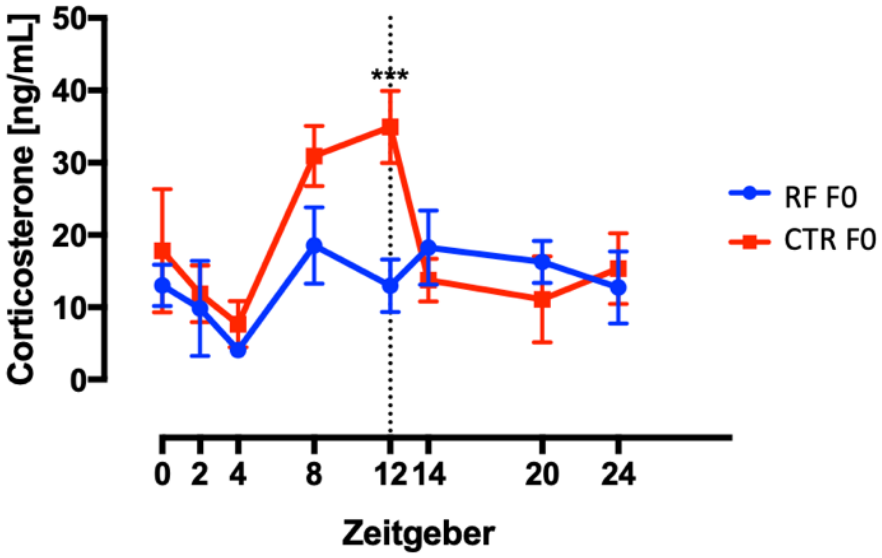

Figure 28: RF and CTR F0 seminal fluid corticosterone levels
RF and CTR F0 seminal fluid corticosterone concentrations at Zeitgeber 0, 2, 4, 8, 12, 14, 20, 24 measured via ELISA. n=4 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

To conclude, we saw alterations in corticosterone concentrations in F0 mice in blood serum as well as in seminal fluid. Thus, we hypothesize that glucocorticoid alterations could be translated or at least transferred as metabolic information from a F0 to a F1 generation.

Metabolic phenotypes of F1 conceived via IVF

We created a new RF F1 generation via in-vitro fertilization (ivRF F1). The seminal fluid was eliminated and only the sperm cells of the RF F0 were used to fertilize the oocytes of wildtype females [173]. Except for using a different method of reproduction, the breeding scheme for the ivRF F0 was identical to that of the RF F0.

We also used the same protocol and phenotyping methods for the ivRF F1 as for the RF F1 cohort. We measured the bodyweight of the ivRF F1 every 2 weeks (Figure 31), their 24-hour food intake at 2, 4, 6 months of age (Figure 29) as well as their energy expenditure via single caged calorimetry (Figure 30). At the age of 16 weeks, we measured the corticosterone levels of the ivRF F1 and ivCTR F1 using ELISA. We then harvested the organs of the ivRF and ivCTR F1 (including blood, liver, hypothalamus, and adrenal gland) for RNA analysis and compared the data to RF F1 and CTR F1. This method offered us the opportunity to verify that, as expected from the unchanged clock genes expression in the sperm cells, sperm cells are not involved in the mechanism.

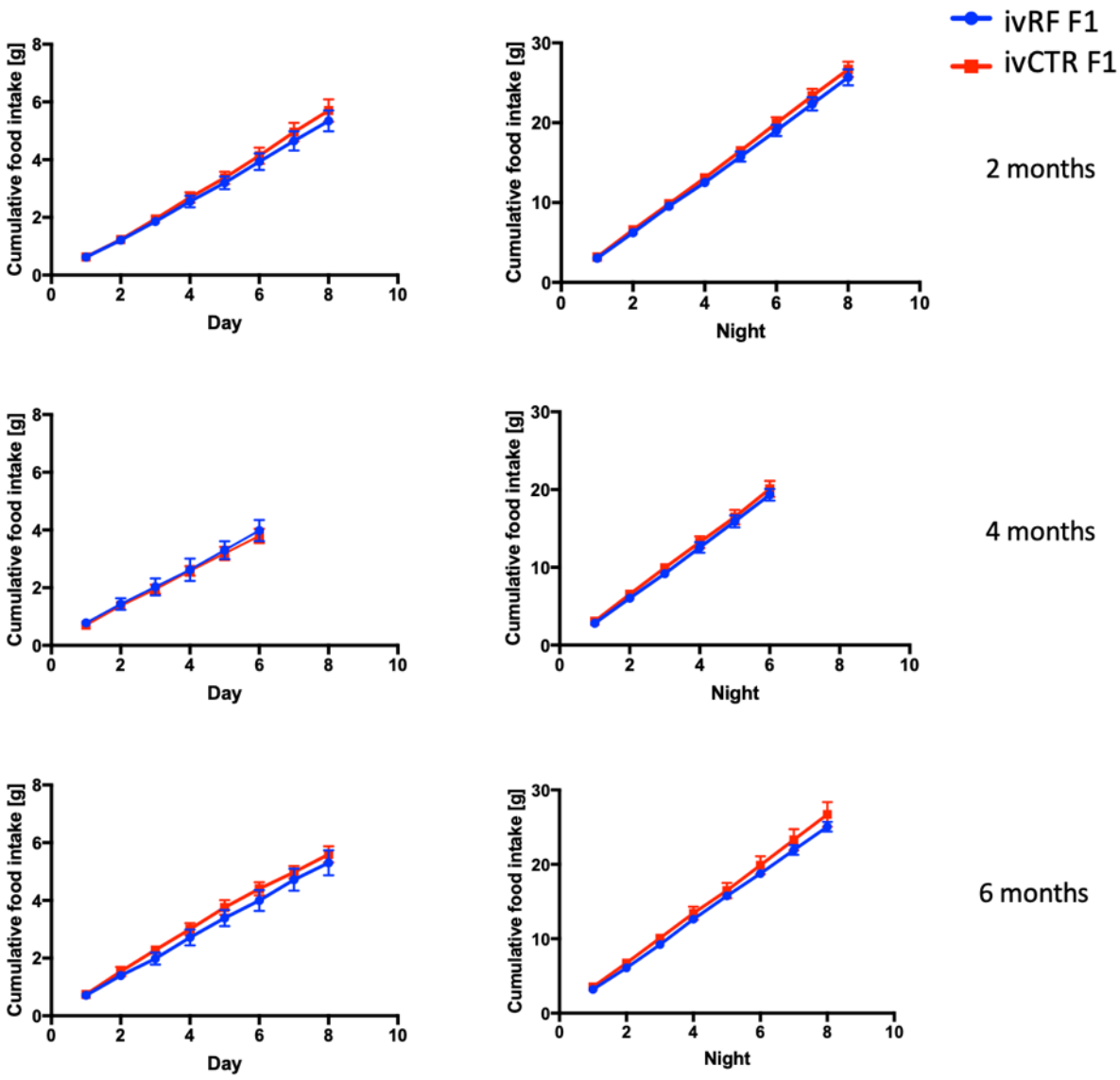

Figure 29: Cumulative food intake of ivRF and ivCTR F1 at different ages
Daily cumulative food intake of ivRF and ivCTR F1 at different ages (2,4 and 6 months) shown on the left. Nightly cumulative food of ivRF and ivCTR F1 at different ages (2,4 and 6 months) shown on the right side, n=5. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05,**<0.01,***<0.001).

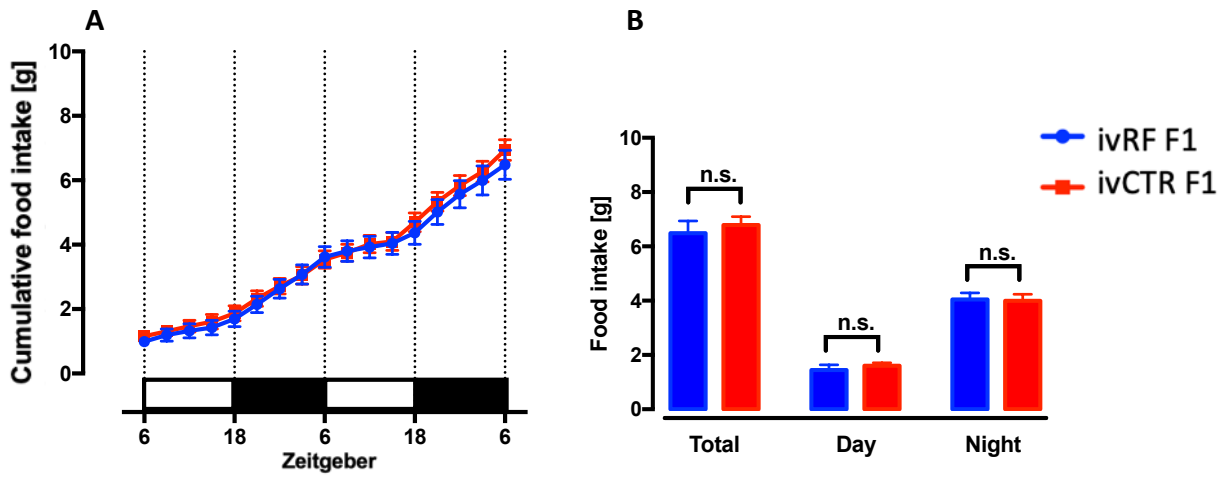

Figure 30: Food intake of ivRF and ivCTR F1
Cumulative food intake of ivRF and ivCTR F1 over 48 hours (A) average total and average daily/nightly food intake of ivRF and ivCTR F1 measured over 24 hours via calorimetric cages (B) n=14. Error bars display SEM values. Data were considered statistically significant at p<0.05 using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001, n.s. = non-significant).

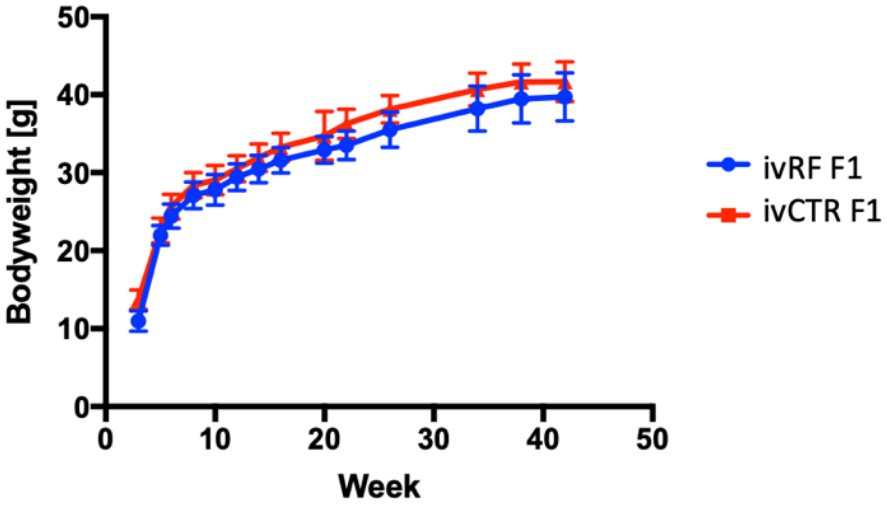

Figure 31: Bodyweight of ivRF and ivCTR F1 at different ages
n=14. Error bars display SEM values.

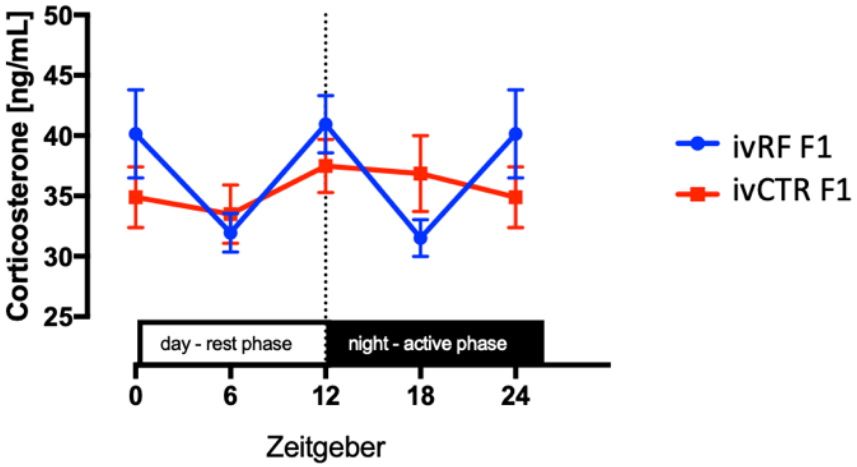

Figure 32: Corticosterone levels of ivRF and ivCTR F1
Blood plasma corticosterone levels of ivRF and ivCTR F1 measured at Zeitgeber 0, 6, 12, 18 and 24 via ELISA. n=4 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

The ivRF F1 cohort showed no signs of altered food intake, neither during the day nor in total (Figure 29 & Figure 30). Contrary to the RF F1, these mice did not show any changes in their food intake pattern that could be attributed to the paternal circadian arrhythmia. There was also no difference regarding the bodyweight (Figure 31) and corticosterone levels (Figure 32) of the ivRF and ivCTR F1. This was expected, as their food intake habit and their activity level did not change, which directly influences the corticosterone release.

The 24-hours glucose levels were significantly higher in ivCTR animals compared to ivRF F1 (Figure 33 - blood glucose at Zeitgeber 0 $p<0.01$, blood glucose at Zeitgeber 18 $p<0.05$). However, if one also compares the difference in blood glucose trend among the CTR and ivCTR F1, the data suggest that the IVF procedure alone has a strong influence on the blood glucose. While maximum blood glucose among the RF F1 was 175 mg/dl, the maximum blood glucose among the ivCTR F1 was 197mg/dl.

At all Zeitgeber the insulin levels were also significantly higher among the ivCTR than among the ivRF F1 (ZT0, 6, 18, 24 $p<0.05$, ZT 12 $p<0.01$). The changes in blood glucose and insulin levels among the ivRF and the ivCTR F1 may therefore be attributed more to the IV procedure than to the parental circadian arrhythmia. This is in line with the observations made among human children conceived by IVF. A Dutch study, among 8 to 18-year-old IVF

singletons and spontaneously born children of subfertile parents, for instance, had shown that pubertal children conceived by IVF treatment had higher fasting glucose levels [174].

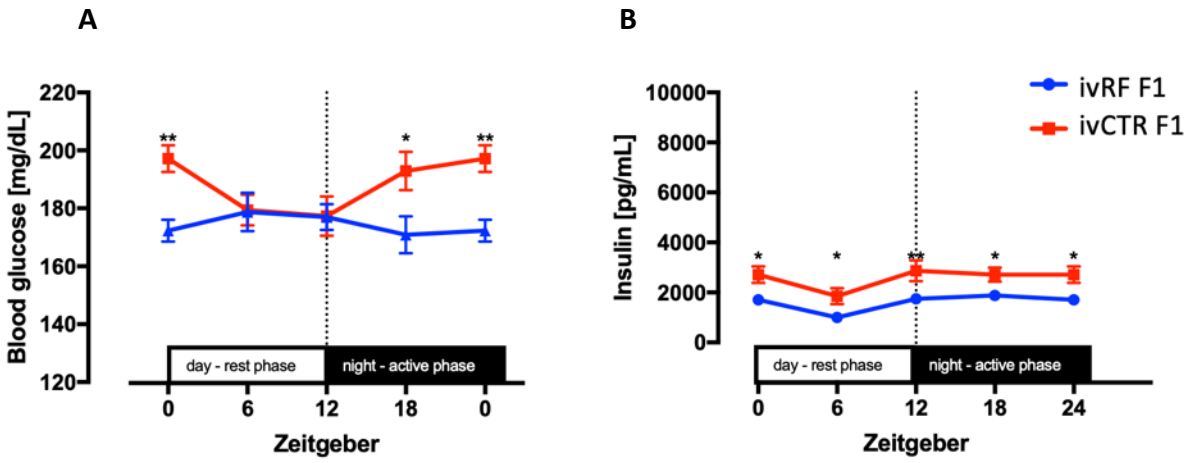

Figure 33: Blood glucose and insulin levels of ivRF and ivCTR F1
ivRF and ivCTR F1 glucose (A) and insulin (B) levels measured in blood plasma at Zeitgeber 0, 6, 12, 18. n=14 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

The food intake phenotype observed in the RF F1 group could not be observed in the ivRF group. This verifies our hypothesis, that the strong food intake phenotype observed among the RF F1 is not transferred by the sperm cells of the RF F0. Previous experiments among the RF F1 show that the food intake phenotype and corticosterone levels are linked together. This also indicates that altered corticosterone levels in the seminal fluid may influence various metabolic key points and lead to specific phenotypes, without directly disrupting the clock genes.

4) EPIGENETIC, INTERGENERATIONAL AND DEVELOPMENTAL MECHANISMS

To further investigate the correlation between corticosterone and key metabolic points we looked at the hypothalamus pituitary adrenal (HPA) axis and the core contributor organs involved in the pathway e.g. the liver, hypothalamus and adrenal gland.

RNA sequencing of liver and hypothalamus samples of RF F1

Stress factors are sensed by the paraventricular nucleus of the hypothalamus. The corticotropin-releasing hormone stimulates the anterior pituitary gland to release adrenocorticotropic hormone (ACTH) into the blood stream. ACTH in turn acts on the adrenal cortex to produce glucocorticoid [139, 175]. Glucocorticoids have been shown to influence crucial clock genes. To understand whether the altered glucocorticoid rhythm is associated with a global alteration of oscillatory transcription, we performed 24-hour liver RNA-Seq from samples harvested at Zeitgeber 0,6,12 and 18 from the RF and CTR F1.

Liver

We used "JTK cycle" [176, 177, 178] to identify mRNA transcripts with robustly oscillating expression profiles. The results showed a global transcriptional shift of approximately 6 hours in the liver of RF F1 in comparison to the liver of CTR F1 (CTRL F1 peak ~ ZT18; RF F1 peak ~ZT0 – Figure 34 A). More than 2000 transcripts lose or gain rhythmicity (Figure 34 B), and 1390 transcripts maintain rhythmicity with an overall 6-hours shift in peak expression (Figure 34 C).

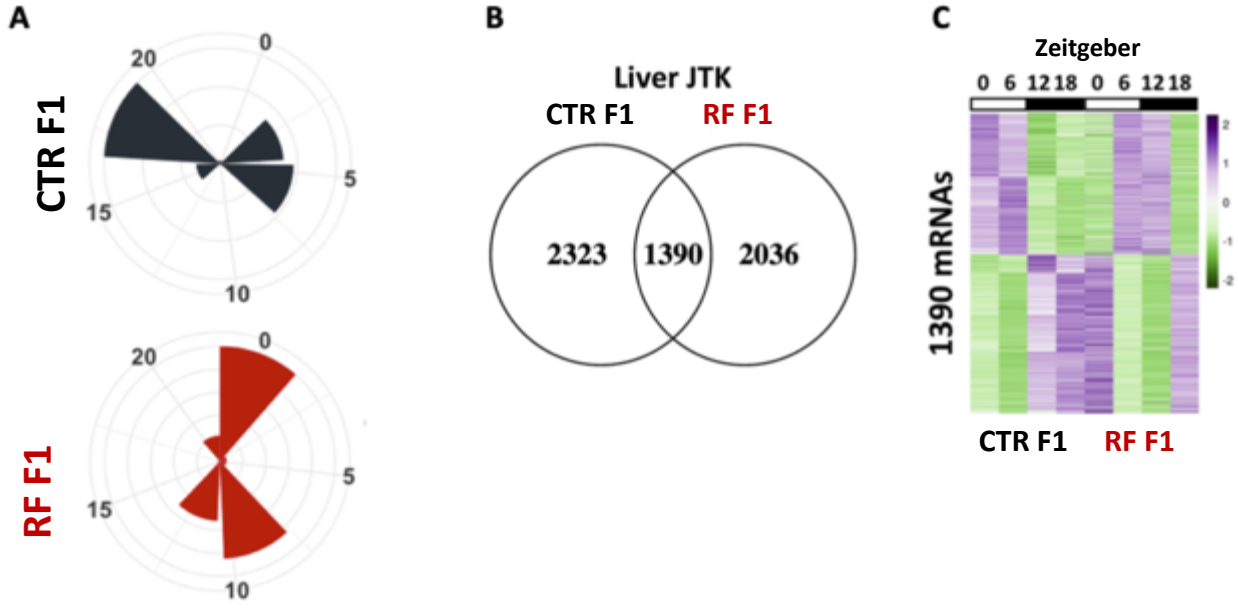

Figure 34: RNA-Seq data of oscillating liver genes of RF and CTR F1
Radar plots showing the circadian gene expression of oscillating genes in the liver of RF and CTR F1 mice (A). Venn diagram of JTK CYCLE analysis showing the number of oscillating genes in the liver of only CTR F1, only RF F1 and in both CTR and RF F1 (B). Heatmap visualization of RNA-Seq-based expression of oscillating transcripts in the liver of CTR and RF F1 (C). Average signal of n=3 biological replicates / Zeitgeber.

Hypothalamus

In addition to the liver samples, we also analysed the hypothalamus of the RF and CTR F1 mice. We profiled the 24-hour hypothalamic transcription (Zeitgeber 0, 6, 12 and 18) by RNA-Seq. We then again used the JTK cycle to identify oscillating transcripts [176, 177]. We observed a profound alteration of rhythmic transcription in the hypothalami of RF F1, with a pattern similar to the one observed in the liver (Figure 34). The expression of oscillating hypothalamic transcripts in RF F1 peaked at ZT0 with a delay of approximately 6 hours compared to the peak in CTR F1 (Figure 35 A). Only 198 of the RF F1 oscillating genes maintained the same rhythmicity as in the CTR F1 (Figure 35 B & C).

In particular and in keeping with the reported feeding phenotype, we found strong and robust upregulation of key orexigenic neuropeptides, such as neuropeptide y and hypocretin (precursor of the two major orexins 1 and 2) and downregulation of anorexigenic neuropeptides, such as Cartpt, Bdnf and Pomc at Zeitgeber 0 (Figure 36). Npy expression increases food intake and glucocorticoid concentrations in plasma. Hypocretin, like Npy, is involved in stimulating food intake and wakefulness. The shift in the oscillation of liver and hypothalamic gene transcripts may explain why, these mice display phenotypes of circadian arrhythmia.

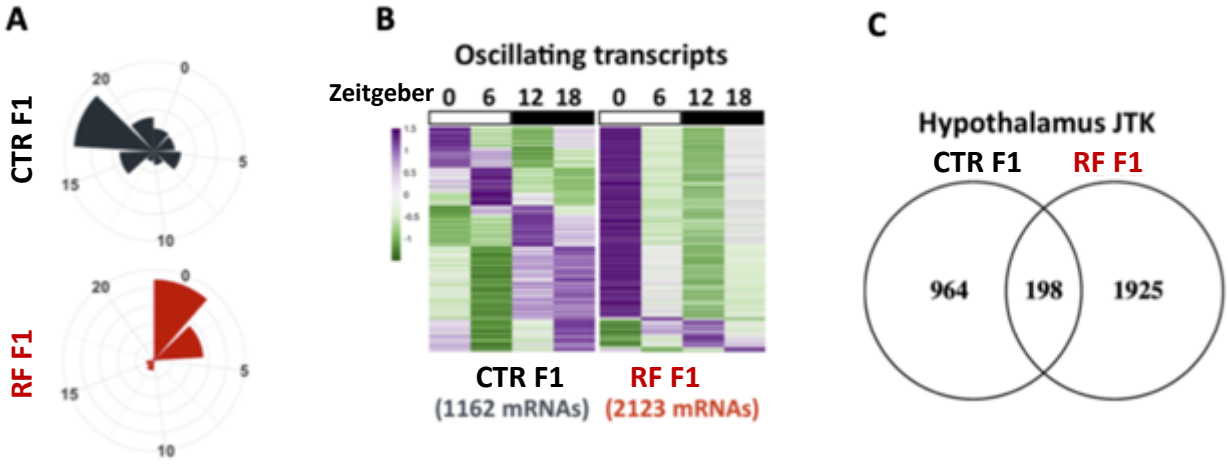

Figure 35: RNA-Seq data of oscillating hypothalamus genes of RF and CTR F1
Radar plots showing the circadian gene expression of oscillating genes in the hypothalamus of RF and CTR F1 mice (A). Heatmap visualization of RNA-Seq-based expression of oscillating transcripts in the hypothalamus of CTR and RF F1 (B). Venn diagram of JTK CYCLE analysis showing the number of oscillating genes in hypothalamus of only CTR F1, only RF F1 and in both CTR and RF F1 (C). Average signal of n=3 biological replicates / Zeitgeber.

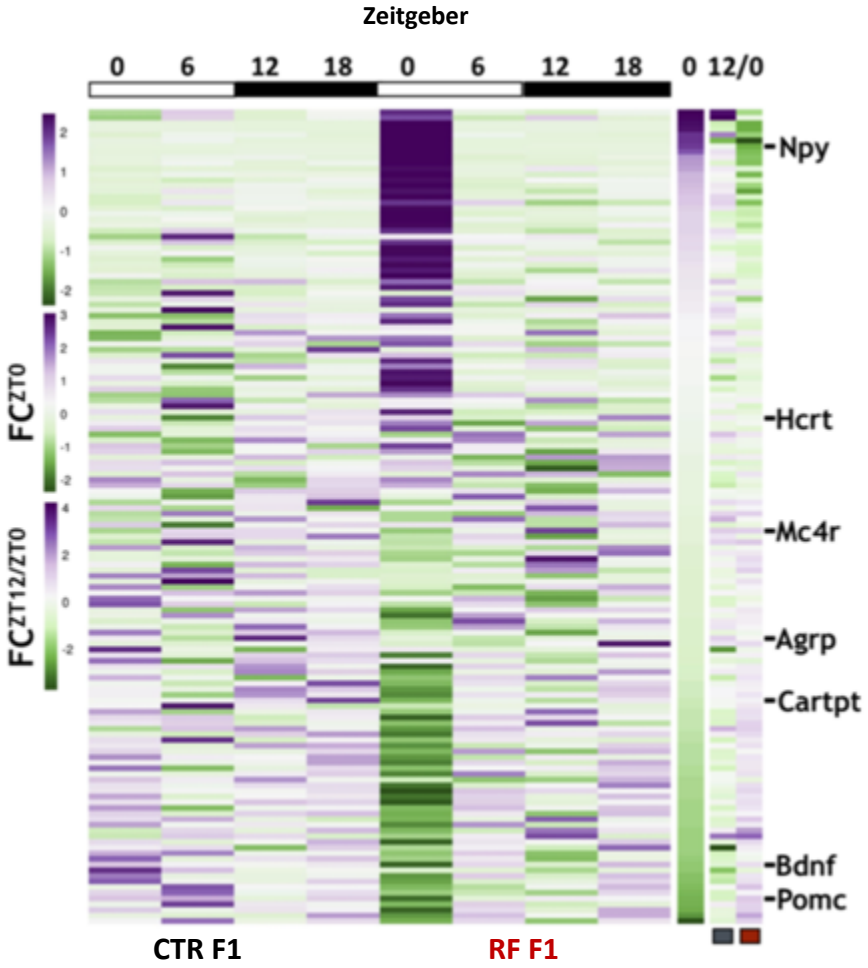

Figure 36: Heatmap visualization of RNA-Seq-based analysis of orexigenic and anorexigenic neuropeptide expression in the hypothalamus of RF and CTR F1
Additional heatmaps represent selected LogF2C at ZT0 (F1 RF / F1 CTR – column "0") or represent the induction of neuropeptides at the day/night transition (Zeitgeber 12 / ZT0 – column "12/0") in the respective groups.

Clustering of liver genes

The analysis of a set of 50 core-clock genes (as reported by the REACTOME database) the liver and hypothalamus confirmed transcriptional alterations of the clock machinery in both tissues (Figure 37, Figure 38, Figure 39 and Figure 40). Clustering of the liver's core clock gene according to similarity in expression pattern identified three distinct clusters of genes in both liver and hypothalamus. Cluster 1 included genes highly expressed during Zeitgeber 0 and 6 in the CTR F0 (eg. Nr1d1 - Figure 37). These genes showed a delayed transcriptional peak in RF F1 i.e. at Zeitgeber 6 and 12. The shift in gene expression of the key gene Nr1d1,

for instance, was statistically significant (p <0,05). Nr1d1 showed a shift in gene expression of 6 hours which confirmed the observations seen in Figure 34 A. Cluster 2 and cluster 3 included genes whose transcription primarily changed during dusk and dawn (e.g. Dbp, Clock and Per2 - Figure 37). The clock genes of RF F1 in cluster 2 showed a dampened transcriptional pattern at Zeitgeber 0 and 12 compared to their expression in CTR F1. The two main genes in this group Clock and Dbp showed a statistically significant change in expression at Zeitgeber 12 (Figure 38). In cluster 3, the expression pattern of the genes of the RF F1 also showed a shift of 6 hours. In the CTR F1 these genes (including Per2) were primarily expressed at Zeitgeber 12 and 18 while in the RF F1 these genes were primarily expressed at Zeitgeber 18 and 0.

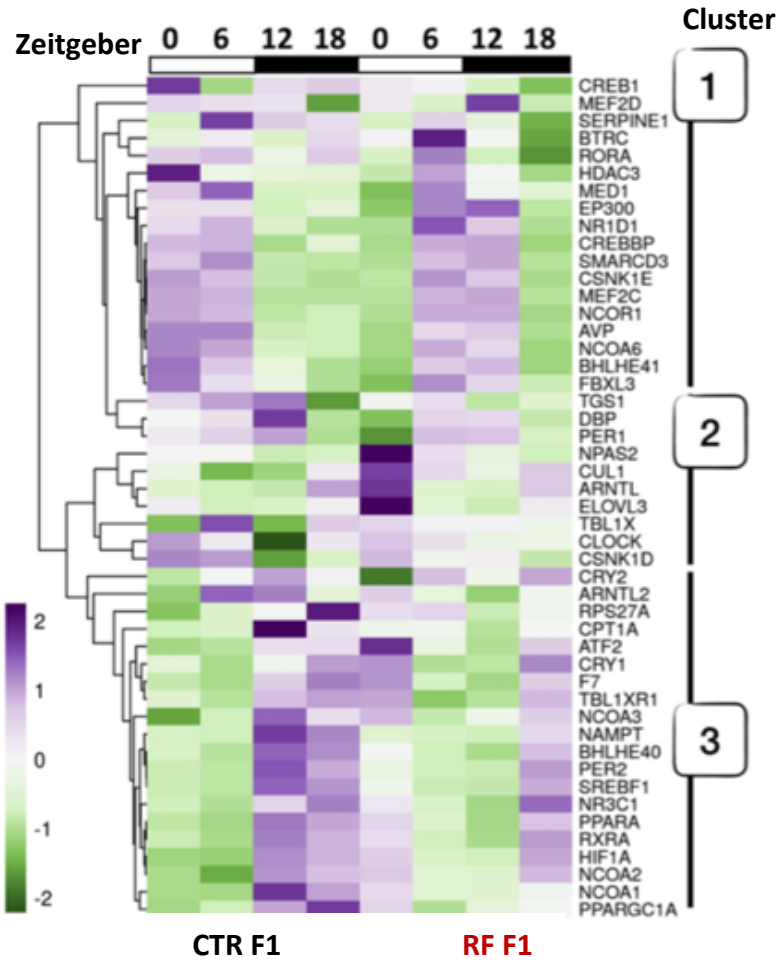

Figure 37: Heatmap visualization of core clock genes in the liver of RF and CTR F1
Analysis of a set of 50 core-clock genes in liver RF and CTR F1 groups describing transcriptional alterations of the clock machinery at Zeitgeber 0, 6, 12, 18, divided into 3 different clusters.

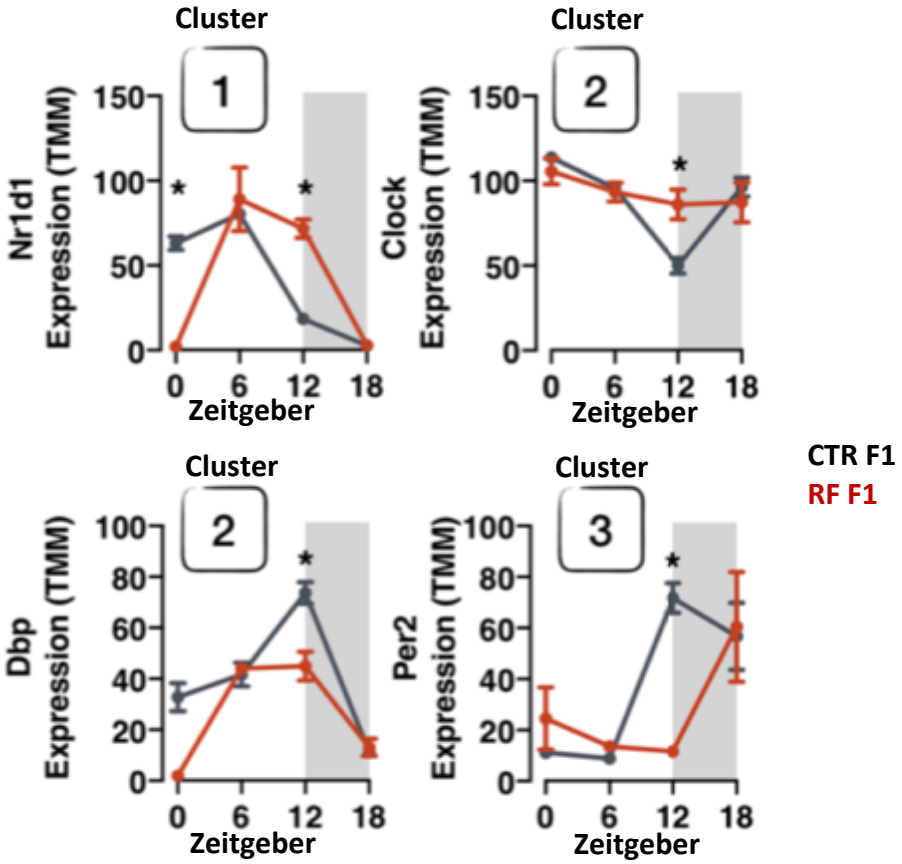

Figure 38: Gene expression of selected core-clock genes in the liver of RF and CTR F1
Expression level of core-clock genes in the liver of RF F1 (red) and CTR F1 (black) at Zeitgeber 0, 6, 12, 18 (via RNA-seq data). Expression in trimmed mean of M-value (TMM) expression.

Clustering of hypothalamus genes

We also clustered the genes of our hypothalamus samples according to their expression level at ZT0 (cluster 1) or ZT12 (cluster 3) (Figure 39). Cluster 1 included genes that were not strongly expressed in the CTR F0 but had a strong expression peak at Zeitgeber 0 in the RF F1. The genes included the key clock genes Dbp and Nr1d1. The increase in expression level of Dbp and Nr1d1 of RF F1 was highly significant ($p < 0,05$) in comparison to the expression level in CTR F1. Cluster 2 included genes that showed no significant change in transcription levels among CTR F1 and RF F1. This group included the main clock gene Clock. Cluster 3 included genes that had a 6-hour shift from high expression at Zeitgeber 0 to high expression at Zeitgeber 6. Per2 was included in cluster 3. While in the CTR F1 the expression

level of Per2 decreased from Zeitgeber 0 to Zeitgeber 6, it increased significantly in the RF F1 (Figure 40).

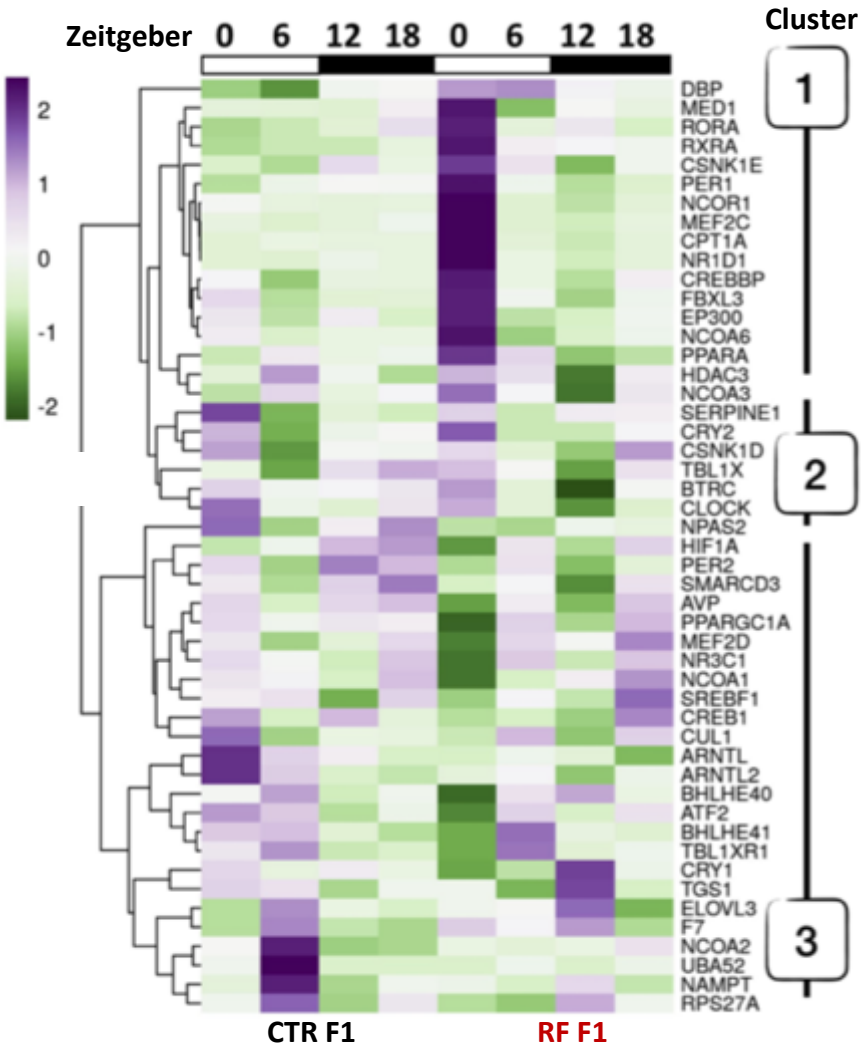

Figure 39: Heatmap visualization of core-clock genes in the hypothalamus of RF and CTR F1
Analysis of a set of 50 core-clock genes in hypothalamus RF and CTR F1 groups describing transcriptional alterations of the clock machinery at Zeitgeber 0, 6, 12, 18, divided into 3 different clusters.

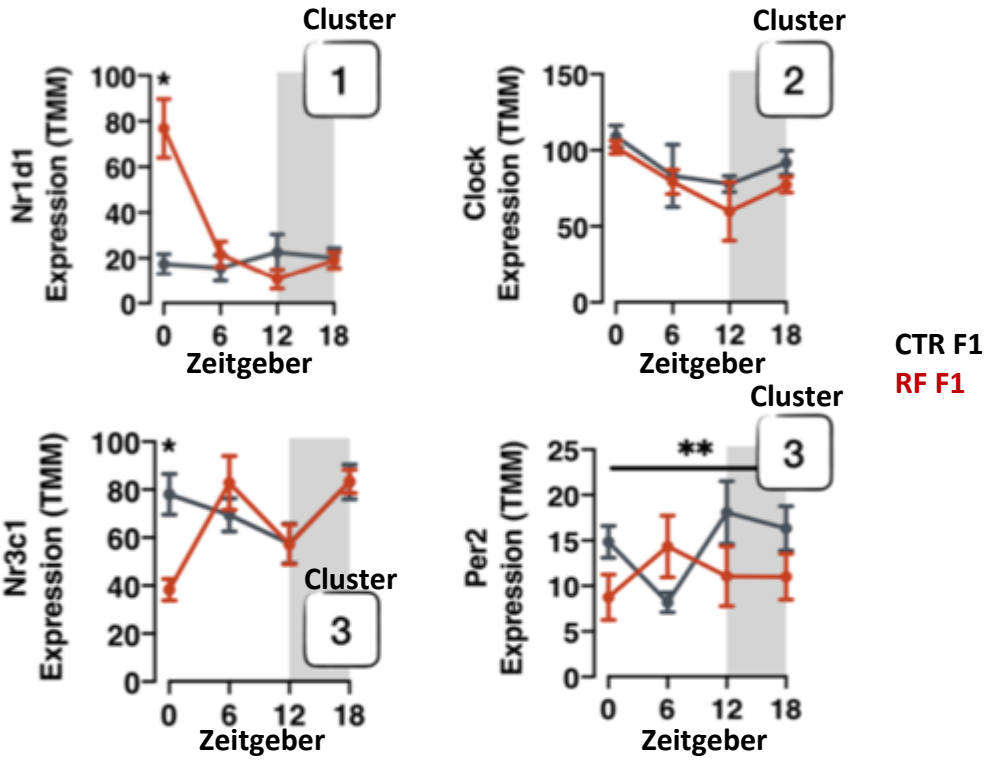

Figure 40: Gene expression of selected core-clock genes in hypothalamus of RF and CTR F1
Expression levels (trimmed to mean of m-value) of single genes at Zeitgeber 0, 6, 12, 18 for RF F1 (red) and CTR F1 (black) (via RNA-seq) data.

Our RNA sequencing data from the liver and hypothalamus are in line with the general findings that paternal circadian disruption leads to alterations of feeding behaviour, corticosterone rhythm and oscillating transcription in male offspring, while maintaining intact whole-body glucose homeostasis and light-entrained circadian rhythm.

5) F1 OFFSPRING OF FATHER'S THAT EXPERIENCED RESTRICTED AND THEN AD LIBITUM FEEDING

The previous data of the RF F1 mice showed that only 30 days of restricted feeding of the RF F0 males leads to highly significant changes in the food intake phenotypes of the RF F1. We wanted to investigate whether the RF F1 phenotype was reversible , if after a period of circadian arrhythmia (restricted food intake) the F0 generation was again exposed to a normal circadian rhythm (ad libitum food access). In the first part of this thesis, we had

found out that, contrary to previous publications where the mice were exposed to a chronic environmental change over several months [40, 41, 43, 54], a period of only 30 days was sufficient to induce an intergenerational phenotype. Therefore, it was of interest to investigate if the phenotype seen in the RF F1 could be reversed and if 30 days of ad libitum access to food after 30 days of restricted feeding was a sufficient time span to cause the reversal of the phenotype.

To test this, we used the same experimental F0 setup as with the RF F0 mice. However, at the end of the 30 days of restricted feeding (12 hours/day during the daytime/resting phase), the F0 mice were given 30 days of ad libitum food access. These mice will be referred to as revRF F0. After this period, we mated the revRF F0 with age-matched isogenic control females and phenotyped the F1 generation (revRF F1), using the same methods previously described in the text for the initial RF F1 cohorts. The revCTR F0 cohort consisted of male mice that were kept on an ad libitum diet throughout the full 60 days and then mated with age-matched isogenic control females to produce the revCTR F1. Figure 41 shows that the overall food intake of the revRF F1 did not differ from the revCTR F1. The feeding habits expressed by food intake during the day and night were also similar in the revRF and revCTR F1.

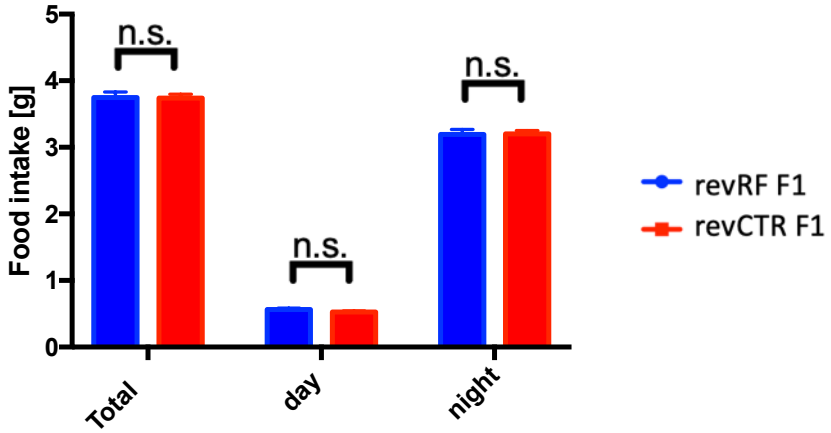

Figure 41: Food intake of revRF and revCTR F1
Total food intake and daily/nightly food intake of revRF and revCTR F1 over 10 days. n=10. Error bars display SEM values. (n.s. = non-significant).

Over a period of 35 weeks, there was no significant difference in bodyweight between the groups (Figure 42).

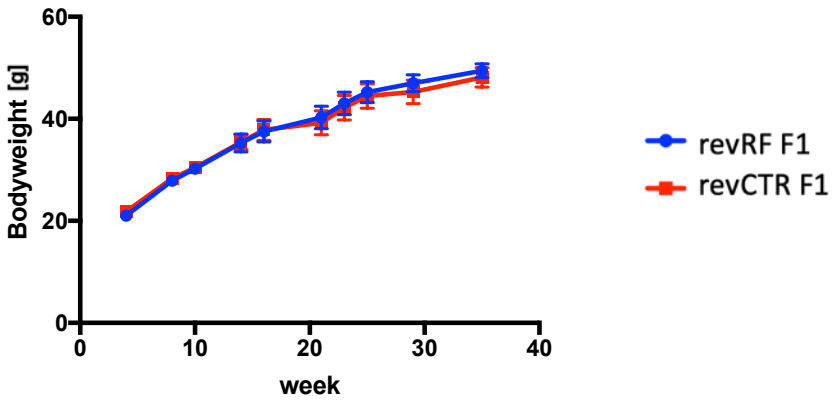

Figure 42: Bodyweight of revRF and revCTR F1 over 36 weeks
n=10. Error bars display SEM values.

There were also no significant differences in glucose levels between the revRF and revCTR F1 (Figure 43).

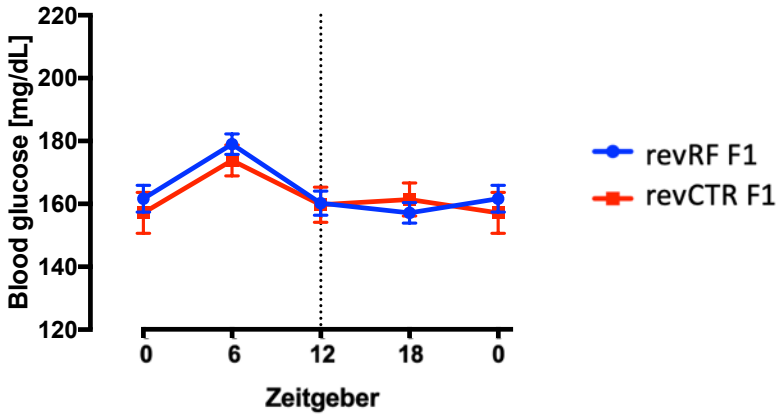

Figure 43: Blood glucose of revRF and revCTR F1 at different Zeitgeber over a total of 48 hours
n=10 / Zeitgeber. Error bars display SEM values.

No significant phenotypic differences were observed between the revRF and revCTR F1. Therefore, we can conclude that 30 days of ad libitum food access are enough to reverse any phenotype in the F1 generation induced by the 30 days of circadian arrhythmia the mice were exposed to prior.

6) F2 GENERATION OF RF F0

After observing distinct phenotypes and elevated glucocorticoid levels in the RF F1 generation whose fathers had been exposed to circadian arrhythmia, we were interested in finding out if circadian arrhythmia was penetrant enough to result in transgenerational phenotypes that would be apparent in the F2 generation. Previously published data had shown that certain environmental exposures, such as the high-fat diet of the F0 cohort, can lead to phenotypes in the F2 generation via transgenerational epigenetic inheritance [43, 44, 179].

In order to answer this question, we generated a RF F1 cohort as described above and mated the RF F1 males with control wildtype females at the age of 16 weeks. We then phenotyped the resulting RF F2 cohort in the same way as previously described for the RF F1 cohort.

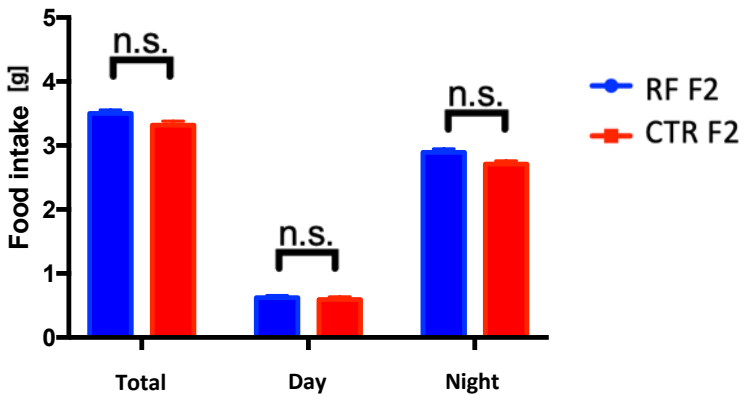

Figure 44: Food intake of RF and CTR F2
Total food intake and daily/nightly food intake of RF and CTR F2 over 10 days. n=8. Error bars display SEM values.

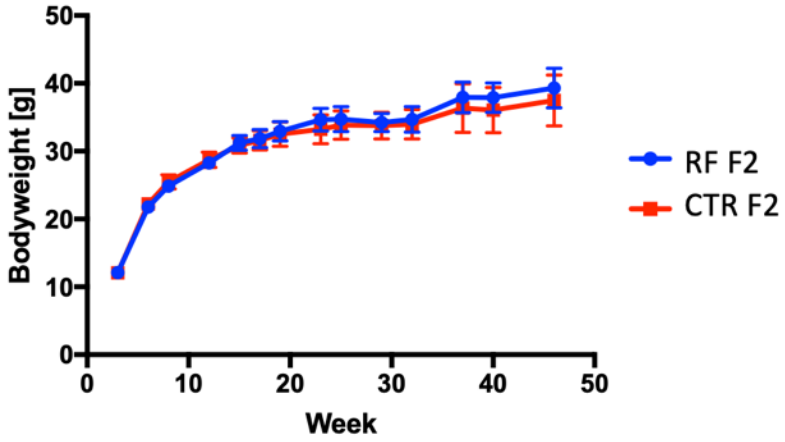

Figure 45: Bodyweight of RF and CTR F2 over 48 weeks
n=8. Error bars display SEM values.

We followed the RF F2 generation for 6 months, measuring the body weight every 2 weeks. No significant differences in food intake habits were observed between the RF and CTR F2 generation (Figure 44). As food intake remained the same between the RF and CTR F2 generation, there were also no differences in body weight (Figure 45).

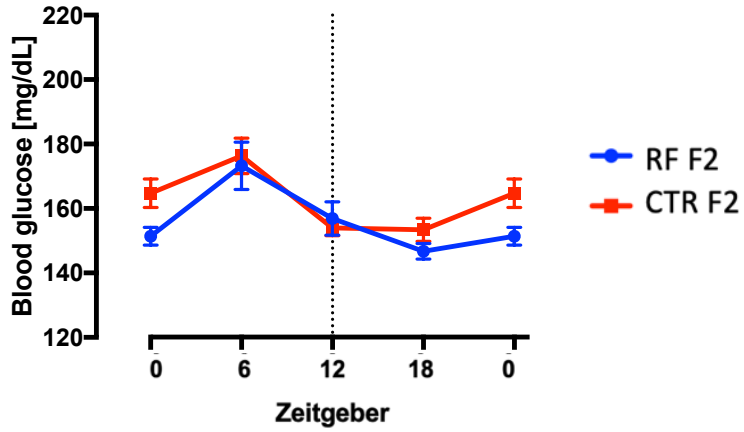

Figure 46: Blood glucose of RF and CTR F2 at different Zeitgeber
Blood glucose of RF and CTR F2 at Zeitgeber 0, 6, 12, 18. n=8 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t test with multiple comparison correction (*<0.05, **<0.01, ***<0.001).

At the age of 16 weeks, we measured the 24-hour blood glucose levels of the RF F2 and CTR F2 mice . Blood glucose was measured every 6 hours. No statistically significant differences in blood glucose between the CTR and RF F2 were observed (Figure 46).

Based on our data, we conclude that none of the observed phenotypes among the RF F1 generation was seen in the RF F2 generation. We noticed no significant differences in the phenotypes of the RF and the CTR F2 generations. This suggests that any possible phenotypes transmitted from the RF F0 to the RF F1 are either not impactful enough, or mechanistically stable enough, to be further transmitted from the F1 to the F2 generation. Our data thus indicates that phenotypes caused by the circadian arrhythmia of the RF F0 are only inherited intergenerationally but not transgenerationally.

7) RF F1 FED A HIGH-FAT DIET

In the past century the incidence of type 2 diabetes in humans has drastically increased. One main reason for this is the excess of caloric intake. In mouse models it has been proven that high fat diet feeding can impact the circadian clock by disrupting behavioral and molecular circadian rhythms in mice [180]. A chronic consumption of a high fat diet in human leads to a variety of phenotypes summarized under metabolic syndrome, including elevated blood pressure, high triglycerides, elevated blood levels and low HDL cholesterol. People suffering from metabolic syndrome have a higher risk of heart attack, stroke and type 2 diabetes [1, 181-183]. We used high fat diet feeding to further investigate how paternal circadian arrhythmia caused by an altered feeding pattern in the F0, in combination with a higher caloric intake (high fat diet) consumed by the F1 affect the metabolic phenotype of the F1 generation [184]. We created a F1 generation using the same method as in the RF F1. However, instead of feeding the RF F1 and CTR F1 a chow diet when they reached 3 weeks of age, the F1 was fed a 60% high-fat diet (hRF F1 and hCTR F1).

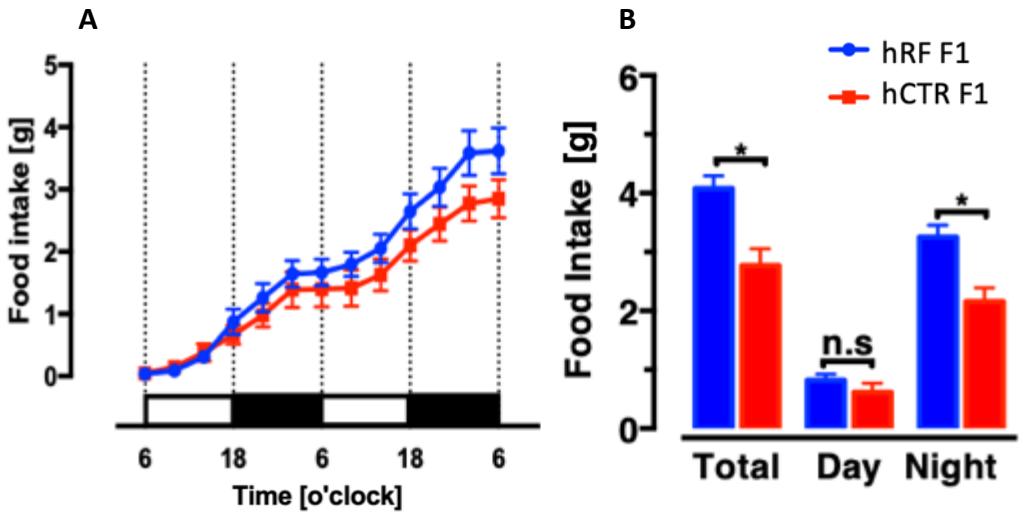

Figure 47: Food intake of hRF and hCTR F1
Cumulative food intake of hRF and hCTR F1 over 48 hours (A). Average total and average daily/nightly food intake of hRF and hCTR F1 is measured over 24 hours in calorimetric cages (B) n=8. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001, n.s. = non-significant).

As in the RF F1, the overall food intake of the hRF F1 was significantly higher than that of the CTR F1 (3,6 g vs 2,8 g, $p<0.05$). While hCTR and hRF F1 mice consume similar amounts of food during the day (0,72 g vs 0,62 g) the hRF F1 mice eat significantly more during the night than the hCTR F1 (2,86 g vs 2,15 g $p<0.05$). This is a different food intake habit than seen in the RF F1 mice, where the statistically significant difference in food intake was seen during the day instead of the night.

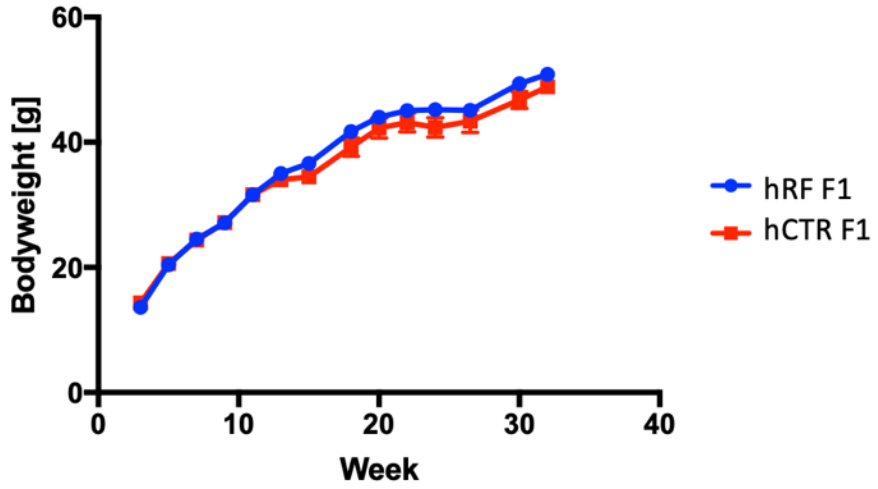

Figure 48: Bodyweight of hRF and hCTR F1
Body weight was measured over 34 weeks. n=8. Error bars display SEM values.

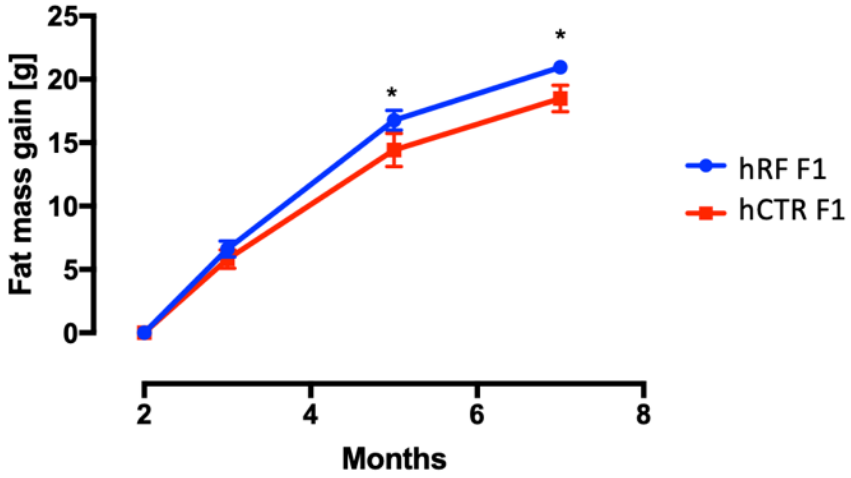

Figure 49: Fat mass gain of hRF and hCTR F1
The gain in fat mass of hRF and hCTR F1 was measured at an age of 2, 3, 5 and 7 months. n=8. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

Although not statistically significant, the trend shows that the body weight increase among the hRF F1 is slightly higher than that of the hCTR F1 (Figure 48). However, the most distinct difference between the two groups is the change in body fat. We measured the body composition of the hRF and hCTR F1 by using non-invasive nuclear magnetic resonance (MiniSpec) at the age of 2, 3, 5 and 7 months. No difference in body fat could be seen when the mice were young (2 and 3 months old). At the age of 5 and 7 months, however, the hRF F1 had a statistically significant higher fat mass gain than the

hCTR F1 (Figure 49, 7 months: hRF F1: 20.9 g vs. hCTR F1 18.4 g $p<0.05$). This may offer an explanation as to why the hRF F1 develop higher bodyweight with increasing age (Figure 48).

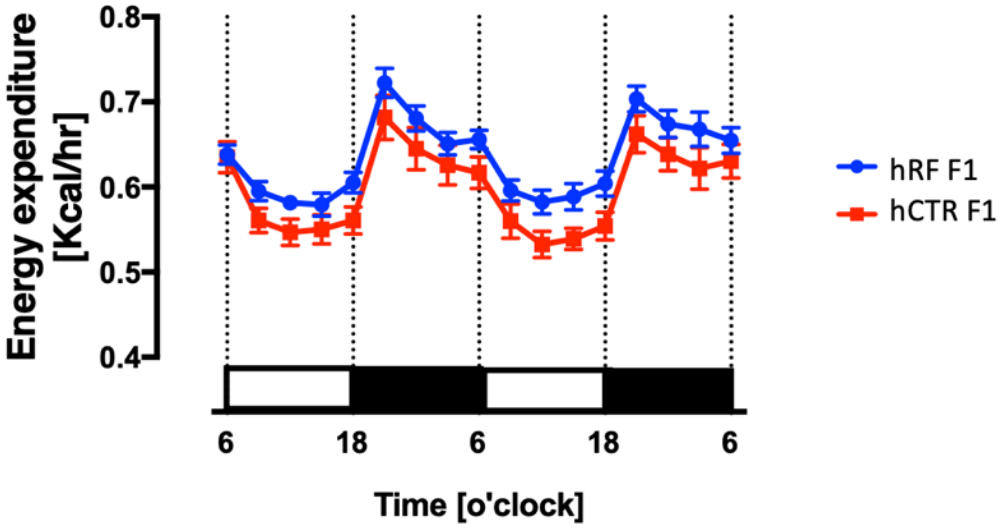

Figure 50: F1 Energy expenditure of hRF and hCTR F1 over 48 hours
Energy expenditure of hRF and hCTR F1 was measured over 48 hours in calorimetric cages. n=8. Error bars display SEM values.

When checking for the 24-hour energy expenditure in calorimetric cages (from 6:00 to 6:00) for a total duration of 48 hours, we noticed elevated energy levels among the hRF and hCTR F1 throughout the whole day (Figure 50). The hRF F1 did not only eat more but they also burned more energy, which may explain why no statistically significant increase in body weight was recorded.

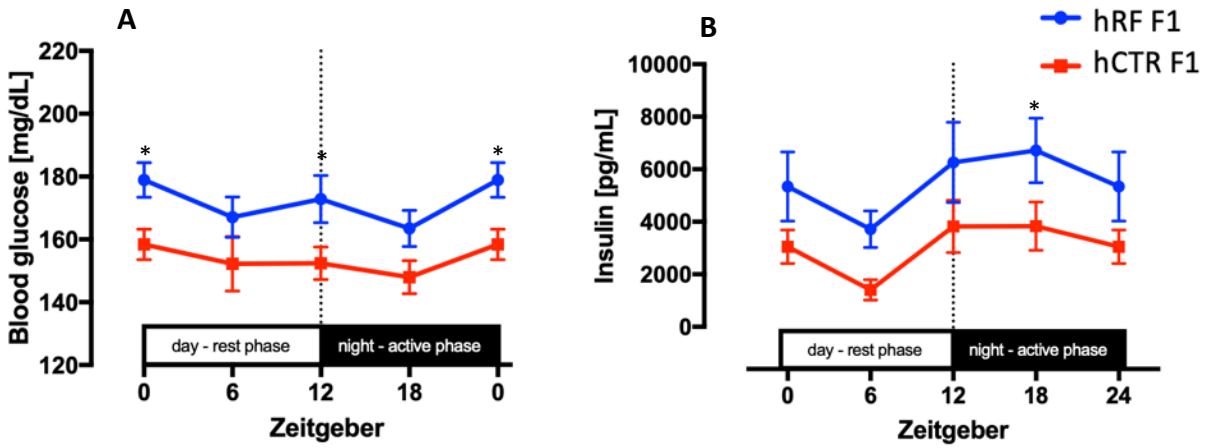

Figure 51: Blood glucose and insulin levels of hRF and hCTR F1 over 24 hours
Blood glucose (A) and insulin (B) levels measured in blood plasma at Zeitgeber 0, 6, 12, 18. n=8 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05,**<0.01,***<0.001).

To investigate the difference in metabolic phenotype, we measured the glucose and insulin levels of the hRF F1 and hCTR F1 every 6 hours over a 24-hour period (at Zeitgeber 0, 6, 12, 18, 24). Similar to the RF F1, the hRF F1 had higher blood glucose levels than the hCTR F1 over the whole 24 hours. The blood glucose values of the hRF F1 fluctuated between 170 and 180 mg/dl, while the levels for the hCTR F1 varied between 153 and 160 mg/dl. At the time points where there was a natural shift in food intake and general metabolic activity i.e. ZT 0, ZT12 and ZT 24, the blood glucose of the hRF F1 was significantly higher than that of the hCTR F1 (Figure 51 – $p<0.05$).

The data indicates that the hRF F1 mice are hyperglycemic. In addition, hRF F1 mice are also hyperinsulinemic. The insulin levels of the hRF F1 were about twice as high as the insulin levels of the hCTR F1. This was not the case for the chow diet cohort (Figure 23).This could mean that F1 mice which are on a high-fat diet are even more affected by paternal circadian arrhythmia than the F1 cohort on a chow diet.

To conclude, we observed that the hRF F1 and the RF F1 mice showed similar behavior. Both mice cohorts had a higher food intake, higher basal glucose levels over the 24 hours and higher insulin secretion. Previous studies had shown that solely a high-fat diet impacts the circadian rhythm, since mice tend to snack more throughout the resting phase, which leads to shorter resting phases and ultimately induces circadian arrhythmia [180, 185].

Our data indicate that paternal preconceptional circadian arrhythmia increases susceptibility to diet-induced obesity and metabolic syndrome. In terms of translation to epigenetic and human relevance, we hypothesize that the results provide evidence that children from fathers working as shift workers already at birth have a higher risk of suffering from strong metabolic misalignments. We also hypothesize that these metabolic phenotypes, however, can be further intensified if the children regularly eat a high fat and high caloric diet.

8) MECHANISTIC MODEL: F1 FROM FEMALE MICE WITH A HETEROZYGOUS GLUCOCORTICOID-RECEPTOR

We could show that paternal circadian arrhythmia induced by restricted feeding in mice leads to reduced total corticosterone levels in the seminal fluid and the disappearance of the typical peaking of corticosterone before the active phase in their offspring (Figure 25). Given the results we obtained by profiling the seminal fluid and phenotyping F1 animals generated by both natural conception and IVF, we hypothesized that reduced corticosterone levels (and glucocorticoid receptor signaling) at conception may be important for the phenotype. To verify this hypothesis, we genetically disrupted GR signaling by using females heterozygous for the glucocorticoid receptor (GRhet mice) [175]. Since a homozygous glucocorticoid receptor knockout is lethal, we had to use a heterozygous model. These mice have approximately half of the glucocorticoid receptor activity recorded in wild type mice.

We generated a GRhet F1 cohort by mating GRhet females with naïve wildtype males, that did not have an altered glucocorticoid receptor activity. The embryo resulting from this mating of the wildtype male with the GRhet female therefore was only exposed to a fraction of the corticosterone of an embryo resulting from the mating of a wild type female and a wild type male (CTR F1). Therefore, we compared the genotyped wildtype offspring of GRhet mothers (mGRhet F1) and wildtype control mothers (mCTR F1). Our cohort setup

allowed us to investigate if the GRhet F1 and RF F1 present the same phenotypes independently of the fact that the lower levels of corticosterone of the F1 embryos were caused by the knockout of corticosterone receptors in the mothers (GRhet experiment) or due to lower levels in seminal fluid corticosterone via circadian arrhythmia of the fathers (RF experiment).

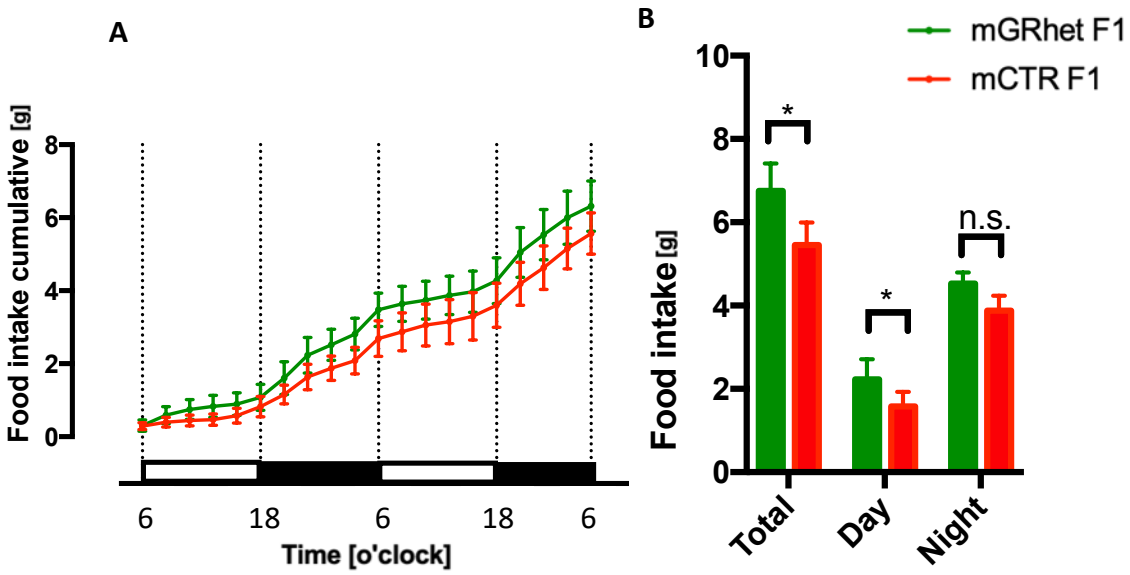

Figure 52: Food intake of mGRhet and mCTR F1
Cumulative food intake of mGRhet and mCTR F1 over 48 hours (A) average total and average daily/nightly food intake of mGRhet and mCTR F1 measured over 24 hours in calorimetric cages (B). n=18. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-test with multiple comparison correction (*<0.05, **<0.01, ***<0.001, n.s. = non-significant).

mGRhet F1 have a statistically signisficantly higher total food intake than mCTR F1 (Figure 52 mGRhet F1: 6.7g vs. mCTR F1: 5.4g, $p<0.05$). It primarily results from a statistically significantly higher food intake during the daytime/resting phase among the mGRhet F1 than among the mCTR F1 (GRhet: 2.2g vs. CTR: 1.6g $p<0,05$). This phenotype therefore cannot only be observed among the RF F1, whose father had been exposed to circadian arrhythmia, but also among the mGRhet F1, where the F0 had had ad libitum access to food, but the mothers had a heterozygous glucocorticoid receptor.

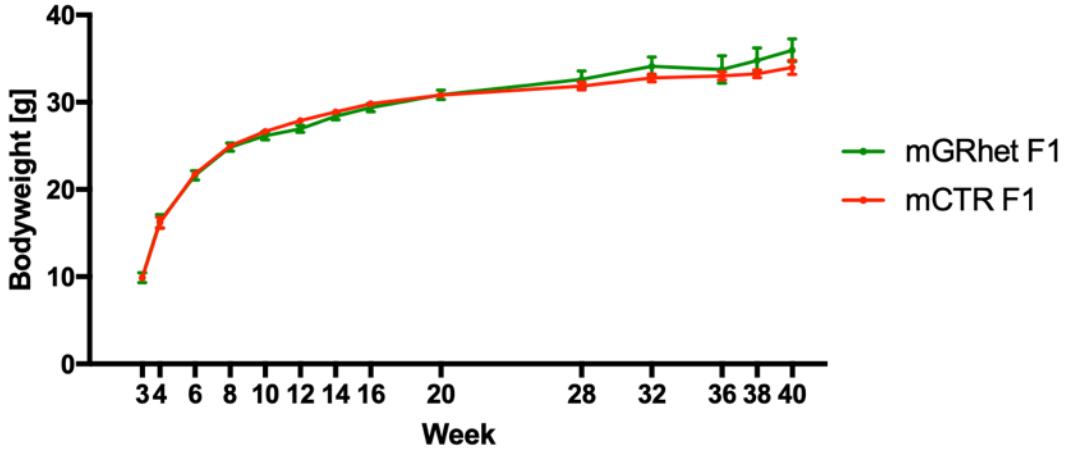

Figure 53: Bodyweight of mGRhet and mCTR F1
n=18. Body weight of mGRhet and mCTR F1 was measured over 40 weeks. Error bars display SEM values.

No significant difference between the body weight of mGRhet F1 and mCTR F1 mice could be recorded (Figure 53). However, the body weight of RF F1 and CTR F1 was also the same. Paternal circadian arrhythmia and a maternal heterozygous glucocorticoid receptor do not have an impact on this phenotype.

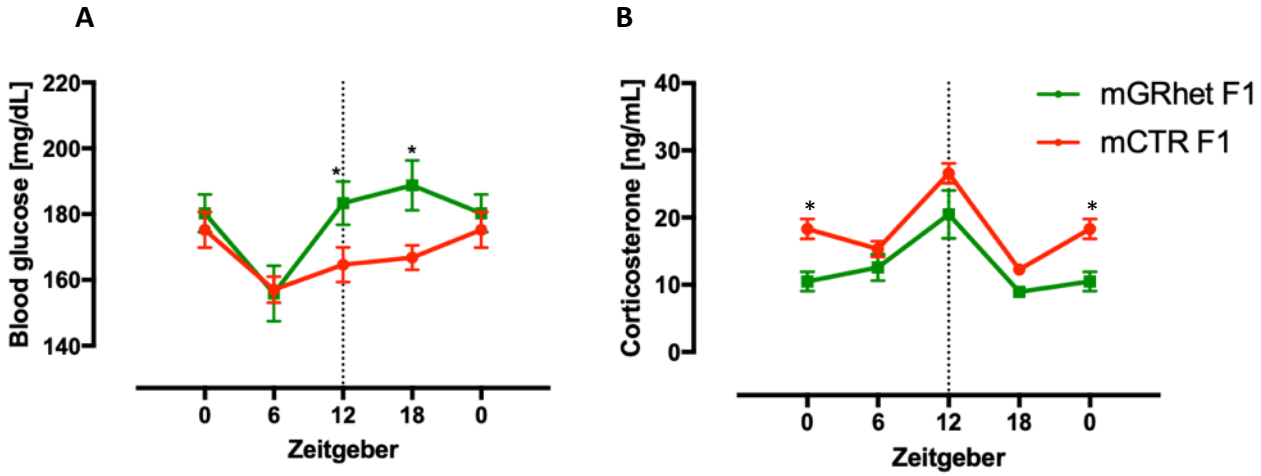

Figure 54: Blood glucose and corticosterone levels of mGRhet and mCTR F1 over 24 hours
Blood glucose (A) and corticosterone (B) levels of mGRhet and mCTR F1 at Zeitgeber 0, 6, 12, 18. n=18 / Zeitgeber. Error bars display SEM values. Data were considered statistically significant at $p<0.05$ using multiple t-tests with multiple comparison corrections (*<0.05, **<0.01, ***<0.001).

Comparing glucose (Figure 54 A) and corticosterone levels (Figure 54 B) at different times of the day, we noticed significant differences between the mGRhet and mCTR F1. mGRhet and mCTR F1 showed elevated blood glucose levels during the active time of the day (Zeitgeber 12 and 18 - mGRhet: 183, 188 mg/dl vs. mCTR: 164, 166 mg/dl $p<0,05$). This trend could also be observed in the RF F1 mice. In addition, for the mGRhet cohort we noticed a decrease in corticosterone concentration during the 24-hour period (Figure 54 B, ZT0 mGRhet: 10 ng/ml vs. mCTR: 18ng/ml $p<0,05$ / ZT 12 GRhet:20 vs CTR: 26 ng/ml $p<0,05$). Unlike the RF F0 mice, where the cyclic peak had been eliminated by the circadian arrhythmia, the RF and GRhet F1 both showed a peak at the beginning of the active phase (ZT12). Our data support the hypothesis that decreased corticosterone levels during embryonic development, caused by lower corticosterone levels in the seminal fluid or by reduced glucocorticoid receptor-binding, can impact the F1 generation's metabolic phenotypes and not only cause higher food intake during the resting phase but also alter glucose and corticosterone levels [186]. This hypothesis would explain the phenotypes seen in the offspring of male mice exposed to circadian arrhythmia.

Discussion

PATERNAL CIRCADIAN ARRHYTHMIA AND ITS INFLUENCE ON THE F1 GENERATION

The circadian rhythm is per classical definition entrained by the light. By repeatedly limiting the feeding schedule of F0 male mice to their resting phase i.e. daytime for 30 days, we saw arrhythmic glucocorticoid secretion and an inverse expression of the core clock genes (Bmal1, Per2, Cry and Rev-erb α). Therefore, the manipulation of the peripheral clocks leads to circadian arrhythmia even with an intact day/night rhythm. No research, however, has been conducted to date on the effect of this circadian arrhythmia on the offspring of these mice. In this PhD project, we therefore mated the male mice with circadian arrhythmia with wild type females. We thereby focused our research on the effect that the paternal circadian arrhythmia has on the offspring's feeding schedule and metabolism. We were able to demonstrate that a relatively short period of paternal circadian arrhythmia (30 days), has an indisputable impact on the metabolic state of the following unexposed F1 generation. Since the F1 generation has an intact light-dark cycle and is not externally manipulated, these phenotypes can be defined as diurnal arrhythmia (representing a one-day phenotype). Paternal circadian arrhythmia has a direct influence on the F1's food intake and feeding schedule. F1 of RF F0, like their fathers, eat more during their resting phase. They mimick the feeding schedule of the RF F0, although the RF F1 have never been directly exposed to the environmental challenge. Since the RF F1 eat more during daytime, in addition to the normal amount they eat during nighttime (their active phase), they have a higher 24-hour food consumption than control mice. This phenotype becomes stronger with increasing age of the RF F1 mice. We were also able to demonstrate that the changes in the feeding schedule of the RF F0 mice have a direct influence on the glucose metabolism of the RF F1. RF F1 mice generally have higher blood glucose levels than control mice and less fluctuation in blood glucose between the active and resting phases of their day. Less

fluctuations in the glucose metabolism may be linked to the higher intake of food during the 24 hours. These RF F1 phenotypes were continuously reproduced in multiple cohorts, which proved the robustness of our data.

When exposing the F1 generation of restricted fed fathers (RF F0) to a high fat diet (hRF F1), we observed even stronger phenotypes. Not only was the overall food consumption of the hRF F1 higher, the hRF F1 mice also had a significantly higher proportion of fat mass. Again the percentage of fat composition was higher with increasing age of the hRF F1 mice. The hRF F1, like the RF F1 mice with a chow diet, were also hyperglycemic. In addition, hRF F1 were also hyperinsulinemic, a phenotype that was not observed among RF F1 mice on a chow diet. These results show that the phenotypes seen among RF F1 mice are more distinct among hRF F1 mice fed with a high fat diet. A metabolically unfavorable diet in the F1 generation therefore enhances the phenotype resulting from the circadian arrhythmia the F0 generation was exposed to.

In our third experiment, we were able to prove that the described phenotypes in the F1 generation are, however, reversible when after 30 days of restricted feeding, the RF F0 mice are fed ad libitum for 30 days and then are mated with the wild mice.
In order to find out if the observed phenotypes are inter- or even transgenerational, we mated RF F1 males with wild type females and obtained the RF F2 generation. In the RF F2 we did not observe the phenotypes described in the RF F1 and hRF F1. The RF F2 mice neither had a higher food consumption overall nor did they metabolically differ from the CTR F2 mice. These results indicate that the observed phenotypes are only inherited intergenerationally and not transgenerationally.

Investigation of the core clock genes shows a reversed expression in the RF F0 generation at Zeitgeber 0 in the liver samples. Our data indicate that the RF F0 mice change their day-night rhythm if their exposure to food is limited to their resting phase. Data of a RNA-seq heat map of the clock gene expression in the liver of RF/CTR F1 at Zeitgeber 0, however, did not indicate highly inverted transcription patterns to be the driving mechanism behind the phenotype seen in the RF F1. Further analysis of the expression of clock genes in the RF F1, however, showed that in the RF F1 gene expression of clock genes was also altered.

Although the core clock gene expression was not completely inverted, separate analysis of the expression of clock genes in RF F1 during different Zeitgebers showed a statistically significantly dampened expression in a great proportion of the key clock genes (Dbp, Clock and Per2) at Zeitgeber 12 in the RF F1. These data suggests that unlike the RF F0 mice whose day and night phases were inverted, the discrepancy in the day and night phases of RF F1 mice was smaller. Expression levels therefore did not vary as much during the transition from active to resting phase. Other main clock genes such as Nr1d1 and Per2 showed a shift of 6 hours in expression levels in the RF F1 vs. CTR F1. These results indicate that due to the circadian arrhythmia of the RF F0, RF F1 have a shifted day-night rhythm.

As seminal fluid and sperm cells are the only two vehicles of transmission between the RF F0 and RF F1, we investigated them separately in order to determine the vehicle of intergenerational inheritance.

SEMINAL FLUID AS A MECHANISTIC TRANSPORTER

We investigated the involvement of sperm cells in our project by creating an RF F1 using in-vitro fertilization (IVF). In the IVF RF F1 cohort we did not see the phenotypes described in the RF F1. Food intake, body weight, glucose levels did not significantly differ between the IVF and CTR F1 which confirmed the hypothesis that the strong food intake phenotype observed among the RF F1 is not transferred by the sperm cells of the RF F0. We therefore focused on the influence of seminal fluid as transmitter of epigenetic inheritance. DNA methylation, histone alterations and small RNAs have been the focus of research but the role of seminal fluid in epigenetic inheritance has not yet been extensively studied.

We were able to show strong alteration in the corticosterone levels of the seminal fluid of RF F0 mice. While RF F0 mice had highly elevated corticosterone levels in their blood, the corticosterone levels in their seminal fluid were significantly lower than that of CTR F0. The RF F0 mice also showed a dampening of the corticosterone peak at Zeitgeber 12, i.e. during the transition from day to night. Altered corticosterone concentrations in seminal fluid have been shown to impact the embryo in the first 3 days after mating, when the seminal fluid

is still in the reproductive tract. This time-window is crucial for the development of the embryo and has been proven to be greatly impacted by environmental factors e.g. toxins [187]. The initial three days after fertilization also represent the embryonal developmental stage (oocytes, zygotes, 2-cell, 4-cell stage) with the highest glucocorticoid-receptor expression (Figure 55).

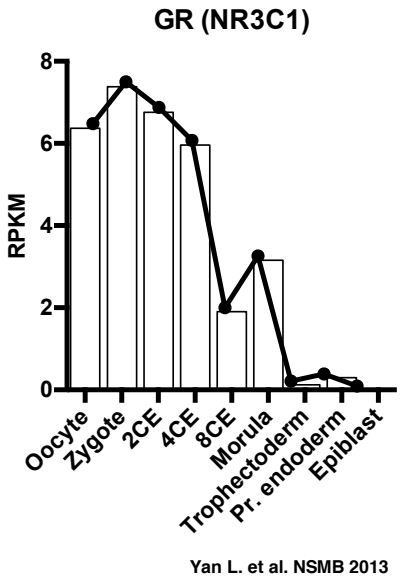

Figure 55: Glucocorticoid receptor expression at different stages of embryonic development – data adapted from Yan et al. [188].

In order to verify our hypothesis, that decreased corticosterone levels in the seminal fluid lead to the phenotypes described above, we used a mouse model of glucocorticoid heterozygous mice (GRhet), to genetically mimic a lack of corticosterone/ cortisone-glucocorticoid receptor interaction. F1 of GRhet mothers mated with wildtype fathers showed similar phenotypes as our RF F1 mice: a statistically significantly higher total food intake, higher food intake during the daytime and significantly elevated blood glucose levels. Our experiments proved that changes in corticosterone levels during embryogenesis, when the oocyte corticosterone receptors are the highest, result in changes in feeding habit and hyperglycemia of the RF F1.

INVOLVEMENT OF THE BRAIN

Since the brain influences the sensation of hunger and subsequently food intake, we assumed a direct involvement of the brain, leading to the described food intake phenotypes. Previous studies have shown that stress and emotions highly influence food intake behavior in rodents [189, 190], as well as in humans [191]. Acute stress can for example severely increase or decrease food intake. According to Rutters et al. "…*acute psychological stress is associated to eating in absence of hunger, especially in vulnerable individuals characterized by disinhibited eating behavior and sensitivity to chronic stress*" [191].

RNA-sequencing of hypothalamic samples at different times of the day (Zeitgeber 0, 6, 12 and 18) showed a six-hour shift in oscillating hypothalamic transcripts in RF F1 animals compared to CTR F1. RNA-sequencing also indicated that in RF F1 a low number of genes maintain rhythmicity. Further functional analysis of differentially expressed genes showed strong enrichment for lipid metabolism and adipocytokine signalling pathways. Key factors in orexigenic pathways (such as neuropeptides Npy and Hcrt) were upregulated. Anorexigenic pathways (such as Cartpt, Bdnf and Pomc), on the other hand, were downregulated in the RF F1. Neuropeptide Y has been reported to mimic the effects of light and to shift the circadian clock when injected into the SCN [192]. Belle et al. also showed that orexin neurons are not only regulated by SCN but orexin neurons reciprocally can also regulate the SCN clock [193]. In the context of our data, this implies that the higher activity seen in the RF F1, expressed via a higher food intake during the resting phase, may also be the result of a higher expression of the orexin neuropeptide. This may explain why, unlike the RF F0, the core clock gene expression was not completely inversed in the RF F1, although the mice showed similar food intake behaviour as the RF F0. As we saw a stronger phenotype among the older RF F1 mice than among the younger mice, it would be interesting for future experiments to conduct RNA sequencing in the liver and hypothalamus of RF F1 at different ages. Higuchi et al. showed that with increasing age, the expression of neuropeptide Y increases [194]. The age-dependant phenotype may therefore also be linked to higher expression of orexigenic neuropeptides.

EXPOSURE TIME OF F0 AND PENETRANCE OF CIRCARDIAN ARRHYTHMIA TO FURTHER GENERATIONS

Before the start of the research project, the exposure period was determined. Our decision to expose the F0 to 30 days of restricted feeding was based on previously published studies. Mukherji et al. had shown that 30 days of exposure to circadian arrhythmia causes metabolic changes in the F0 cohort and we aimed to investigate, if 30 days of circadian arrhythmia were impactful enough to not only influence the F0 but also the F1 and possibly even the F2 generation [47, 152]. By choosing a short exposure period, we were able to show that a period of only 30 days is impactful enough to cause phenotypes in the next generation. Whereas genetic modifications need long-time exposure or relatively impactful influences (including toxic chemicals and irradiation) in order to cause biological alterations which are passed from one generation to the next, epigenetic factors constitute a way for the body to quickly react to short-time changes in the environment.

Publications by Mukherji et al. also show that longer periods of exposure, 60 days (twice as long) and 90 days (thrice as long), lead to more distinct phenotypes in the F0. Further studies need to be conducted to see if these longer periods of exposure not only cause more distinct phenotypes in the F0 but also in further generations. Longer exposure periods with a more distinct phenotype may also lead to phenotypes that are not only transmitted inter- but also transgenerationally. Future research projects should therefore be conducted to examine how the different durations of circadian arrhythmia impact the phenotypes.

LIMITING FACTORS AND FUTURE EXPERIMENTS

Mice and humans

Mice share 78,5% of their DNA with humans [195]. Although many breakthroughs in medicine have come from studies carried out in mice, scientific findings in mice cannot be directly transferred to humans. Some of the main pitfalls of rodent models are:

Firstly, mice, unlike humans, are nocturnal animals. Most researchers perform their experiments during daytime, which corresponds to the resting time of the animals. As a result, the timing of experiments should be re-evaluated for some methods. Chronotherapeutic approaches, such as in cancer therapy have to be adapted to the different sleep and night rhythms of humans and rodents. Tumor models in rodents, for instance, may express proliferative targets relevant to cancer cell DNA synthesis or cancer cell division at different time points than in humans.

Secondly, rodents are polyphasic sleepers. Contrary to humans, rodents have short sleep and wake periods during their resting phase [196].

To adapt to these pitfalls, we measured selected parameters - such as blood glucose, insulin and corticosterone - not only once per day at a certain timepoint, but 4 to 6 times per day at different Zeitgeber - both during day and night times. Strong fluctuations were observed in certain expression levels over 24 hours. The corticosterone levels of restricted fed F0 fathers for example fluctuated highly (Figure 25).

Paternal circadian arrhythmia and the impact on the offspring

For our experimental setup we chose to focus our investigation on paternal circadian arrhythmia because to date research in mice and humans has primarily focused on maternal circadian arrhythmia. Focusing on paternal circadian arrhythmia was therefore a novel approach which allowed us to minimize factors that could influence our F1 generation. Paternal circadian arrhythmia has only has two possible vehicles of inheritance: seminal fluid and sperm cells. All other possible vehicles of transfer are eliminated, thereby ensuring robust data. Nevertheless, future experiments with a similar setup using maternal

circadian arrhythmia could be used to strengthen our findings and could also lead to more clarity about sex differences for various phenotypes and the molecules involved between males and females.

Differences between male and female offspring

Recent studies have shown that the characteristics of the human circadian system and energy metabolism differ between males and females [197]. Direct circadian misalignment primarily leads to disturbances in the energy homeostasis in females, including a decrease in satiety hormone and an increase in hunger hormone. In males, on the other hand, circadian arrhythmia is expressed via elevated cravings for energy-dense and savoury foods [197]. Interestingly, in our study we also saw strong gender differences in the offspring of male mice with circadian arrhythmia. Data from our first experiments, that were not further investigated for this thesis, showed that while the male RF F1 showed the previously described phenotypes, female RF F1 phenotypes were not as pronounced. These sex-dependent phenotypic differences may be the result of sex hormones influencing or buffering the phenotypes.

Influence of seminal fluid on placenta environment

We feel confident in our results, as the same phenotypes in RF F1 cohorts were seen repeatedly. We also identified the seminal fluid as the vehicle of intergenerational transmission. Our data confirms previously published research that identified seminal fluid as transmitter of information to the female uterus and as driving force for changes in the offspring [198-201].

Our research indicates that dampened corticosterone levels in seminal fluid induce changes in the RF F1. Further experiments, however, are required to verify our hypothesis. The experiment could proceed as follows: feeding of the RF F0 males would be restricted for 30 days, then the RF F0 males would be mated with wild type super-ovulated females. After confirmed pregnancy, the zygote would be transferred into a petri dish with medium.

Enriching the medium with different dexamethasone levels could mimic altered corticosterone levels in the embryonal environment. After reaching the morula stage, the embryos would be transferred to foster mothers and examined for food intake, glucose, insulin and corticosterone levels as well as for clock gene expression phenotypes. This experiment could confirm if the reported phenotypes are indeed induced by corticosterone levels in the embryonic environment or by other mechanisms.

THE INFLUENCE OF CIRCADIAN ARRHYTHMIA ON MICE AND HUMANS

Technological advances have given humans the possibility to not only work and enjoy recreational activities during daytime but also at night, thereby changing our society from a 12-hour to a 24-hour society [202]. 80% of the world's population is now exposed to light during the night [203], and 20% of European and 29% of US employees work in shift work [204]. According to 2004 data from the Bureau of Labor Statistics, almost 15 million Americans work full time on evening shift, night shift, rotating shifts, or other employer arranged irregular schedules [61].

While extending an organization's operational time from 8 hours up to 24 hours per day, means more profit for the organization, shift work and night work pose many health and safety dangers. The human is a diurnal creature and synchronized to a 24-hour light/dark cycle. If we are forced to stay awake and active at night and to be resting and sleeping during daytime, this change strongly impacts our body-homeostasis and metabolism [47, 65, 205 - 207]. Long-term studies such as the NHS nurses studies have demonstrated that they belong to a vulnerable group of people who have a greater risk of developing various diseases including metabolic syndrome and type 2 diabetes [208, 209].

While we already have proof from historic events such as the "Dutch Hunger Winter" that maternal food consumption directly influences the metabolism of future generations, no studies to date have been performed on the effects of eating habits caused by paternal circadian arrhythmia on the metabolism of future generations. Our mouse data suggests that children from shift working fathers have a higher risk of impaired food intake as well

as circadian and metabolic desynchronization. This means that children of fathers who suffer from circadian arrhythmia and who have their highest caloric intake during the time when they normally would be resting, may also be prone to eat during the night. As a result, these children have a higher risk of developing hyperglycemia and showing changes in the 24-hour corticosterone secretion. The rising numbers of global shift workers may offer an explanation as to why the prevalence of type 2 diabetes among children and teenagers is rising. Furthermore, our results demonstrate that eating a diet high in calories (HFD cohort experiment in the mice) even potentiates this pre-determined disadvantage. Our mouse data shows that the consumption of a high fat diet leads to a more pronounced phenotype in the RF F1.

Our findings suggest that children of shift workers should make sure that they are eating a healthy and balanced diet. However, from a social perspective this is often challenging since shift workers have been shown to often eat high caloric meals i.e. fast food [210]. Balancing shift work and family life is often very demanding and so quick and easy lunch and dinner options are often preferred by shift workers. Therefore, with an increasing prevalence of shift workers in the society, children should be educated about healthy food choices and school lunches should offer healthy food. Our experiment, where male mice first received 30 days of restricted feeding followed by 30 days of ad libitum eating, showed that the observed phenotypes, however, may be reversed.

CONCLUSION AND RELEVANCE OF THE RESULTS FOR HUMANS

Research on the circadian clock has led to the insight that it controls many important physiological processes such as wake/sleep cycles, hormonal levels, metabolism, and immune system. Disruption of the circadian rhythm contributes to several diseases including cardiovascular disease, obesity and type 2 diabetes that pose a significant public health challenge in to our society. In order to raise productivity, many people no longer have a ”9-to-5” business day but are required to work (night) shifts. Numerous publications show that shift work disrupts the circadian rhythm and increases the risk for several diseases including obesity and type 2 diabetes.

Research on the circadian clock has also led to the emergence of chrononutrition, a discipline that investigates the relation between metabolism and circadian rhythm. In the past few years, Panda et al. for example published several studies on the benefits of chrononutrition [48, 211, 212]. He proposed that time-restricted feeding for two-thirds of the day (16 hours out of 24) may reduce overweight and fat distribution. Restricted feeding of a high fat diet showed less negative metabolic impact in mice than a diet that is eaten ad libitum. This explains why intermittent fasting and “16/8 eating” have become popular diet trends in the past few years [49, 50, 98, 213]. The value of chrononutrition on our metabolism and on the metabolism of future generations is also highlighted by our data. As more and more people in our 24-hour society work (night) shifts, we need to further investigate the link between circadian arrhythmia and metabolic diseases.

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PUBLICATIONS, TALKS AND POSTERS

Award

DZD Award 2019 (Deutsches Zentrum für Diabetesforschung)
for presenting this research project at
Epigenesys London 22.-24. September 2019
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Talk:"Paternal circadian arrhythmia affects offspring health and feeding behavior"

Publications related to this thesis

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"Paternal circadian arrhythmia affects offspring health and feeding behavior"
***Lassi M**, Gerlini R, Tomar A, Darr J, Scheid F, Gailus-Durner V, Fuchs H, Hrabé de Angelis M, Torres Padilla ME, Marschall S, Rozman J, Teperino R*

Other Publications

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"Paternal overweight controls transgenerational metabolic health via Polycomb"
*Gerlini R, Tomar A, Darr J, **Lassi M**, Kiess W, Scheid F, Calzada-Wack J, Gailus-Durner V, Fuchs H, Hrabé de Angelis M, Marschall S, Kotaja N, Toppari J, Körner A, Teperino R*

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"FNDC4 controls systemic glucose homeostasis through binding to the orphan receptor GPR116"
*Georgiadi A, Mourão A, Ma X, Klepac K, Jimena Alfaro A, Salazar Lopez V, Bosma M, Shilkova O, Ritvos O, Nakamura N, Hirose S, Sharma S, Grallert H, **Lassi M**, Teperino R, Scheideler M, Dietrich A, Blüher M, Herzig S*

Cell Reports, 2020
"In-vivo targeted tagging of RNA isolates cell specific transcriptional responses to environmental stimuli and identifies liver-to-adipose RNA transfer"
*Darr J, **Lassi M**, Tomar A, Gerlini R, Scheid F, Hrabé de Angelis M, Witting M, Teperino R*

Molecular Metabolism, 2018
"Glucose tolerance and insulin sensitivity define adipocyte transcriptional programs in human obesity"
*Gerlini R, Berti L, Darr J, **Lassi M**, Brandmaier S, Fritsche L, Scheid F, Boehm A, Koenigsrainer A, Grallert H, Haering HU, Hrabé de Angelis M, Staiger H, Teperino R*

Nature Structural & Molecular Biology, 2017
"MacroH2A1.1 regulates mitochondrial respiration by limitting nuclear NAD+ consumption"
*Marjanović MP, Hurtado-Bagès S, **Lassi M**, Valero V, Malinverni R, Delage H, Navarro M, Corujo D, Guberovic I, Douet J, Gama-Perez P, Garcia-Roves PM, Ahel I, Ladurner AG, Yanes O, Bouvet P, Suelves M, Teperino R, Pospisilik JA, Buschbeck M*

Oral presentation

Epigenesys London 22.-24. September 2019
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46. Österreichische Diabetes Gesellschaft Jahrestagung 15-17.November 2018
Salzburg, Austria
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***Lassi M**, Gerlini R, Tomar A, Darr J, Scheid F, Gailus-Durner V, Fuchs H, Hrabé de Angelis M, Torres Padilla ME, Marschall S, Rozman J, Teperino R*

Keystone; Drivers of Type 2 Diabetes: From Genes to Environment, 7-11 Oktober 2018
Seoul, South Korea
"Paternal circadian arrhythmia affects offspring health and feeding behavior"
***Lassi M**, Gerlini R, Tomar A, Darr J, Scheid F, Gailus-Durner V, Fuchs H, Hrabé de Angelis M, Torres Padilla ME, Marschall S, Rozman J, Teperino R*

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Berlin, Germany
"Intergenerational inheritance of circadian arrhythmia in mice"
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