TEMPORAL FASTING AND REDUCED CALORIES INDEPENDENTLY CONTRIBUTE TO METABOLIC BENEFITS OF CALORIC RESTRICTION

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DEDICATION

I would like to dedicate my work in the loving memory of my late maternal grandparents, Mr. Narayan and Mrs. Gulabi Narayan Amin, and late paternal grandparents Mr. Hari and Mrs. Anuradha Velingkar, for their immense love and blessings, my parents, Mr. Sanjay and Mrs. Jyothi Velingkar, maternal aunt and uncle, Mrs. Vasundhara and Mr. Jagannath Das Gujrati, for their constant support, love and encouragement.

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TEMPORAL FASTING AND REDUCED CALORIES INDEPENDENTLY CONTRIBUTE TO METABOLIC BENEFITS OF CALORIC RESTRICTION

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ABSTRACT

Caloric restriction (CR) intervention has been demonstrated to improve health and extend lifespan. CR in mammals, imposes a short interval of feeding, called time restricted feeding (TR), which is followed by prolonged interval of fasting. TR or mealtime (MT), a form of periodic fasting, without reducing caloric intake, may contribute to improvement in metabolism. To dissect the contributions of reduced caloric intake and periodic fasting in health benefits mediated by CR, we measured physiological and metabolic parameters in mice subjected to CR and TR (without reduction in caloric intake). CR reduced blood glucose and insulin, and increased ketone levels across the day, significantly improved glucose and insulin sensitivity. TR did not affect blood glucose and glucose sensitivity, in contrast to CR, but reduced blood insulin and partially improved insulin sensitivity. Both the diets had little to no effect on phases of circadian clock genes, and CR significantly induced the expression of glucose metabolic genes, whereas TR did not, which correlates with modest effect of TR on glucose homeostasis. Therefore, we concluded that, TR is metabolically different from CR, and that periodic fasting contributed to some of the metabolic improvements on CR, independent from caloric intake. This may help provide a mechanistic explanation to differences in lifespan extension observed under CR and TR.

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4EBP1	4E binding protein1
ACC	Acetyl CoA Carboxylase
Akt	Protein kinase B
AL	Ad libitum
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
ARNTL	Aryl Hydrocarbon Receptor Nuclear Translocator Like
ATP	Adenosine triphosphate
Bmal1	Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-like 1
C.elegans	Caenorhabditis elegans
CALERIE	Comprehensive Assessment of the Long Term Effects of Reducing Intake of Energy
cDNA	complementary Deoxy ribonucleic acid
Clock	Circadian Locomotor Output Cycles Kaput
CR	Caloric Restriction
CREB	cAMP response element binding protein
Cry1	Cryptochrome 1
Cyp2a5	cytochrome P450, family 2, subfamily a, polypeptide 5
Cyp4a12b	cytochrome P450, family 4, subfamily a, polypeptide 12B
Cyp7a1	Cytochrome P450 Family 7 Subfamily A Member 1
Dbp	D-Box Binding PAR BZIP Transcription Factor

DIO	Diet induced obesity
DR	Dietary restriction
DTT	Dithiothreitol
E.coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
Fas	Fatty acid synthase
Fbp1	Fructose 1,6 bisphosphatase 1
Fmo3	Flavin containing monooxygenase 3
FOXO	Forkhead Box O
G6pc1	Glucose 6 phosphatase
G6pd	glucose-6-phosphate dehydrogenase
Gck	Glucokinase
GH	Growth hormone
Gk	Glycerol kinase
GTT	Glucose tolerance test
HDL	High density lipoprotein
Hmgcs2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2
i.p.	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IGF-1	Insulin like growth factor-1
IL-6	Interleukin-6

IS	Insulin sensitivity
ITT	Insulin tolerance test
LDL	Low density lipoprotein
MOP3	Member Of PAS Protein 3
mRNA	messenger RNA
MTF	Mealtime feeding
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
mTORC2	Mammalian target of rapamycin complex 2
Mup4	Major urinary protein 4
Na_3VO_4	Sodium orthovanadate
$Na_4P_2O_7$	Tetrasodium pyrophosphate
NaCl	Sodium Chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NIA	National Institute of Aging
NPAS2	Neuronal PAS Domain Protein 2
Pck1	Phospho-enol pyruvate carboxykinase 1
PCR	Polymerase Chain Reaction
pCREB	phosphorylated cAMP response element binding protein
Pcx	Pyruvate carboxylase
Per1	Period 1

Per2	Period 2
Per3	Period 3
Pfk1	Phosphofructokinase 1
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PKCα	Protein kinase C alpha
Pparα	Peroxisome Proliferator Activated Receptor Alpha
PVDF	Polyvinylidene difluoride
RAPTOR	Regulatory-associated protein of mTOR
Rev-erbα/β	Nuclear Receptor Subfamily 1 Group D Member 1
RNA	Ribonucleic acid
Rorα/β/γ	Retinoic Acid Receptor-Related Orphan Receptor Alpha
rRNA	Ribosomal ribonucleic acid
S.cerevisiae	Saccaromyces cerevisiae
S6K1	Ribosomal protein S6 kinase1
S6K2	Ribosomal protein S6 kinase2
SCN	Suprachiasmatic nucleus
SD	Standard deviation
SDS	Sodium dodecyl sulfate
Serpina12	Serpin Family A Member 12
SF	Spontaneous fasting
SGK	Serum/Glucocorticoid Regulated Kinase
Sir2	Silent information regulator 2
SIRT1	Sirtuin1

SIRT2	Sirtuin2
SIRT3	Sirtuin3
SIRT5	Sirtuin5
SIRT6	Sirtuin6
SIRT7	Sirtuin7
SOD2	Superoxide dismutase 2
Srebp1	sterol regulatory element-binding protein 1
TBS	Tris-buffered saline
TNFα	Tumor Necrosis Factor alpha
TR	Time restricted feeding
TSC1	Tuberous sclerosis 1/Hamartin
TSC2	Tuberous sclerosis 2/Tuberin
US	United States
WHO	World Health Organization
WNPRC	Wisconsin National Primate Research Center

CHAPTER I

INTRODUCTION

1.1 Global Impact of Obesity

Obesity is a complex disease that is increasing at an alarming rate across the world. Since 1980, the incidence of obesity has doubled (GBD 2015 Obesity Collaborators et al. 2017). According to WHO 2015 report, with body mass index (BMI) of 25-29 being used as cut-off for overweight, and ≥ 30 used for obesity, it was reported that there were approximately 1.9 billion people who were overweight, of whom, approximately 600 million adults and 100 million children were obese (GBD 2015 Obesity Collaborators et al. 2017). According to the National Center for Health Statistics report, about 39.8% of American adults aged 20 and above were obese and about 31.8% overweight. If this trend continues, by the year 2030, approximately 38% of the population in the world will be overweight and around 20% will be obese (Kelly et al. 2008), while in the US it is predicted that around 51% of adults will be obese (Wang et al. 2008). One of the major causes of obesity-related deaths is cardiovascular disease, with diabetes being the second cause; other causes include, osteoarthritis and cancer. There are many factors that contribute to increased incidence of obesity worldwide; two of the most widely known factors include: first, consumption of energy rich foods and drinks which exceed the daily

caloric expenditure; second, decrease in physical activity and increase in sedentary lifestyle; others include genetics, wherein if one or both the parents were obese, there is likelihood that you may inherit obesity from them, medications or medical conditions, and lack of sleep. In order to successfully treat obesity, people need to make changes to their lifestyle, adjust their dietary intake by consuming less amount of energy rich food and increase their physical activity. In the recent years, multiple studies have demonstrated that restricting the dietary intake has multiple benefits on health by improving several physiological parameters and has potential to extend life-span too. Dietary restrictions are of several types, which involve either reduction in total amount of calories consumed (caloric restriction), or restricting the time window for availability of food (time-restricted feeding), intermittent fasting, fasting mimicking diets such as ketogenic diets, or modulating either one or multiple macronutrients in a diet (example high protein diet, high-fat diet). Of these, two are of major interest in my thesis: Caloric restriction and Time restricted feeding and are discussed in detail below.

1.2 Caloric Restriction

A. Background

Dietary restriction (DR) is a type of non-genetic, non-surgical intervention that has been practiced and investigated for decades and is known to increase lifespan in living organisms. When DR is implemented as modest reduction in the total amount of calories without causing malnutrition, it is called caloric restriction (CR). Generally, CR that is implemented, is usually 10-40% reduction in ad libitum amount of food. 10-40% reduction in calories implies equivalent reduction in macronutrients (carbohydrates, fats

and proteins) and micronutrients (vitamins and minerals). The initial aim of implementation of CR intervention was to analyze survival of living organisms such as lab-bred rats (McCay, Crowell & Maynard 1935; Osborne, Mendel & Ferry 1917; Turturro et al. 1999; Weindruch et al. 1986) and in turn served as foundation for studying mechanisms of lifespan extension in primates (rhesus monkeys) and humans (Colman et al. 2009; Holloszy & Fontana 2007; Ingram et al. 2006). CR has also been demonstrated to extend lifespan in other model organisms such as yeast (Jiang et al. 2000; Lin, Defossez & Guarente 2000), worms (Klass 1977; Lee et al. 2006), fruit flies (Chapman & Partridge 1996; Chippindale et al. 1993), and even dogs (Kealy et al. 2002; Lawler et al. 2008).

Implementation of CR in many organisms has a range of benefits. In rodents, it has been demonstrated to either reduce the severity or delay the onset of several diseases such as cancer, cardiovascular diseases, nephropathy, type II diabetes, auto-immune diseases and neurodegenerative diseases (Anderson, Shanmuganayagam & Weindruch 2009; Blackwell et al. 1995; Fontana & Klein 2007; Ingram et al. 1987; Martin, Mattson & Maudsley 2006; Masoro 2005). Extension of lifespan by CR observed in yeast, worms, flies, non-human primates (monkeys), rodents and humans are outlined below.

C. Caloric restriction in yeast

The best experimental model to study mechanisms of aging and improved lifespan upon caloric restriction was a single cell budding yeast, *Saccharomyces cerevisiae*. Caloric restriction was implemented in the form of reduction in the amount of glucose (0.5%) in growth media, and was found to increase the replicative life and lifespan of *S.cerevisiae* (Kaeberlein et al. 2005; Lin, Defossez & Guarente 2000).

However one of the main disadvantages of yeast is that it is a unicellular organism and hence the beneficial effects of caloric restriction cannot be extrapolated to multicellular organisms such as humans.

D. Caloric restriction in nematodes

Caenorhabditis elegans is a convenient experimental model which has allowed researchers to investigate changes at the tissue and cellular level when subjected to caloric restriction. One of the biggest advantage of using *C.elegans* as model organism stems from the fact that they have a short life-span compared to other multicellular organisms. Research has demonstrated that by reducing the source of food (*E.coli*), there is increase in life-span of *C.elegans* (Houthoofd et al. 2002; Klass 1977; Lee et al. 2006; Magwere, Chapman & Partridge 2004; Wei et al. 2008). In fact, Houthoofd et al. 2002 study demonstrated that upon complete removal of *E.coli*, the reproductive capacity of *C.elegans* increases to 150% , which suggests that increase in life-span is synonymous with increase in feeding restriction (in this case starvation). But because the increase in lifespan was observed under starvation conditions, hence caloric restriction benefits cannot be compared between *C.elegans* and other multicellular model organisms.

E. Caloric restriction and fruit flies

Drosophila melanogaster is one of the most widely studied model of fruit fly and a convenient model organism due to short life span (approximately 70 days) thus making it easier to study mechanisms of aging in these models. Generally, caloric restriction studies on Drosophila are performed by diluting the nutrients in growth media and reports suggest that it is quality of the diet rather than amount of calories that have beneficial

effects on life-span extension in Drosophila (Mair, Piper & Partridge 2005) whereas other studies reported that ratio of protein versus non protein sources in growth media is the determinant in life-span extension in Drosophila (Simpson & Raubenheimer 2009).

F. Caloric restriction in non-human primates

To understand the benefits of caloric restriction in non-human primates, rhesus monkeys (*Macaca Mulatta*) were used. Three separate groups namely, National Institute of Aging (NIA), Wisconsin National Primate Research Center (WNPRC) and University of Maryland used rhesus monkeys for their study. The investigating team at University of Maryland implemented short-term caloric restriction and studied its effects on obesity and diabetes in rhesus monkeys. Indeed, short-term caloric restriction did reduce body weight, improved insulin sensitivity and decreased incidence of diabetes in these monkeys (Bodkin et al. 2003; Bodkin, Ortmeyer & Hansen 1995). On the other hand, WNPRC and NIA studied effects of caloric restriction on life-span extension in rhesus monkeys. The duration of both studies was for 20 years. WNPRC, who started their experiment in 1989, after 20 years in 2009, reported that long-term caloric restriction was able to increase the median life-span of rhesus monkeys and delayed the onset of aging related diseases (Colman et al. 2009). However this group was not able to complete their study because approximately 37% of their ad libitum (control) monkeys died due to aging related disease whereas 17% of their control monkeys died due to non-aging related disease. NIA group, on the other hand, started their study in 1987 and their published report in 2012 suggested that there was no difference in health benefits between caloric restricted and ad libitum (control) monkeys. Such strikingly different results in the studies have been attributed to type of diet, amount of calories supplemented to these

animals or genetic background of these monkeys. However the overall conclusion drawn from these studies was that implementation of caloric restriction on rhesus monkeys could potentially improve health and increase life-span.

G. Caloric restriction in humans

Multiple studies have been published reporting benefits of caloric restriction in humans. These include fasting for religious purposes, Okinawan population study, Biosphere 2 and CALERIE (Comprehensive Assessment of the Long Term Effects of Reducing Intake of Energy) studies. While it is ethically not possible to check life-span benefits on humans, physiological and metabolic health benefits present a more convincing data.

Some of the communities that perform religious fasts include Greek orthodox, who consume only vegetarian food in a restricted manner for approximately 200 days per year; and Christians perform Daniel fast, wherein they eat vegan food for 21-40 days. In Greek orthodox, total cholesterol and LDL are reduced after implementation of fast versus prior to start of fast (Papadaki et al. 2008). Similar improvements in physiological parameters such as total cholesterol, LDL, blood pressure and insulin were found to be improved in Christians who followed the Daniel Fast (Bloomer et al. 2010). However additional studies are required to investigate the effects of indulgence in overeating to compensate for period of fasting. Another study of epidemiologic nature was conducted on Okinawan population residing in the Japanese island of Okinawa. People belonging to this island had the highest life expectancy as high number of people lived above the age of 100. In this epidemiologic study conducted on elderly population of Okinawa, it was reported that Okinawans began intake of reduced calories very early in life, thus,

resulting in reduced BMI throughout their life-span and decreased pre-disposition to aging related diseases which contributed to increased life-span compared to Japanese population living on the mainland (Willcox et al. 2007). Both, the studies on religious fast and Okinawan population, did demonstrate improvement of physiological parameters upon implementation of restricted diet that mimicked caloric restriction, however there was not enough attention paid to amount of calories consumed, and the study was not carried out in a controlled environment (which is not known if it positively contributes to improved health and life-span). Hence to control this variance factor, Biosphere 2 study was conducted in a controlled environment which included 4 men and 4 women and the duration of trial was 2 years. In this trial study, participants were fed low caloric, nutrient rich, vegetarian diet (between 1800-2000 kcal/day). Participants indeed showed significant reduction in weight, better glucose and insulin sensitivity, improved total cholesterol, LDL, triglycerides and blood pressure. Several months after the study concluded, it was observed that the subjects returned to their original weight and physiological parameters measured prior to the start of the study, which suggests inability of humans to maintain life-long CR diet (Walford et al. 2002). Another two year randomized clinical study under controlled conditions was conducted on humans, which was named as CALERIE (Comprehensive Assessment of the Long Term Effects of Reducing Intake of Energy). A total of 225 volunteers participated in this multicenter study wherein they were subjected to either control diet (to maintain their body weight) or 25% caloric restricted diet. Results showed that CR subjects had reduced body weight, reduced fat in both entire body as well as viscera (Heilbronn et al. 2006; Racette et al. 2006), their fasting insulin levels, LDL, total cholesterol to HDL ratio and C-reactive

protein were found to be improved (Fontana & Klein 2007). However, like the previous Biosphere 2 study, CALERIE study too encountered issues with ability of subjects to sustain a caloric restricted diet. But the overall conclusion of these studies is that caloric restriction does improve health by either reducing or preventing or delaying aging related biomarkers and diseases, while we do know the beneficial effects of CR, the mechanism by which CR induces these changes remains elusive. The potential metabolic benefits by which CR improves health and extends life-span are discussed later in section I.8.

1.3 Time Restricted Feeding

Time restricted feeding (TRF) is a term that is often used when effect of timing of food is to be studied on circadian rhythms of clock, clock controlled and metabolic genes. It is known that circadian clock is influenced by two important external cues (zeitgebers): light and food. When food is presented in an unrestricted manner, the master clock (suprachiasmatic nucleus) present in the hypothalamus entrains the peripheral clocks and produces biological rhythms. However it was demonstrated that upon implementation of time restricted feeding, TR was able to entrain the rhythms of peripheral clock (such as liver, lungs) independent of the master clock (Damiola et al. 2000; Hara et al. 2001; Satoh et al. 2005). One of the behavioral aspects of time restricted feeding is that mice learn that food will be presented for a specific interval of time and hence they quickly learn and adapt to this restricted feeding schedule by resorting to locomotor behavior in the anticipation of food. This locomotor behavior usually occurs 1-2 hours prior to the presentation of food, such an activity is termed as food anticipatory activity. Duration of time restricted feeding extends anywhere between 2 hours to 15 hours, either during the day or night. If the duration of restricted feeding is less than 6 hours, then mice cannot

consume equivalent quantity of food as their ad libitum (control) counterparts. However if the duration is more than 8 hours, then restriction fed mice are able to consume equivalent amount of food as their control group. Hence feeding window of greater than 8 hours serves as an appropriate model to distinguish the effects of periodic feeding/fasting cycles from control group without compromising on nutritional quality or quantity.

A. Time restricted feeding in Drosophila

TR studies performed on Drosophila involved feeding flies for 12 hours during the day phase, whereas during the night phase, they were provided with 1% agar only to maintain humid conditions. When compared with control counterparts (AL), TR fed fruit flies ate equivalent amount of calories as AL group, but did not gain body weight, with no change in activity levels. Flies develop age associated symptoms similar to humans when fed ad libitum food; they have dampened sleep and diurnal activity and increased cardiac arrhythmia. When flies were fed for 12 hours during the day, their sleep activity at night was better and had improved cardiac rhythms, however it did not alter gene expression analyzed in head, body and heart (Gill et al. 2015; Melkani & Panda 2017).

B. Time restricted feeding in humans

Studies have been conducted on humans to assess the benefits of time restricted feeding. A few of the studies evaluated how implementation of time restricted feeding affected body weight in humans. Those studies which had a restricted feeding period between 4-8 hours, did not observe any change in body weight in their subjects, irrespective of whether they were overweight or normal weighted males and females

prior to the start of the experiment (Aksungar, Topkaya & Akyildiz 2007; Halberg et al. 2005; Ravanshad et al. 1999; Soeters et al. 2009). With the exception of three studies (Gabel et al. 2018; Gill & Panda 2015; Temizhan et al. 2000), wherein they reported a 3-5% decrease in body weight in subjects after 4-16 weeks of restricted feeding; the reason why subjects lost body weight in Gabel and Gill study was due to degree of energy restriction (subjects ate 300 – 400 kcal less per day); however in Temizhan study the cause is not known. Studies that implemented restricted feeding hours of 10-12 hours have reported a 1-3% decrease in body weight of their subjects (Adlouni et al. 1997; Fakhrzadeh et al. 2003; LeCheminant et al. 2013; Nematy et al. 2012; V. et al. 2006; Zare et al. 2011), however, it is to be noted that these 10-12 hours feeding studies were Ramadan trials, wherein individuals fasted during the day and consumed food at night. In such trials, feeding periods are generally limited, as participants maintained a sleep duration of 7-8 hours. Restricting the feeding window may influence intake and expenditure of energy by the participants and in turn may contribute to changes in their body weight. Unfortunately majority of the studies did not measure energy intake and expenditure, and thus is one of the few pitfalls of these trial studies.

C. Time restricted feeding in mice

Time restricted feeding regimens that were implemented on mice for different durations of time, had contrasting effects on their body weights. Mice subjected to a 3-4 hour time restricted feeding schedule demonstrated a 9-18% reduction in body weight (Sherman et al. 2011, 2012). In the 2012 study, Sherman group fed mice with either high-fat (60% kcal of energy from fat) or low-fat (10% kcal energy from fat) diet in a time restricted manner for a period of 4 hours. Mice on both these diets showed 17-18%

decrease in body weight compared to their ad libitum control groups. Another study by the same lab in 2011 fed their mice a normal chow diet in the form of time restricted feeding for 3 hours, these mice reported only a 9% decrease in body weight, despite consuming equivalent amount of calories as their ad libitum control group. A different group of studies implemented time restricted feeding for 8-9 hours. Hatori et al. 2012 study group fed their mice a high-fat diet (60% kcal energy from fat) for a period of 8 hours and observed that although the TR fed mice consumed equivalent amount of calories as their control group, TR mice had 28% reduced body weight. This is in contrast to the Fonken et al. 2010 study, wherein they implemented 8 hour time restricted feeding, and neither the dark phase nor the light phase fed mice reported any changes in body weight compared to their control groups. Studies were also performed on mice with a time restricted feeding duration of 10-12 hours. These studies too reported contrasting effects on body weights of rodent models. Farooq et al. 2006 and Salim et al. 2007 studies demonstrated that rats when fed for 12 hours either during the dark or light phase for a period of 4 weeks, do not show any significant change in body weight compared to their controls. However, Salgado-Delgado et al. 2010 study reported a 13% increase in body weight when rats were fed during the light phase, as opposed to no change observed in the dark phase. On the other hand, Arble et al. 2009 study reported that when mice were fed a high-fat diet during the dark phase, there was a 19% decrease in body weight compared to those fed only during the light phase. Studies by Bray et al. 2010 and Tsai et al. 2013 compared the effects of high-fat diet (45% kcal energy from fat) versus low-fat diet (10% kcal energy from fat); while Bray group did not observe any change in body weight, Tsai group reported an 18% decrease in mouse weight when on high-fat diet but

no change when on low-fat diet. The reason for this difference in results is not known, it could be attributed to the duration of the study (Tsai group – 16 weeks vs Bray group – 12 weeks), which may have caused a decrease in body weight of TR mice. Another factor could be housing of mice for these studies. Most of the studies have not reported how they housed their mice. If mice are placed singly in a cage versus group, mice have a tendency to lose more body heat and thus decrease in body weight compared to group caged mice. Therefore, it can be concluded from these studies that duration of feeding, time of feeding, duration of the study and housing of mice are important factors for effects of TR in mice.

1.4 Circadian clock

A. Introduction

Circadian clocks are cell autonomous timekeeping systems that produces circadian rhythms in behavior, physiology and metabolism with a periodicity of 24 hours. These rhythms are conserved across various species from cyanobacteria to humans (Bass & Takahashi 2010; Eckel-Mahan & Sassone-Corsi 2013; Maury, Ramsey & Bass 2010; Panda, Hogenesch & Kay 2002). Circadian rhythms are generated by multiple circadian clocks that are located in every tissue of living organisms (Albrecht 2004; Dibner, Schibler & Albrecht 2010). The organization of circadian clock is such that there is master/central clock present in the suprachiasmatic nucleus of the anterior hypothalamus that receives photic signal through the retino-hypothalamic tract, which then entrains the peripheral clocks located in peripheral tissues such as heart, liver, and skeletal muscle, which in turn generates rhythms to maintain physiology and metabolic homeostasis (Bass

& Takahashi 2010). Photic cues are dominant cues for the central clock (Dibner, Schibler & Albrecht 2010), whereas feeding cues (which are an example of non-photic cues) have a different effect on both central and peripheral clocks. When feeding period is unlimited, food has the ability to synchronize both central and peripheral clocks; however, if feeding period is restricted, food has the ability to de-couple peripheral clock from central clock and peripheral rhythms that are generated are primarily dictated by the feeding period (Damiola et al. 2000).

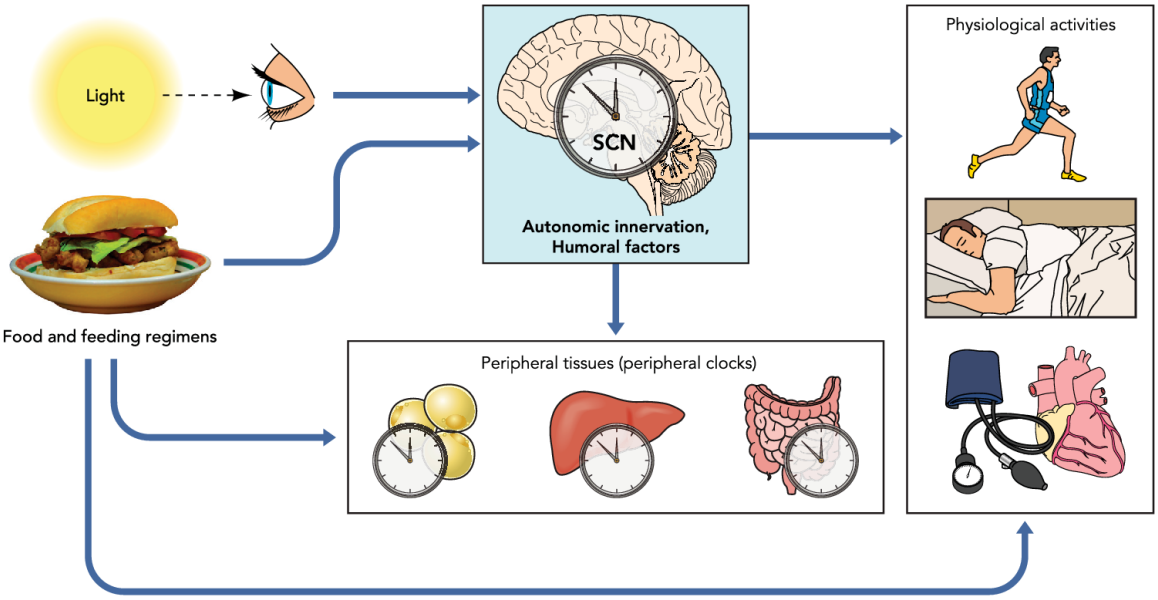

Figure I.4A: Overview of circadian clock

The master circadian clock present in the SCN of the hypothalamus and is predominantly entrained by the light entering the retina, which in turn synchronizes peripheral clocks present in different tissues such as the liver, muscle and heart. Peripheral clocks can also be entrained by feeding cues, and produce circadian rhythms independent of master clock. (Image courtesy Froy, O. 2011. Circadian rhythms, aging, and life span in mammals. *Physiology*, *26 4*, 225-35)

B. Molecular Organization of Circadian clock

Circadian rhythms in every mammalian tissue are generated by circadian clock genes and their downstream targets via transcription-translation feedback loops. At the core of this clock mechanism are two transcriptional factors BMAL1 (MOP3 or ARNTL) and CLOCK (NPAS2) that heterodimerize and bind to the E-box element located on the promoter of their downstream targets *Periods* (*Per1,2,3*) and *Cryptochromes* (*Cry1,2*) and drive their transcription. PERIODS and CRYPTOCHROMES form heterodimeric complexes in the cytoplasm and enter the nucleus, where they bind to both BMAL1 and CLOCK and repress their transcriptional activity, thus in turn inhibiting their own gene expression. Such a feedback loop is termed as negative feedback loop. Another feedback loop that is formed consists of retinoid-related orphan receptors (RORs) and REV-ERB family of transcription factors. Both these factors compete with each other to bind to ROR-E box element located in the promoter region of *Bmal1*. ROR (RORα, RORβ, RORγ) proteins bind to these elements and positively regulate *Bmal1*, whereas REV-ERB (REV-ERBα, REV-ERBβ) proteins compete with RORs to bind to the same element box and negatively regulate the transcription of *Bmal1* (Dunlap 1999; Eckel-Mahan & Sassone-Corsi 2013; Mohawk, Green & Takahashi 2012; Zhang & Kay 2010).

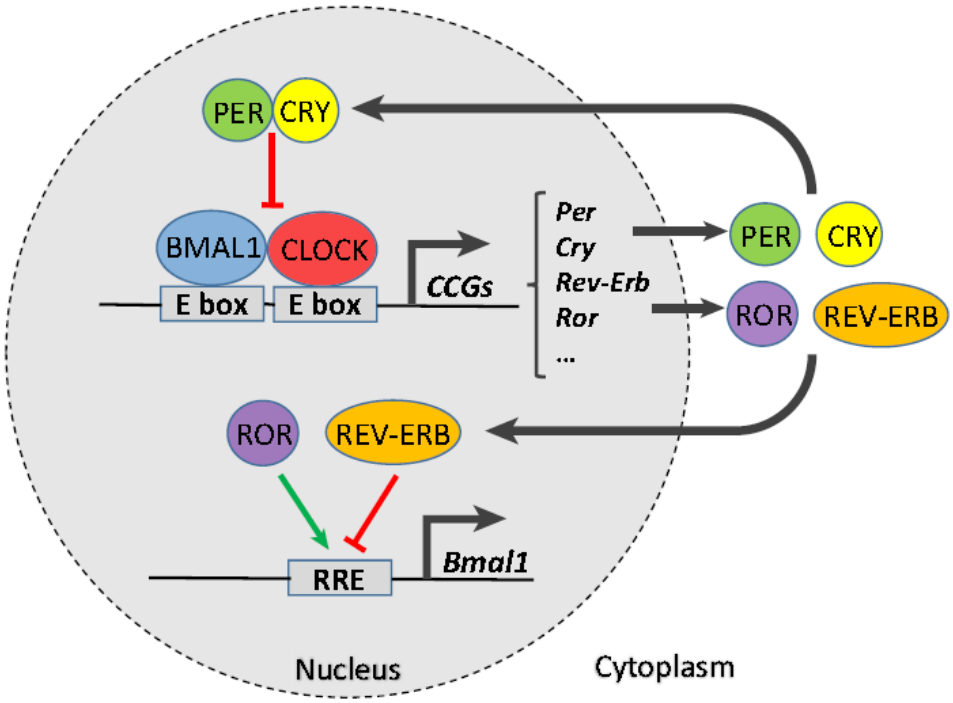

Figure I.4B: Molecular mechanisms of circadian clock

The circadian clock in mammals constitutes transcription-translation feedback loops. Core clock transcription factors CLOCK and BMAL1 bind to E-box elements of their downstream targets *Periods* and *Cryptochromes* and drive their transcription. PERs and CRYs then translocate into the nucleus to inhibit the transcriptional activity of CLOCK and BMAL1. CLOCK and BMAL1 also drive the transcriptional activity of *Ror*s and *Rev-erb*s, however, *Ror*s positively regulate *Bmal1*, whereas *Rev-erb*s negatively regulate transcription of *Bmal1*. (Image courtesy Gaucher et al 2018. Molecular Cogs: Interplay between Circadian Clock and Cell Cycle. Trends in Cell Biology, Vol. 28, No. 5)

1.5 Glucose Homeostasis

Metabolism of glucose is very important for normal functioning of the body. Besides being a major source of energy, glucose is also used as a starting material for

many biochemical reactions. Among the various tissues that uptake glucose, brain is the biggest consumer of glucose; about 60-70% of the total body glucose is used by the brain every day. When glucose levels fall below 40 mg/dl, functioning of the brain is seriously affected, which may lead to permanent damage and ultimately result in death. Therefore, it is necessary that plasma glucose levels are tightly regulated between 90-120 mg/dl. There are two ways of producing glucose; one, through intake of diet, and second, by the liver by a process called gluconeogenesis. Liver is the main metabolic organ that produces more than 90% of glucose, not derived from diet. This large amount of glucose is produced via non-carbohydrate sources such as amino acids, lactate and glycerol, that are released into circulation by other tissues. Besides producing glucose, liver also stores glycogen, which is used under conditions of fasting. During prolonged conditions of starvation, liver produces glucose as well as ketone bodies, both of which serve as source of energy for the brain. There are several biochemical pathways that occur in the liver, but the two main pathways that help maintain glucose homeostasis are glycolysis and gluconeogenesis and are discussed in brief below.

A. Glycolysis

Glycolysis is the main pathway that produces energy by catabolizing glucose. Intermediates and products that are generated in this pathway, can be used as a source of carbon in almost every biochemical reaction. The pathway begins with entry of glucose in the cell followed by immediate phosphorylation of glucose to glucose 6-phosphate. The enzyme that catalyzes this reaction is hexokinase that is present in most of the tissues. Due to its high affinity for glucose, the rate of hexokinase reaction is limited by

the amount of glucose entering the cell and its end product glucose 6-phosphate. In tissues such as liver and pancreas (β cells), another isoform of this enzyme (glucokinase/hexokinase IV) catalyzes the same reaction. Since glucokinase (GCK) has lower affinity for glucose and is not allosterically inhibited by glucose 6-phosphate, thus it allows the liver to use an unlimited amount of glucose for the process of glycolysis during feeding conditions. The first committed and irreversible step in glycolysis is catalyzed by phosphofructokinase (PFK-1) that converts fructose 6-phosphate to fructose 1,6-bisphosphate. PFK-1 is allosterically inhibited by ATP and citrate, whereas it is allosterically activated by ADP/AMP and fructose 2,6-bisphosphate. Pyruvate kinase (L-PK) is the last key regulating enzyme in this reaction that converts phosphoenol pyruvate to pyruvate. Pyruvate kinase is allosterically activated by fructose 1,6-bisphosphate, which suggests active glycolysis, whereas it is allosterically inhibited by ATP, acetyl CoA and long chain fatty acids. L-PK is phosphorylated under fasting conditions by cAMP-dependent protein kinase which inhibits its enzyme activity.

B. Gluconeogenesis

Gluconeogenesis is the biochemical reaction that produces glucose from non-carbohydrate sources. Liver and kidneys are two main organs that carry out gluconeogenesis. Many of the steps in gluconeogenesis are reversible due to reversible enzymes common to both glycolysis and gluconeogenesis. However, there are four regulatory and irreversible enzymes that are unique for gluconeogenesis. The first enzyme is pyruvate carboxylase (PC) that catalyzes pyruvate to oxaloacetate in the mitochondria. Acetyl CoA generated from fatty acid oxidation under fasting conditions,

is a positive allosteric activator of PC. Second regulatory enzyme is phosphoenolpyruvate carboxykinase (PEPCK) that converts cytoplasmic oxaloacetate to phosphoenol pyruvate. Fructose 1,6-bisphosphatase is the third regulatory enzyme that converts fructose 1,6-bisphosphate to fructose 6-phosphate. This enzyme is allosterically inhibited by AMP and fructose 2,6-bisphosphate. Lastly, glucose 6-phosphatase dephosphorylates glucose 6-phosphate to yield glucose. This enzyme is regulated by the level of its substrate glucose 6-phosphate, as the level of glucose 6-phosphate increases, enzymatic activity of glucose 6-phosphatase increases and hence more glucose is produced.

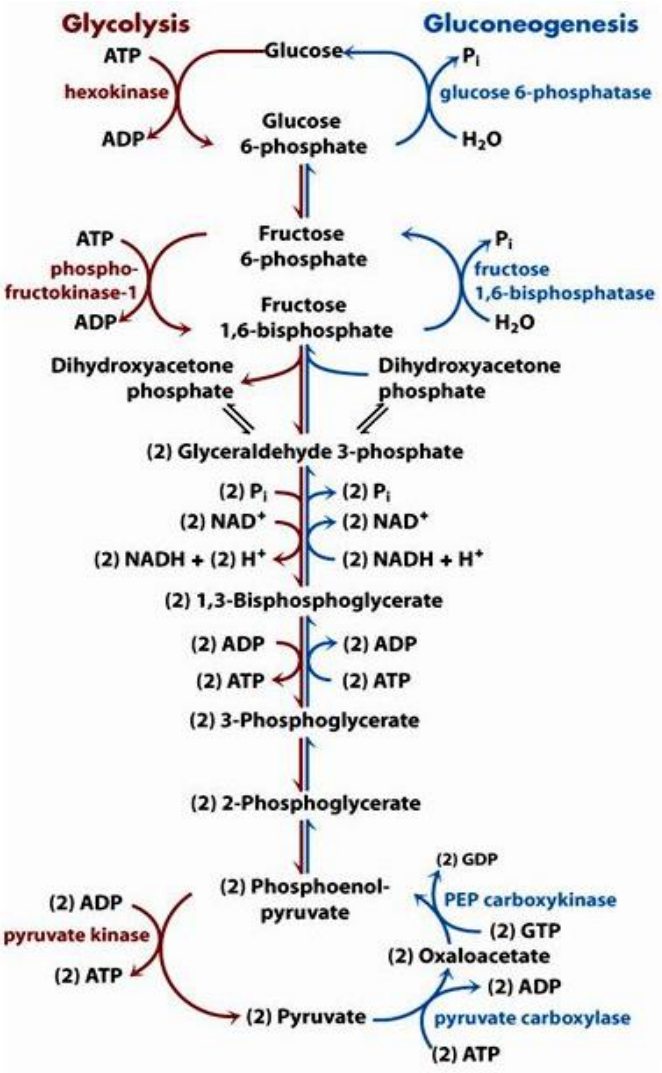

Figure I.5: Overview of Glycolysis and Gluconeogenesis pathways (Image courtesy Lehninger Principles of Biochemistry, 5th Edition, 2008, W.H. Freeman and Company)

1.6. Circadian regulation of Glucose Metabolism

Several studies have demonstrated that circadian clock regulates many metabolic pathways (Bass & Takahashi 2010; Eckel-Mahan & Sassone-Corsi 2013; Panda, Hogenesch & Kay 2002). This regulation may be either directly by the clock (via transcription) or indirectly (wherein endocrine factors regulated by circadian clock are released into the bloodstream in a time of the day dependent manner which further regulates tissues that are metabolically active such as liver and pancreas). Among the several metabolic pathways under circadian control, glucose metabolism is the subject of interest in this study and is discussed below.

Human and mice model studies have shown that glucose metabolism exhibits time of the day dependent oscillations in whole body and tissue specific context. There are two ways by which glucose homeostasis is maintained in the body: one, by exogenous route (such as consumption of carbohydrate rich food, digestion and absorption) and, two, by endogenous route (such as gluconeogenesis where in glucose is generated from non-carbohydrate sources). Liver plays an important role in maintaining glucose homeostasis; during feeding time, liver stores energy in the form of glycogen whereas during fasting, liver uses alternate sources of energy such as glycogen, fatty acids and ketone bodies to generate glucose and maintain energy homeostasis. Diurnal oscillations have been observed not only in glycogen levels in number of organisms such

as mice, rats and humans (Sollberger 1964), but also enzymatic activities of glycogen metabolism related enzymes such as glycogen synthase (which peaks during the active/dark phase) and glycogen phosphorylase (peaks during rest/light phase) in rodents (Ishikawa & Shimazu 1980; Peret, Chanez & Pascal 1976) and these oscillations were reported to be mediated potentially by circadian cock wherein ClockΔ19 mutant mice demonstrated decreased in oscillations of glycogen levels and glycogen synthase mRNA and protein levels in the liver (Doi, Oishi & Ishida 2010). Just as glycogen metabolism shows diurnal oscillations, similar observations were made in context of gluconeogenesis too, wherein increase in enzyme activity of phosphoenolpyruvate carboxykinase (PEPCK), which is a regulatory enzyme in the process of gluconeogenesis, was observed during the transition from sleep to wake cycle in the liver of rats (Kida et al. 1980), and that an intact circadian clock in the hepatocytes was required for maintaining rhythms of PEPCK activity (Lamia, Storch & Weitz 2008). Zhang et al. 2010 study also reported that CRYs were involved in the regulation of gluconeogenesis in the liver in a time-of-the-day dependent manner via β-adrenergic signaling pathway and activation of cAMP response element binding protein (CREB).

Maintenance of glucose homeostasis by circadian clock also involves regulation at the level of release and secretion of endocrine hormones. Two of the most important hormones involved in glucose homeostasis are Insulin and Glucagon. A study by Peschke & Peschke in 1998 demonstrated that secretion of insulin occurred in a circadian manner in vitro in pancreatic islets in rats. Kalsbeek & Strubbe 1998 study reported a similar observation wherein they observed oscillations in plasma levels of insulin across the day in rats in response to feeding. A group of studies showed that when circadian clock is

disrupted, it impairs secretion of insulin and thus results in hypoinsulinemia (Coomans et al. 2013; Marcheva et al. 2010), thus indicating that plasma insulin is under the regulation of circadian clock. In addition to regulating insulin secretion, circadian clock also has been involved in the regulation of insulin sensitivity, which occurs in a time-of-the-day dependent manner. Impairment of insulin tolerance and glucose tolerance have been observed in rats with SCN (master clock) ablation, ClockΔ19 mutants and BMAL1 null mice (La Fleur et al. 2001; Rudic et al. 2004). Besides insulin release and secretion, regulation also is impaired at the level of insulin signaling in multiple tissues obtained from BMAL1 germline null, PER2 mutant and mice with ClockΔ19 mutation in cardiomyocytes (Anea et al. 2009; Carvas et al. 2012; Durgan & Young 2010). Similar to insulin, glucagon too is involved in glucose homeostasis and shows diurnal oscillations in the plasma and liver of humans as well as mice (Gagliardino et al. 1978; Tasaka et al. 1980) and that these oscillations are modulated by feeding and circadian clock (Ruiter et al. 2003). Therefore the conclusion from these studies is that circadian clock is involved in glucose homeostasis either by regulating genes and/or enzymes critical for metabolic pathways or though release and/or secretion of endocrine hormones.

1.7 Circadian clock and Caloric restriction

A number of studies have reported the inter-relation of circadian clock with caloric restriction. Caloric restriction has been demonstrated to affect the amplitude of expression of several circadian clock genes, both in flies and mammals. In Drosophila, it was demonstrated that, upon implementation of caloric restriction, there was significant induction of circadian clock genes *tim* and *per* in the head and body of these flies (Katewa et al. 2016). Our lab also demonstrated similar effects in mice (Patel, Chaudhari,

et al. 2016). We subjected our mice to caloric restricted feeding during the dark (active) phase, to prevent dissociation of central and peripheral clocks. We reported that caloric restriction significantly affected the amplitudes of *Bmal1*, *Per1* and *Per2* in the liver of mice. Another similar study conducted by Mendoza group in 2005 reported that calorie restriction affected the expression of clock genes in the SCN of mice. In addition to reporting the effects of caloric restriction on gene expression, Katewa et al. 2016 and Patel, Chaudhari, et al. 2016 studies also demonstrated effects on lifespan extension. Indeed, in Katewa study, it was demonstrated that *tim* and *per* mutant flies showed a decrease in lifespan upon implementation of CR, compared to the control group. In Patel study too, similar results were observed wherein *Bmal1* knockout mice failed to show an increase in lifespan when subjected to caloric restriction. Implementation of caloric restriction has multiple benefits which include reduction in insulin and igf-1 levels; and shift to fatty acid synthesis and breakdown which thus contributes to improved fat turnover. In case of flies, circadian clock is necessary for improved fat turnover mediated by caloric restriction, whereas in mice, knockout of *Bmal1* fails to reduce the plasma levels of insulin and igf-1 upon CR. Thus, we can conclude that circadian clock and caloric restriction are interlinked and both are required for improved metabolism and extension of lifespan mechanisms. However, it is not known, what is the extent of the roles each of them play in mediating these beneficial effects on living organisms.

1.8 Circadian clock and Time restricted feeding

Circadian clocks are integral to maintaining metabolic homeostasis. Tissue specific or whole body genetic disruptions of circadian clock result in de-synchronization of circadian rhythms which ultimately predisposes animals to metabolic diseases. Peripheral

clocks such as liver, are entrained to cycles of time restricted feeding and produce their own rhythms independent of the master clock in the SCN (Damiola et al. 2000; Hara et al. 2001; Stokkan et al. 2001). Mice are nocturnal animals and most of their feeding and physical activity occurs during the dark (night) phase, which is in contrast to humans (Satoh et al. 2005; Sherman et al. 2011, 2012). Studies conducted on mice and rodent models have demonstrated that time restricted feeding has the ability to change or restore the phase of peripheral clock genes without affecting the phase of master clock genes under three conditions: 1) when no food is provided during the active (dark) phase, but unlimited access to food is given for 12 hours during the light (inactive) phase ; 2) when an unlimited amount of food is presented between 2 to 12 hours during the light (inactive) phase and 3) when an unlimited amount of food is provided for 12 hours during the subjective light phase, but, mice are placed on a constant darkness schedule (Damiola et al. 2000; Hara et al. 2001; Satoh et al. 2005; Sherman et al. 2011; Stokkan et al. 2001). On the other hand, when unlimited amount of food is presented only during the dark phase or mice are fed ad libitum, there is no change in phase of clock gene expression. For example, study conducted by Damiola et al in 2000 demonstrated that when unlimited amount of food was presented only during the light (inactive) phase, it significantly changed the phase of expression of circadian clock and clock controlled genes namely, *Per1*, *Per2*, *Per3*, *Cry1*, *Dbp*, *Rev-erb α* and *Cyp2a5* in the mouse liver. Study conducted by Hatori et al in 2012 demonstrated that when high-fat diet is provided in a time restricted manner only during the dark (active) phase for 8 hours, this resulted in increase in the amplitude of mRNA expression of clock genes which include *Per1*, *Per2*, *Bmal1*, *Rev-erb a*, *Cry1*, *Clock*, *Ror α* and *Dbp* compared to mice that were fed ad

libitum amount of high-fat diet. Hara et al study in 2001 demonstrated that when food is limited to only 4 hours during the light (inactive) phase or 4 hours during the subjective light phase when mice are on 24 hours constant darkness schedule, it increased the amplitude of mRNA expression of clock genes *Per1* and *Per2*. In addition to regulating the expression of several clock genes, time restricted feeding was demonstrated to restore the rhythmic expression of hepatic transcripts in the absence of clock. Vollmers et al. 2009 study demonstrated that when time restricted feeding of 8 hours was performed during the day phase on *Cry1,2-/-* mice, it drives rhythmic expression of genes regulated by transcription factors such as CREB, AKT, SREBP1/2 and ATF6. Another study conducted by Chaix et al. 2019, performed time restricted feeding for 8 hours during the dark phase and reported that high-fat fed TR diet reduced serum triglyceride between 17 – 42% and cholesterol levels between 22 – 35%, improved glucose and insulin sensitivity, and induced diurnal rhythms of nutrient sensing pathways such as AMPK and mTORC1 in the liver-specific *Bmal1*, *Rev-erbα/β* and *Cry1/2* knockout mice. Thus the conclusion from these studies is that when periodic feeding/fasting cycle is imposed, it has the ability to drive rhythmic expression of transcripts and metabolic pathways, which is otherwise dysregulated in the absence of a functional circadian clock.

1.9 Metabolic effects of Caloric Restriction

A. Insulin/Igf-1 Signaling

Glucose, which is the main source of energy for the cells, which is either obtained through consumption of carbohydrate rich food or through internal biochemical mechanisms such as gluconeogenesis or glycogen breakdown. When glucose is present in

excess, it results in cancer, type II diabetes and cardiovascular diseases and promotes aging. To counteract excess glucose in the blood, β-cells of pancreatic islets release a hormone called Insulin, which decreases the amount of glucose in the blood by promoting enhanced uptake of glucose by various tissues such as skeletal muscle, adipose tissue and brain. Increased uptake of glucose results in increased production of ATP which further contributes to cell growth and proliferation. Increased glucose metabolism also results in increased production of reactive oxygen species thus allowing less time for repair mechanisms. When insulin binds to Insulin receptor, it activates pathways that promote cell growth and proliferation. Caloric restriction was demonstrated through previous studies to reduce blood glucose and insulin and improves glucose and insulin sensitivity in many species (Kalant, Stewart & Kaplan 1988; Masoro et al. 1992; Mitchell et al. 2015; Wang et al. 1997). Thus reduction in glucose and insulin through caloric restriction promotes results in improved metabolism and health.

Growth hormone – Igf-1 signaling axis is another hallmark of CR. Increase in GH-Igf-1 signaling pathway also promotes cell growth and proliferation which ultimately contributes to advance in the process of aging. Implementation of CR reduces GH-Igf-1 signaling pathway thus changing the preference from cell growth and proliferation to repair, replacement and maintenance mechanisms in mammals (Fontana et al. 2016). Some strains of dwarf mice, when provided with unlimited access to food, show increase in lifespan (Bartke, Sun & Longo 2013), which is similar to mice that are subjected to caloric restricted diet. Ames and Snell are two examples of dwarf strains of mice, which have defective anterior pituitary development and hence are unable to produce hormones such as prolactin, thyrotropin and growth hormone (GH). Defects in the production of

GH alone, reflects on decreased IGF-1 production, which results in reduced size of body and increase in life-span. Other effects of defective GH production include decrease in insulin secretion, increase in insulin sensitivity, and decrease in plasma glucose levels and increase in resistance to oxidative stress (Brown-Borg & Bartke 2012). Although we observe promising results of application of CR in mice, studies on humans suggest otherwise. Fontana et al in 2008 and 2016 reported that when humans were subjected to a two year CR, they did not observe any changes in plasma IGF-1 levels, which suggests that CR mediated improvement in human health may not involve lowering of plasma IGF-1 levels, but instead, CR may affect alternative pathways that will help improve health-span, which would further suggest that extension of life-span by CR mediated decrease in plasma IGF-1 levels is species-specific, i.e. it is observed only in mice but not in humans. Additional factors that could potentially explain differential effect on plasma IGF-1 levels in both mice and humans could be due to the fact that mice are nocturnal (active during the night) whereas humans are diurnal (active during the day). Another factor could be feeding and fasting patterns: mice tend to eat continuously across the day (feeding activity higher during the day compared to night), whereas humans consume 1-3 meals at periodic intervals during the day.

B. Adenosine Monophosphate-activated Protein Kinase

Adenosine tri-phosphate (ATP) is the energy currency of the cell that is generated from Adenosine mono-phosphate (AMP) in the mitochondria during oxidative phosphorylation. Activation of AMPK occurs when AMP:ATP ratio rises, which signifies energy deficit and thus promotes increase in uptake of glucose, oxidation of fatty acids and in turn shuts down energy consuming process such as fatty acid synthesis

and protein synthesis, an adaptive response to maintain energy balance (Cantó & Auwerx 2010). AMPK activates a number of downstream targets; such as phosphorylation of PGC1a in skeletal muscle, thus promoting mitochondrial energy metabolism, phosphorylation and activation of SIRT1, which further deacetylates PGC1a and thus promotes utilization of fatty acids as a source of fuel. SIRT1 also deacetylates LKB1 (an activating upstream regulator of AMPK) and induces its activity, thus serving as positive feedback loop. mTOR signaling is also downstream of AMPK. Under energy deficit conditions, AMPK phosphorylates RAPTOR, which is a binding partner of mTOR, and promotes binding of RAPTOR with 14-3-3, thus inhibiting mTOR signaling. Another mechanism involves AMPK phosphorylating TSC1 and 2 (GTPase activating protein) and thus in turn inhibiting mTORC1. Therefore, AMPK is implicated in playing an important role by regulating the expression of several proteins that are necessary for CR mediated increase in health and life-span.

C. Sirtuins

Sirtuins are evolutionarily conserved histone deacetylase proteins that require Nicotinamide adenine dinucleotide (NAD) as a co-substrate. NAD+ is converted to NADH which is useful in oxidative phosphorylation and fatty acid oxidation occurring in the mitochondria. Because availability of NAD is important in metabolism, Sirtuins too share a direct link to metabolism. Levels of NAD are regulated partially by NAD salvage pathway, which has been demonstrated to play a role in aging process and CR mechanisms in yeast and mammals (Anderson et al. 2003; Song et al. 2014). Expression and activity of Sirtuins too are increased in several tissues such as adipose tissue and brain (Nisoli et al. 2005). Sir2 (silent information regulator 2), which is the founding

member of sirtuin family, and is present in yeast, like its homologues in worms and flies, plays an important role in life-span extension (Rogina & Helfand 2004; Tissenbaum & Guarente 2001). Both, overexpression of SIRT1 or use of SIRT1-specific activators have been demonstrated in separate studies to mimic the improvement in physiological parameters observed when caloric restriction is implemented (Mitchell et al. 2014; Pearson et al. 2008). In addition to this, downstream targets of SIRT1 are involved in multiple cellular processes such as cell fate, gluconeogenesis, circadian clock and inflammation which suggests pivotal role of SIRT1 in metabolic processes (Chang & Guarente 2014; Li 2013). Other than SIRT1, there are six additional sirtuins whose role in the process of aging and caloric restriction are not well known. However, independent studies have demonstrated that some of them may be linked to positive effects of caloric restriction. For example, SIRT3 has been shown to activate fatty acid oxidation and improve antioxidant defense by activating and regulating Superoxide dismutase 2 (SOD2) and glutathione system, both of which are observed in CR conditions (Hebert et al. 2013; Qiu et al. 2010; Someya et al. 2010). SIRT5 expression is higher in brain of animals subjected to CR along with increased expression of SIRT1 (Geng et al. 2011).

D. mTOR (mammalian target of Rapamycin)

The main regulator of nutrient sensing pathways is mammalian target of Rapamycin (mTOR). mTOR is a serine threonine protein kinase that is conserved across all species (Lamming & Sabatini 2013). mTOR exists as two complexes: mTORC1 and mTORC2. mTORC1 signaling is activated in response to number of upstream signals such as amino acids, glucose, growth factors, as a result of which downstream target such as ribosomal protein S6 kinase (S6K1 and S6K2) is phosphorylated, which in turn

phosphorylates ribosomal protein S6 and induces ribosome biogenesis. mTORC1 also phosphorylates eukaryotic initiation factor 4E binding protein (4EBP1), which allows 5'-cap dependent protein translation. On the other hand, mTORC2 signaling is activated by insulin, IGF-1 and other growth factors and promotes phosphorylation of AKT, SGK and PKCα, which in turn help mTORC2 to regulate number of processes such as glucose metabolism, ion transport, actin reorganization, cell growth and proliferation.

Inhibition of mTOR protein or proteins associated with mTORC1 or downstream targets of mTORC1 have been demonstrated to increase life-span in yeast, worms, flies and mammals (Harrison et al. 2009; Kaeberlein et al. 2005; Kapahi et al. 2004; Powers et al. 2006; Vellai et al. 2003). Studies have demonstrated that restricting caloric intake too results in reduced mTORC1 signaling (Blagosklonny 2010; Kaeberlein et al. 2005; Kapahi et al. 2010; Powers et al. 2006); in case of mammals, we showed that the effect is time of the day dependent (Tulsian, Velingkaar & Kondratov 2018). Rapamycin, an immunosuppressant and an anti-cancer agent, in itself, can inhibit mTORC1, however when combined with CR and supplemented to fruit flies, it adds to the beneficial effects of CR via extension of life-span (Bjedov et al. 2010). However in case of mice, it was observed that both CR and rapamycin, in addition to extension of life-span, regulated transcription of genes differentially from each other, thus suggesting that mechanisms of life-span extension by rapamycin and CR are very distinct from each other (Fok et al. 2014).

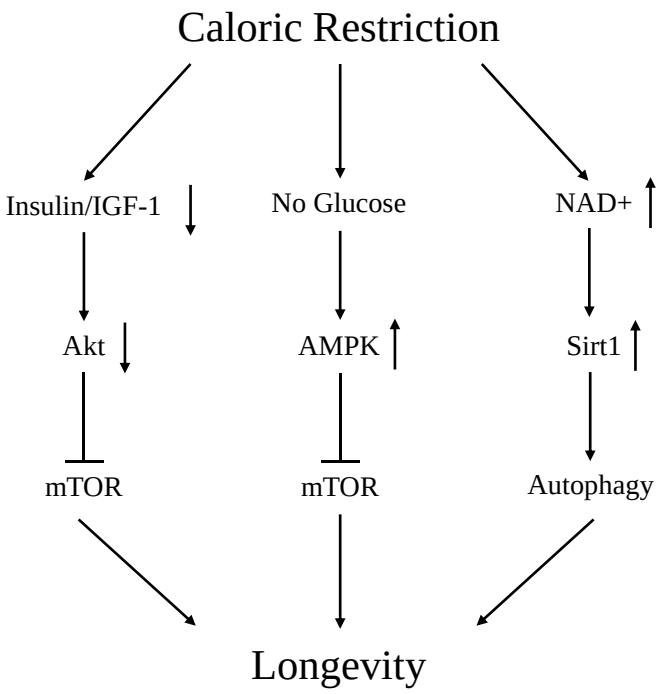

Figure I.9: Schematic representation of metabolic effects of Caloric restriction (Figure adapted from Mangan, 2019).

1.10 Metabolic effects of Time restricted feeding

A. Humans

In addition to body weight, studies also measured fasting glucose and insulin levels as well as insulin sensitivity. Studies that had a restricted feeding window of 4-8 hours produced contrasting results. 1985 study by Halberg and 2009 study by Soeters employed 4 hour restriction feeding for 2 weeks, however Halberg group reported a 16% increase in insulin sensitivity, whereas Soeters group did not observe any change in insulin sensitivity; the difference in results could be attributed to the type of meal used in these studies; Halberg study participants consumed a solid meal, whereas in Soeters study, participants had atleast 40% of their daily intake of energy in the form liquid

meals. Ravanshad et al 1999 study observed a 27% decrease in fasting glucose. However, these changes in fasting glucose were independent of the effect on body weight, as the Ravanshad study reported no change in body weight in participants, however study by Temizhan et al in 2000, reported a 5% decrease in body weight but observed a 20% increase in fasting glucose in their participants. On the other hand, 10-12 hour restricted feeding studies reported a decrease in fasting glucose levels in the range of 10-30% in their respective participants (Adlouni et al. 1997; Fakhrzadeh et al. 2003; V. et al. 2006). Moro et al. 2016 study implemented time restricted feeding of 8 hours for 8 weeks and observed a decrease in fasting insulin, igf-1 and glucose levels. Sutton et al. 2018 study performed a supervised trial study with controlled feeding wherein they provided the participants with restricted access to food for 6 hours of the day for 5 weeks. As a result of this study, participants had reduced plasma insulin levels, blood pressure and oxidative stress levels, whereas insulin sensitivity and β-cell responsiveness were improved. Although both studies suggest that time restricted feeding has beneficial effects, there are a few limitations to these studies. Moro et al study implemented dietary restriction on resistance trained subjects but they did not mention what proportion of subjects were males and females. Sutton et al study, on the other hand, had only 8 participants and all were overweight males with condition of prediabetes. This study also did not measure glucose levels which may have been affected by the restricted feeding schedule.

Time restricted feeding involved consumption of a single meal over the course of the day. One study investigated how meal frequency affected metabolism in humans in comparison to time restricted feeding. Stote et al. 2007 study compared effects of 3 meals a day versus 1 meal a day and demonstrated that consumption of 1 meal a day within a

time window of 4 hours in the evening induced a modest decrease in body weight and fat mass, decreased plasma cortisol concentrations, however, neither of the two diets changed fasting blood glucose levels.

B. Mice

Time restricted feeding studies that have been conducted on rodent models employ high-fat diet and has been demonstrated to be effective in preventing and treating metabolic disorders. In order to the study the mechanisms of metabolic disorders, these studies used diet induced obese (DIO) mice. When mice have unlimited access to high-fat diet across the entire day, they turn obese and hence are termed as diet induced obese (DIO) mice. In addition to being obese, mice also have their circadian oscillations disrupted in metabolic tissues such as liver (Pendergast et al. 2013). Such disruptions in circadian oscillations derives analogy from shift workers, which also results in circadian arrhythmias, thus tipping the balance off the metabolism and thus contributing to metabolic diseases. Multiple studies have demonstrated that when mice are fed a high-fat diet during the active (dark) phase for durations ranging from 8-15 hours, the mice consume equivalent amount of calories as their control (ad libitum) group, but they do not suffer from any metabolic disorders, thus emphasizing the beneficial effects of time restricted feeding (Chaix et al. 2014; Chaix & Zarrinpar 2015; Sherman et al. 2012).

Liver is an important metabolic tissue where majority of the biochemical reactions occur. Most of the time restricted feeding studies were performed on liver tissue and majority of the beneficial effects of time restricted feeding were reported in liver, which include gene expression and metabolites (Hatori et al. 2012), however some of these beneficial effects also extend to other tissues such as muscle, adipose tissue (brown

and white) and gut (Zarrinpar, Chaix & Panda 2016). When compared to DIO mice fed a control diet, TRF fed DIO mice had significantly distinct and improved metabolic profile, among the metabolites that were enriched in TRF mice belong to glucose and lipid metabolism. TRF also affects key nutrient sensing pathways in the liver such as mTOR, AMPK and CREB. pCREB peaks are observed during the daytime, which suggests active gluconeogenesis during the day, whereas pS6 peaks during the nighttime, which suggests utilization of glucose via pentose phosphate pathway. Activation of pentose phosphate pathway results in substrates for nucleotide metabolism. Indeed, implementation of time restricted feeding results in increase in nucleotide levels (Hatori et al. 2012). Time restricted feeding ensures periodic entrainment to fasting cycles, which potentially increases AMP levels in the liver. AMP activates AMPK, which phosphorylates and deactivates acetyl CoA carboxylase (ACC), which acts to inhibit fatty acid oxidation. Fatty acid metabolism in the liver is associated with cholesterol and synthesis of bile acids, and *Hmgcs2*, *Srebp1c* and *Cyp7a1* play an important role in bile acid synthesis (Le Martelot et al. 2009). Time restricted feeding in mice fed a high-fat diet, alters the phase of expression of *Hmgcs2* and *Srebp1c* and increases the amplitude of *Cyp7a1* gene, which when present in high amounts, causes increase in bile acids in the liver which further reduces the serum levels of cholesterol in mice (Hatori et al. 2012). TR also affects adipose tissue, by reducing the adipocyte size, production of inflammatory cytokines, however it increases number of mitochondria in both brown adipose and white adipose tissue (Hatori et al. 2012). In addition to high-fat diet, TR shows beneficial effects on other high energy diets such as high fructose, high-fat + sucrose diet (Chaix et al. 2014). Mice fed normal chow do not show any reduction in body weight, however

when these mice are subjected to long term TR (more than 26 weeks), they have tendency to be lean and reduce adiposity. Also DIO mice that are fed TR have an endurance capacity of 40% more than their control (AL) counterparts (Chaix et al. 2014).

Besides composition of the diet, time of the day when these mice are fed and the duration of intake of food also have profound effects on metabolism. Mice fed TR during the dark (active) phase have better physiological and metabolic outcomes compared to inactive (day) phase. Study conducted by Hatori et al in 2012 demonstrated when mice are fed an ad libitum amount of high-fat diet, have a tendency to develop obesity whereas mice fed high-fat diet only 8 hours during the dark phase did not develop obesity. As a result of time restricted feeding for 8 hours, the mRNA expression of key metabolic enzymes involved in the process of glucose and fatty acid metabolism namely, *G6pd* and hepatic lipase was increased, whereas *Pcx, G6pc* and *Fas* expressions were decreased compared to ad libitum mice fed high-fat diet, which suggested a decrease in inflammation and steatosis in the liver. Sherman et al in 2012 demonstrated that when high-fat dietary intake was restricted to 4 hours a day only during the light phase, it did not cause obesity, it decreased levels of cholesterol and improved insulin sensitivity compared to mice fed ad libitum amount of high-fat diet. Another study by the same group demonstrated that when mice were fed for only 3 hours during the light (inactive) phase for a period of 16 weeks, it increased the amplitude of key metabolic genes (e.g. *Pparα*), decreased the mRNA expression of candidate inflammatory markers (*IL-6*, *TNFα*, *NF-κB*), and decreased cholesterol and triglyceride levels in the serum compared to mice on ad libitum diet. Yasumoto et al. 2016 study demonstrated that when mice are fed a diet rich in fat or sucrose for a period of 8 hours during the light (inactive phase), it dramatically increased

amount of food intake, body weight, levels of triglycerides, cholesterol and free acids in the liver than in mice that were fed the same diet for 8 hours only during the dark (active) phase, thus suggesting that feeding during the wrong phase of the day can lead to obesity and metabolic disorders. The conclusion from these studies is that not only phase of feeding, but the duration of intake of food are important factors to combat obesity and metabolic disorders associated with obesity.

CHAPTER II

MATERIALS AND METHODS

2.1 Details of Experimental Animals

All the animals in research work were used in accordance with the Federal and University guidelines and protocols approved by IACUC and Cleveland State University. C57BL/6J mice used in my study were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and were bred in-house at Cleveland State University. Mice were maintained at Cleveland State University on 12 h light: 12 h dark cycle (LD12:12) with lights turned on at 7am and lights turned off at 7pm. All mice had free access to food and water unless otherwise stated. Animals were maintained in groups of four in cages (Micro-VENT System Caging, # PC7115HT; Overall dimension: 7 3/4"W x 12"D x 6 1/2"H, Allentown, NJ) throughout the experiment. The temperature for animal rooms was maintained at 20 ± 5^0C and humidity between 30-70%. All mice were fed 2018 Teklad global 18% protein rodent diet (Envigo (formerly Harlan), Cat# 2018, Madison, WI). The diet composition by weight: 18.6% protein, 6.2% fat, 44.2% carbohydrate. The diet composition by energy: 24% calories from protein, 18% calories from fat, 58% calories from carbohydrate. Male mice used in the experiments were 12-16 weeks of age.

2.2 Study Design

Prior to the start of the experiment, all mice were fed ad libitum amount of food. When the mice were of 12-16 weeks of age, they were randomly assigned to three feeding groups. One group of mice continued on Ad libitum diet (AL), wherein mice received unlimited amount of food across the day; second group of mice were subjected to Caloric restriction (CR30%). In this group, restriction in the total amount of calories was introduced gradually; 10% reduction in calories was implemented in the first week, followed by 20% reduction in the second week and finally 30% reduction in the third week until the termination of experiment. Third group of mice was assigned to Time restricted feeding (TR). In this group, mice were fed ad libitum amount of food for 12 hours. CR30% group received food once per day as a single meal at zt14 whereas TR group were fed at zt14 and food withdrawn at zt2 on a daily basis. All male mice in this study were group caged and had unlimited access to water. Body weights of mice on all three feeding paradigms were measured once every day at the same time for first ten days from the start of experiment, then switched to once every week for 2 months and or until the termination of experiment. All body weight measurements were performed at the same time every day. Food intake measurements for three feeding groups was performed in the manner as mentioned for body weights.

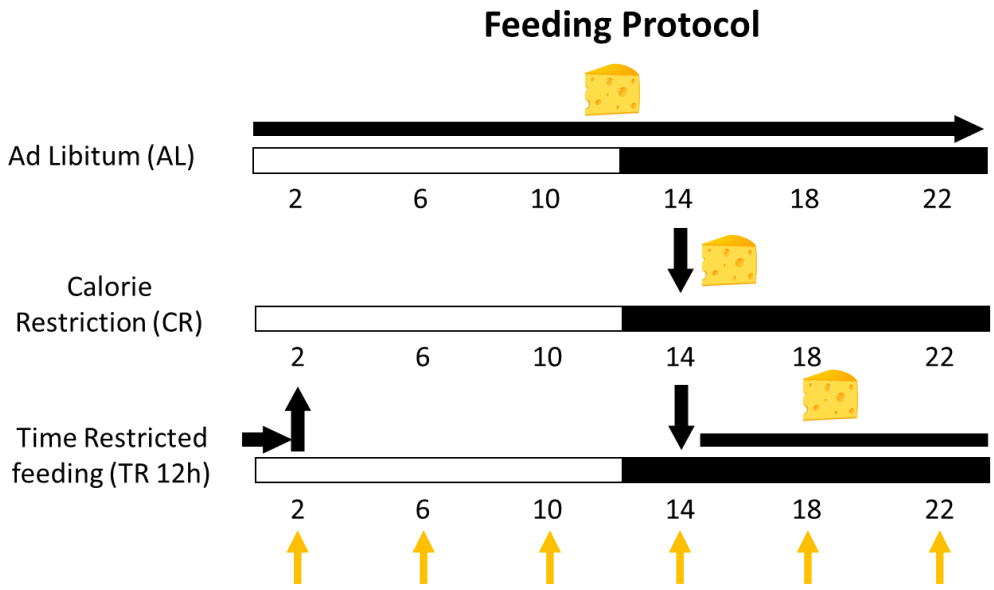

Figure II.1: Overview of feeding protocol

All mice were maintained on 12 hour light: 12 hour dark cycle in standard housing conditions and were provided with access to food and water. AL group had an unlimited access to food; CR group had gradual reduction in calories (10% less than daily average intake for 1st week, 20% less for 2nd week and 30% less starting 3rd week until the end of experiment); TR group received ad libitum access to food for 12 hours. Mice in CR and TR group were fed at zt14 (2 hours after lights were switched off), except for TR group, food was removed at zt2. Mice were euthanized at the end of experiment at times indicated by yellow arrows.

2.3 RNA isolation and processing

After 8 – 10 weeks of subjecting mice to AL, CR30% and TR feeding regimens, mice were euthanized with carbon dioxide gas followed by cervical dislocation. Liver tissues were harvested at times indicated by yellow arrows in Figure II.1. The collected liver tissues were frozen immediately on dry ice and stored at -80^{0}C. Total RNA was

extracted from frozen liver tissue using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Briefly, frozen tissue was mixed with 1ml of TRIzol reagent, homogenized using sonicator, centrifuged at 12,000 rpm for 10 mins. Supernatant from this solution was transferred to new tube and mixed with 200 µl Chloroform, mixed and centrifuged at 11,500 rpm for 15 minutes at 4^0C. The aqueous phase was separated and mixed with 500 µl of Isopropanol, mixed and centrifuged at 14,000 rpm for 10 minutes at 4^0C. The RNA pellet was washed with 1ml of 70% ethanol and centrifuged at 6000 rpm for 10 mins at 4^0C. The RNA pellet obtained after performing this step was air dried and re-suspended in 30 µl RNase-free water. Quantification of RNA was performed using Nanodrop-2000 and quality of total RNA checked by gel electrophoresis.

2.4 Real Time quantitative PCR

After quantification and quality check of total RNA, a reverse transcription reaction was setup to convert RNA to cDNA. For this, a 20 µl reaction mix was setup using 1000ng of RNA, 50ng of 50µM Random Hexamers (N8080127, Invitrogen), 10mM dNTP (DD0058, Biobasic). Then the RNA mix was treated with 200U/µl of Superscript IV Reverse Transcriptase, 5x Buffer and 0.1M DTT. The incubation conditions for reverse transcription are as follows: 65^0C for 5 minutes, followed by ice incubation for 1 minute, 23^0C for 5 minutes, 50^0C for 10 minutes and 80^0C for 10 minutes. Real Time quantitative PCR was performed using iTaq Universal SYBR Green Supermix (1725125, BioRad) on CFX Connect Real Time PCR detection instrument. Thermal cycling conditions were used in accordance with instructions for SYBR Green mix protocol. Values were normalized to 18s rRNA expression levels and measurements

performed in triplicates. Fold change was determined by ΔΔCt method. List of primers and their sequence details are mentioned in Table I.

Table I: Primer sequences for Real Time PCR

Gene	**Forward Primer Sequence (5'-3')**	**Reverse Primer Sequence (5'-3')**
Bmal1	CACTGTCCCAGGCATTCCA	TTCCTCCGCGATCATTCG
Per1	AGGTGGCTTTCGTGTTGG	CAATCGATGGATCTGCTCTGAG
Per2	AGGCACCTCCAACATGCAA	GGATGCCCCGCTTCTAGAC
Rev-erbα	TGGCCTCAGGCTTCCACTATG	CCGTTGCTTCTCTCTCTTGGG
Fmo3	CACCACCATCCAGACAGATTAC	CCTTGAGAAACAGCCATAGGAG
Serpina12	ACCGTGATGATTCTCACAAA	AACATCATGGGTACCTTCAC
Mup4	ACCAAAACCAATCGCTGCCT	GCTGTATCGATCGGAAGAGAGG
Cyp4a12b	CTGATGGACGTTCTTTAC	TCAAACACCTCTGGATT
Gck	CACAATGATCTCCTGCTACT	TTCTGCATCTCCTCCATGTA
Pfk1	AGAGGACCTTTGTTTTGGAG	TCTGCGATGATGATGATGTT
Pcx	GGAGCTAACATCTACCTTCTG	TAGGCTTATACTCCAGACGC

Fbp1	TGACCTGGTGATCAATATGC	CAAAAATGGTTCCGATGGAC
Pck1	TGAGATCTAGGAGAAAGCCA	CCTTGAAGTGGAACCAAAAC
G6pc1	CTAAAGCCTCTGAAACCCAT	ATGACTCAGTTTCCAGCATT
Gk	TCTTGAACCTGAGGATTTGT	TATGGGATACCACTTTCTGGA
18s rRNA	GCTTAATTTGACTCAACACGGGA	AGCTATCAATCTGTCAATCCTGTC

2.5 Pattern of Food intake assessment

Mice were acclimatized for 4 weeks on all three regimens prior to the experiment. Measurements of food intake were performed on a random day simultaneously in all three groups. For AL group, mice were provided with pre-weighed amount of food, whereas CR and TR group received their regular amount of food. Amount of food consumed by each group was measured every hour for 24 hours. Data for amount of food consumed every hour was normalized to daily food intake.

2.6 Glucose, Ketones and Insulin assessment

After 4 weeks on the feeding regimen, blood glucose and ketones (β-hydroxybutyrate) levels were measured in mice on all three groups. Mice were placed in a restrainer and bled through the tail vein nick around the clock at an interval of four hours (zt2, 6, 10, 14, 18 and 22). Blood Glucose was measured using CVS Advanced Health Blood Glucose Meter, while ketone (β-hydroxybutyrate) levels were measured using Precision Xtra Blood Glucose and Ketone meter (Abbott, IL). For blood glucose

and ketone kinetics, measurements were made at one hour intervals between zt12-18. For blood glucose and ketone measurements around the clock and kinetics, mice on all three diets were not fasted prior to the start of the experiment.

To measure plasma insulin levels, mice on three feeding regimens were bled through tail vein at times of collection across the day. Blood was mixed with EDTA (50mM) (anticoagulant) to prevent coagulation, centrifuged at 6300 rpm for 20 minutes at 4^0C. Plasma insulin was measured using commercially available mouse ultra-sensitive ELISA kit (Catalog No 90080; Crystal Chem, Downers Grove, IL). Briefly, 5 µl of plasma for each diet group was added to antibody coated microplate along with mouse insulin standard and incubated for 2 hours at 4^0C. After 2 hours incubation, the plate was washed five times with 300 µl of 1X Wash buffer, then incubated with anti-insulin enzyme conjugate (100 µl per well) for 30 minutes at room temperature. Then, the plate was washed seven times with 1X Wash buffer and later incubated with enzyme substrate solution (100 µl per well) for 40 minutes in dark at room temperature. The enzyme reaction was stopped by addition of 100 µl of stop solution in each well and measured for absorbance reading at 450 nm using Victor3 plate reader (Perkin Elmer, MA, USA). Mice on all three diets were not fasted prior to the start of this experiment. Mice = 4 per time point per diet group.

2.7 Glucose and Insulin Tolerance Tests (GTT and ITT)

After 4 weeks of entrainment to each of the three feeding regimens, mice were subjected to glucose tolerance and insulin tolerance tests. For GTT, mice were fasted for 12 hours and intraperitoneally injected with Glucose (0.4g/kg body weight) and ITT, mice were fasted for 6 hours and intraperitoneally injected with Insulin (0.06U/kg body weight)

respectively. Blood glucose measurements were performed using CVS Advanced Health Glucose meter at times 0, 15, 30, 60, 90 and 120 mins.

2.8 Western Blotting

For protein expression analysis, four mice per time point per diet were used. For total liver lysates preparation, frozen liver pieces were sonicated and homogenated in Cell Signaling Buffer (1M Tris Base pH 7.5, 5M NaCl, 0.5M EGTA, 0.5M EDTA, Triton-X, 0.1M $Na_4P_2O_7$, 1M β-glycerophosphate, 1M Na_3VO_4) containing protease and phosphatase inhibitor cocktails (Sigma). The homogenate mixture was centrifuged at 12000 rpm for 10 mins at 4^0C. Supernatant obtained in this step was used to analyze protein concentration using Bradford method with 1% gamma globulin as standard for determination of protein concentration. After determination of protein concentration, the final lysate mixture was prepared in 2X SDS loading mix (300 mM Tris HCl ph 6.8, 10% SDS, 50% Glycerol, 10% 2-mercaptoethanol and 0.0025% Bromophenol blue) so as to have equal concentration of protein in all lysate mixture. 45 µg of protein was loaded in each well of 4-12% Bis-Tris NUPAGE gels (Thermo Scientific), and electrophoretic run was adjusted to constant voltage at 100V. After the electrophoretic run, proteins were transferred onto PVDF membrane (Thermo Scientific) and a wet transfer was performed at constant amperes at 100 mA for 70 minutes using Transfer Buffer (20% methanol, 3g/L Tris Base and 14.4 g/L Glycine). After wet transfer, the membrane was blocked in 5% Milk prepared in 1X TBST (60.57 g/L Tris Base, 87.66 g/L NaCl, pH adjusted to 7.4 with HCl and finally mixed with 0.1% Tween-20) for 1 hour at room temperature. Then membranes were incubated overnight with primary antibodies with gentle shaking at 4^0C. Next day, membranes were washed three times in 1X TBST for 5 minutes at room

temperature to remove any excess of primary antibodies, then incubated with secondary antibodies at room temperature for 1 hour with gentle shaking. After secondary incubation, membranes were washed three times for 15 minutes with 1X TBST at room temperature to remove excess secondary antibodies. Developing of blot image was performed using Clarity ECL Substrate (BioRad), and Odyssey FC Imaging system (Li-Cor). β-actin was used as internal control for this experiment. Quantification of protein bands done using Image Studio Lite software (Version 5.2). List of antibodies used for western blot are mentioned in Table II

Table II: List of antibodies used for immunoblot analysis

List of Antibodies	Company	Catalog Number
Phospho-S6 Ribosomal Protein (Ser235/236)	Cell Signaling Technology	Cat# 4858S
Ribosomal Protein S6 Antibody (C-8)	Santa Cruz	Cat# sc-74459
β-actin Monoclonal Antibody	Sigma	Cat# A5441
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7074; RRID: AB_2099233
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7076; RRID: AB_330924

2.9 Statistical Analysis

Data for all three groups was analyzed using ordinary Two-way ANOVA (multiple comparison correction was done using Bonferroni method). For GTT, ITT, percent of food consumed during Dark phase and First meal experiments, analysis was performed using ordinary One-way ANOVA (Bonferroni correction for multiple comparison). All statistical analysis was performed using GraphPad Prism 7.0 (San Diego, CA). Data in all figures is denoted as Mean ± SD with *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$. Letters indicate significant effect of diets ($p \leq 0.05$); a – AL vs CR, b – AL vs TR and c – CR vs TR.

2.10 Interpretation of results

It is a known that implementation of caloric restriction results in health benefits and extension of lifespan. Caloric restriction involves two aspects; one, reduction in caloric intake, and two, periodic fasting. Time restricted feeding is a form of periodic fasting regimen. If Time restricted feeding demonstrated significant effect (denoted by letters of significance a,b), then we concluded that periodic fasting is the major contributor to metabolic changes on CR; if not, then the major contributor was reduced calories (denoted by letters of significance a,c). In case of letters of significance (b,c) or (a,b,c), we concluded that both periodic fasting and reduced calories contributed to metabolic benefits of CR.

CHAPTER III

RESULTS

3.1 Time restricted feeding did not change body weight and food intake

To assess the effect of time restricted feeding on physical parameters in mice, we measured their body weight and food intake for first ten days from the start of the experiment and then continued to measure both parameters once every week until the end of the experiment. Prior to dividing mice into three feeding groups, we measured their food intake. Food intake in all mice prior to start of experiment was found to be 3.4 ± 0.3 grams. For the first ten days, we observed no changes in food intake for mice in AL group. CR group received fixed amount of food: 10% reduction in amount of food in the first 7 days, followed by 20% reduction for the next 3 days. Mice in TR group consumed 50% less food on day 1, approximately 250-300% on days 3-4 and then finally return to 100% amount of food, similar to AL group for remainder of the days (Figure 1B). The reduced intake of food on day 1 by TR group may be attributed to the fact that prior to the start of the experiment, mice had a tendency to eat their food around the clock. When this group of mice were exposed to new TR regimen, they did not anticipate that the food would be taken away from them after a period of 12 hours. Hence, within couple days, TR mice learn that they will receive food for only 12 hours during the day. For the rest of

the experiment, amount of food consumed remained the same between AL and TR groups; CR mice on the other hand receive 30% less amount of food starting week 3 until the termination of the experiment (Figure 1A). Similar to food intake, we measured body weight of mice in all three groups for the first ten days and then once every week until the end of the experiment. We observed significant reduction in body weight in mice on CR group, which is evident due to reduced intake of food; however we did not observe any difference in body weight between AL and TR groups during the first ten days (Figure 1D). For the rest of the experiment, we found that both AL and TR groups gained some weight, and by the end of the experiment, both groups had a 10% increase in body weight, compared to their original weights. On the other hand, mice in the CR group weighted 20% less than their original weights (Figure 1C). Thus from this experiment, we concluded that time restricted feeding (TR) did not affect body weight nor food intake. Hence we decided to use TR as a model to study the contribution of periodic fasting to metabolic benefits of CR.

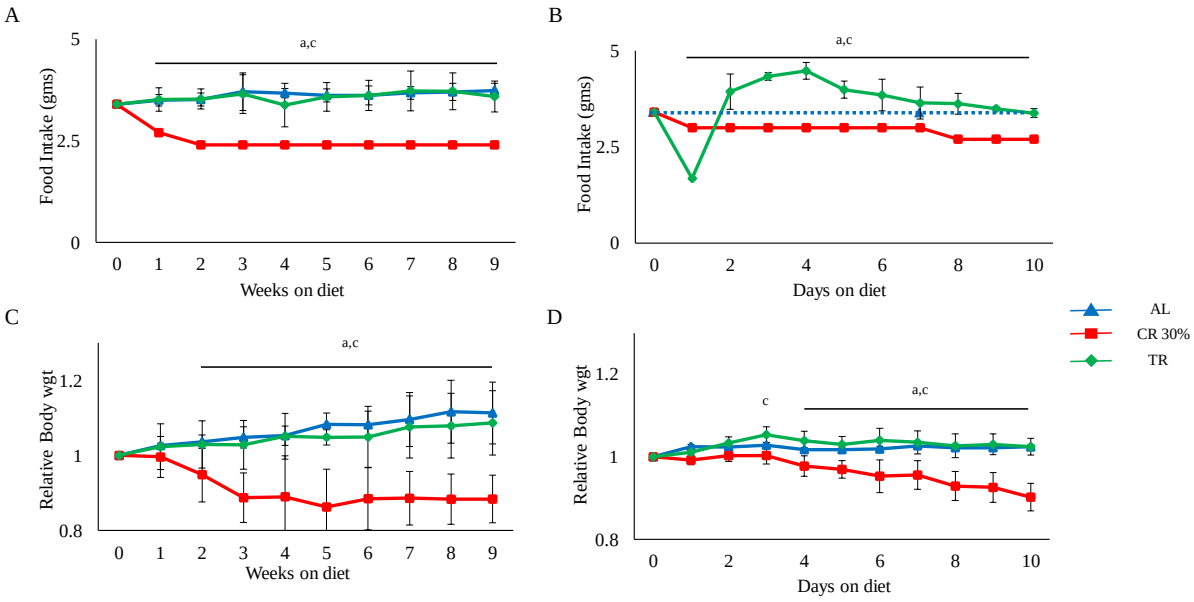

Figure III.1: Food intake and Body weight does not change in mice fed TR diet

Weekly average intake of food (A) and relative body weight (C) was measured in mice subjected to AL, CR and TR diets for the entire course of experiment. The measurements were made at the same time, once a week. Daily average intake of food (B) and body weight (D) for mice on CR and TR was measured for the first ten days from the start of the experiment. Measurements for these experiments were made at the same time, once every day for ten days. For mice in AL group, food intake was not measured for first ten days, hence in (B) AL group is represented as dotted blue line. Body weight measurements were performed as mentioned above. AL - blue dotted/solid line, blue triangles; TR -green solid line, green solid diamonds; CR - red solid line, red squares. Letters signify significant effect of the diets; a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ considered statistically significant.

3.2 Daily patterns of food intake of mice on AL, CR and TR feeding regimens

After measuring food intake, we decided to measure the pattern of food intake in these mice. We know for a fact that in AL group, mice receive an unlimited amount of

food for unlimited period of time, in CR group, mice receive restricted amount of food (in the form of calories) without any time constraints, whereas in TR group, mice receive an unlimited amount of food for a short period of time. With these manipulations in the amount of food and time window, we believed that it might affect the daily pattern of food intake in these mice, which in turn might affect physiology of the mice. Hence we measured pattern of food intake in mice subjected to three different dietary regimens (Figure 2A). Mice in AL group consumed about 70% of their food during the dark (active) phase and remaining 30% during the light (inactive) phase (Figure 2B), which is in agreement with previously published data (Ellacott et al. 2010). In the dark phase, AL mice consume their food in the form of 2 meals. The first major meal is consumed between zt13-18, and contributes to approximately 40% (1.54 grams) of their daily intake of food. Second meal during the dark phase is consumed between zt22-24. Mice in TR group were entrained to have access to food for only 12 hours, they consumed approximately 90% of their food during the dark phase; however, in the dark phase, they too consumed food in the form of 2 meals; first major meal was had between zt14-17 and constituted approximately 60% (1.79 grams) of their daily intake of food, second meal was consumed between zt22-24. Mice on CR group consumed all their food (2.4 grams) between zt14-16 (Figures 2B – D); this is in accordance with two previously published reports (Acosta-Rodríguez et al. 2017; Mitchell et al. 2019). Therefore from this experiment we concluded that, mice on all three diets consume their first major meal at around the same time (between zt14 and zt17).

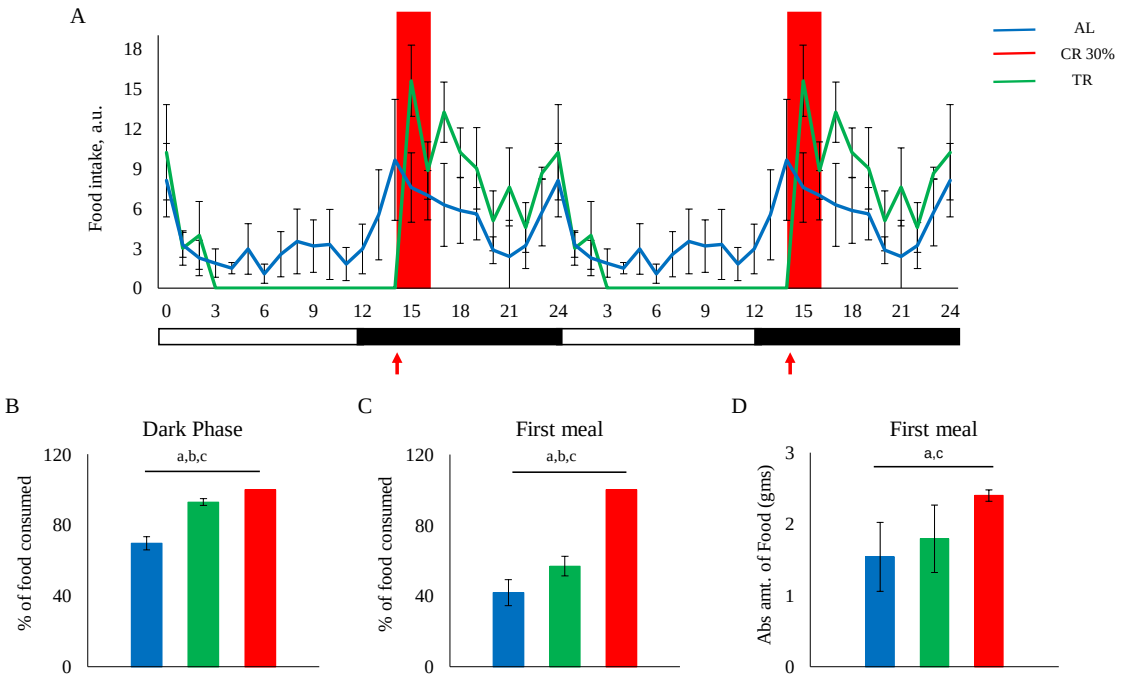

Figure III.2: Effect of diets on daily pattern of food intake

Measurements of pattern of food intake in mice on AL, CR and TR were made at one hour intervals (A) for a period of 24 hours. Normalization of data for every hour was performed by dividing the individual value with daily food intake. Total daily intake of food was considered as 1.0. For illustration purpose, data in (A) is double plotted. Time when food was provided for CR and TR is indicated by red arrow. AL – blue solid line, TR – green solid line and CR – red rectangular box. (B) Represents percent of food eaten by mice on three diets, only during the dark phase (zt12-24). (C) Represents percentage of food eaten by AL, CR and TR mice during their first meal in the dark phase. (D) Represents actual amount of food (in grams) eaten by mice on all 3 diet groups during the first meal in the dark phase. AL – blue line and bar, TR – green line and bar and CR – red bar. Letters signify significant effect of the diets; a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ considered statistically significant

3.3 Reduced calories and periodic fasting do not disturb the mechanism of circadian clock in the liver

Besides light, food is another zeitgeber (external cue) that has the ability to entrain circadian clock. When food is presented in unlimited amounts, central clock (suprachiasmatic nucleus) can entrain the peripheral clocks (such as liver) and synchronize peripheral rhythms in physiology and metabolism. However when food is presented in a limited time frame, peripheral clocks uncouple from central clock and produce their own rhythms. When mice are fed CR in the dark phase, CR does not affect the phase of expression of circadian clock genes in liver, however it does affect the amplitude of gene expression (Mendoza 2005; Patel, Velingkaar, et al. 2016). TR has also been demonstrated to affect the amplitude of clock genes when mice are fed a high-fat diet, but also has the ability to affect the phase of expression if restricted feeding is performed in light (inactive) phase (Damiola et al. 2000; Hara et al. 2001; Satoh et al. 2005). We wanted to investigate whether TR (which represents periodic fasting component of CR) in our experiment affected the phase of circadian clock genes in the liver, hence we measured mRNA expression of several clock genes. Results for *Bmal1*, *Per1*, *Per2* and *Rev-erbα* are depicted in Figure 3A-D. In accordance with previously published reports, CR did cause change in phase of expression of circadian clock genes. In TR group, we observed that there was no change in phase of expression of *Per2*, whereas there was a small 2-4 hour shift in phase of expression of *Bmal1*, *Per1* and *Rev-erbα*. Since we did not observe any major shift in phase of expression of clock genes in the liver, we concluded that implementation of dietary restrictions did not disrupt the mechanism of circadian clock in the liver of mice. In case of CR group mice, we

observed a significant increase in gene expression of *Per1*, *Per2* and *Bmal1*. Similar to CR, TR too demonstrated upregulation of *Per2* between zt14 – 22, *Bmal1* at zt22, and *Rev-erbα* between zt10-18, and this magnitude of expression was similar to CR. However, there was no effect observed on *Per1* expression. Thus from this experiment, we concluded that both reduction in caloric intake and periodic fasting contributed to CR induced changes observed in *Bmal1* and *Rev-erbα* gene expression, periodic fasting contributed to *Per2* expression, reduction in caloric intake contributed to *Per1* mRNA expression, and all the observed effects were time of the day dependent.

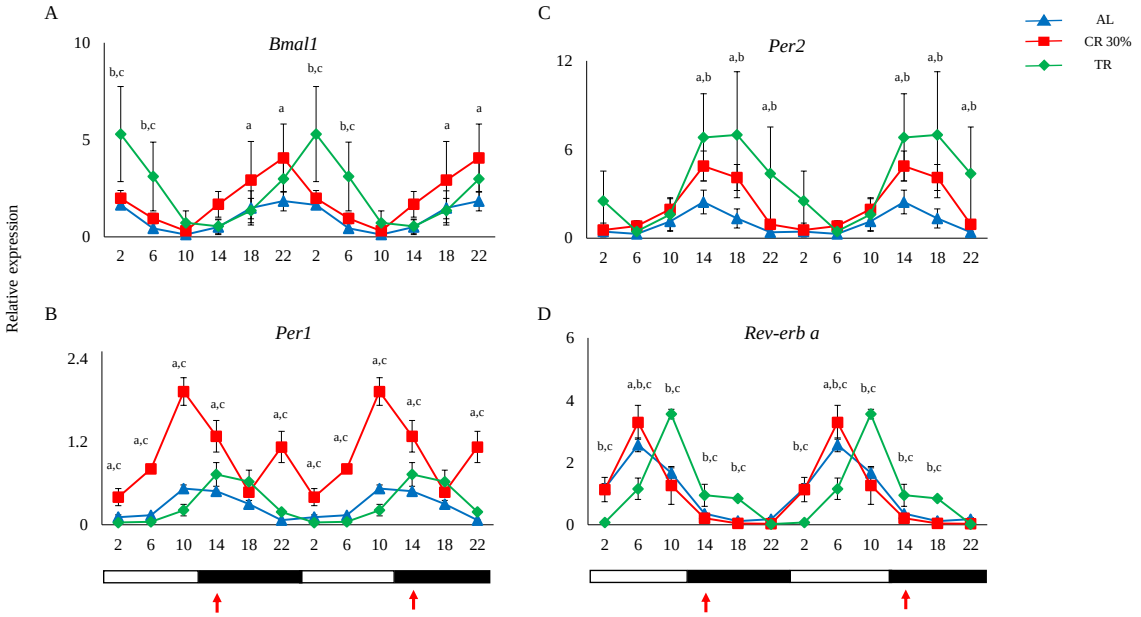

Figure III.3: Effect of reduced calories and periodic fasting on circadian clock genes mRNA expression.

mRNA expression of circadian clock genes *Bmal1* (A), *Per1* (B), *Per2* (C) and *Rev-erb α* was measured in the liver of mice on AL, CR and TR diets. 18s rRNA was used as internal control. Time when food was provided to CR and TR groups is indicated by red arrow. White and black bars indicate day and night phases of the day. Data is double plotted to illustrate circadian rhythmicity of genes. AL – blue solid line, blue triangles;

TR – green solid line, green diamonds and CR – red solid line, red squares. Letters indicate significant effect of the diets; a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ considered statistically significant

3.4 Reduction in caloric intake and periodic fasting have differential effects on mRNA expression of candidate markers of CR

Swindell 2007 study demonstrated that there were several genes whose expression are changed universally by CR not only in the liver but also in other tissues in several mice models, thus these genes could potentially serve as molecular markers for CR. In our study, we assayed the mRNA expression of four candidate genes in AL, CR and TR groups: *Fmo3*, *Mup4*, *Serpina12* and *Cyp4a12b* (Figures 4A – D). In CR group we observed 100,000 fold induction of *Fmo3* mRNA expression whereas in case of *Mup4*, *Serpina12* and *Cyp4a12b*, mRNA expression was decreased dramatically. Since the mRNA expression of 4 genes was up and down-regulated across the day at multiple time points, therefore we can say that these 4 genes serve as molecular markers for CR. However, mRNA expression of these 4 genes was different in TR group compared to CR group, for instance, *Fmo3*, there was a moderate induction in mRNA expression upon implementation of TR, the fold of induction varying between 2 fold to 100 fold across the day, versus CR wherein the fold of induction was 100,000 fold. In case of *Serpina12* and *Cyp4a12b*, we could not observe any difference in mRNA expression between AL and TR groups. On the other hand, TR induced mRNA expression of *Mup4* at majority of time points when compared to CR. Therefore we concluded from this experiment that expression of *Fmo3*, *Serpina12* and *Cyp4a12b* is regulated by reduced in caloric intake, whereas *Mup4* is regulated by reduced calories and periodic fasting components of CR.

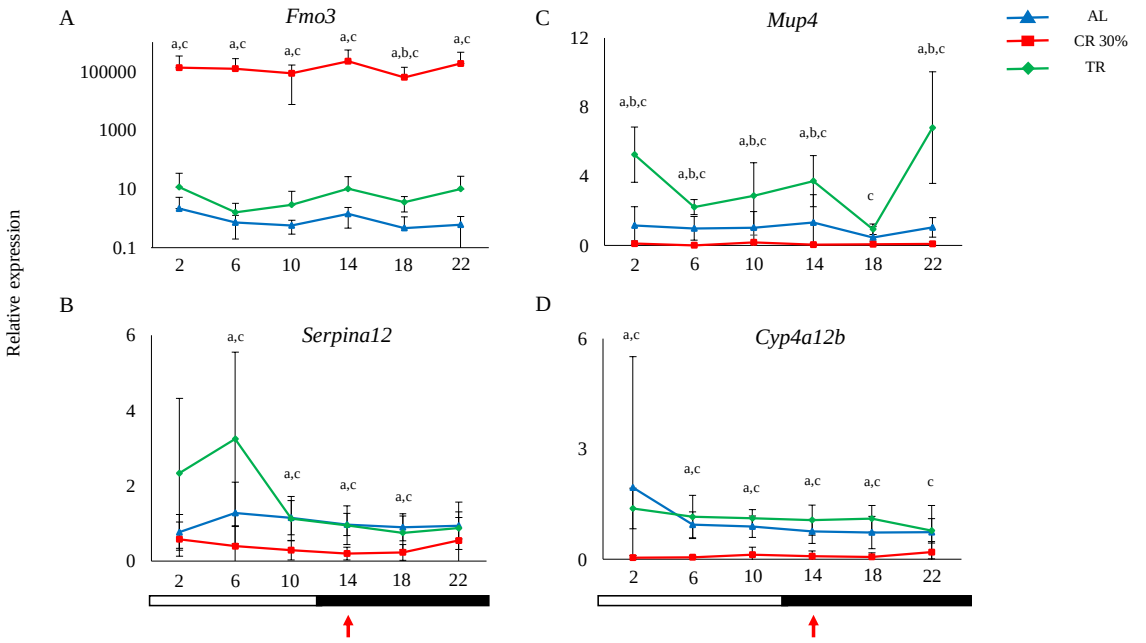

Figure III.4: Effect of reduced calories and periodic fasting on molecular markers of CR mRNA expression

Fmo3 (A), *Serpina12* (B), *Mup4* (C) and *Cyp4a12b* (D) mRNA expression was measured in the liver of mice on AL, CR and TR diets at six time points across the day. White and black bars indicate light and dark phases of the day. Time when CR and TR mice were fed is indicated by red arrow. Letters indicate significant effect of the diets; a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ considered statistically significant. AL – blue solid line, blue triangles, TR – green solid line, green diamonds and CR – red solid line, red squares.

3.5 Differential effects of caloric reduction and periodic fasting on blood glucose and ketones

It has been demonstrated that CR improves glucose homeostasis (McCarter et al. 2007; Mitchell et al. 2016; Pires et al. 2014), we wanted to investigate what would be the effect of TR on blood glucose compared to CR, and hence we measured blood glucose

from tail vein of mice at six time points across the day. Mice on all three diets were not fasted prior to the start of this experiment. As expected, blood glucose levels were reduced upon CR at all six time points across the day. In contrast to CR, TR group showed only moderate reduction in blood glucose levels at time points zt2 and zt6, however blood glucose levels were still higher compared to CR group. Between time points zt10-22, there was no difference between AL and TR groups (Figure 5A). The most surprising observation was that blood glucose levels did not show huge peak after feeding at zt14 neither in CR nor TR group, the reason may have been due to 4 hour intervals between each blood glucose measurement. We have also known from daily feeding pattern experiment, that mice in all three groups have their first major meal between zt13-17, hence we decided to measure blood glucose levels at increased resolution i.e. between zt12-16 with one hour resolution between each time point (Figure 5B). The rationale for using one hour resolution for measurement of blood glucose kinetics, was that we expected to see a huge peak in blood glucose after meal presentation. Mice on CR group demonstrated an increase in blood glucose levels between zt15-16, however this increase was very modest and quickly returned to normal levels at zt18. On the other hand, AL and TR groups did not show any increase in blood glucose. Thus from this experiment using increased resolution of time points for blood glucose, we concluded that, one, blood glucose uptake by the tissues upon feeding was a strong and very fast response, and two, blood glucose levels were tightly controlled at all times across the day in mice subjected to all three diets.

Besides glucose, ketones are other source of energy for tissues such as brain, heart and skeletal muscle. Liver is the main organ that produces ketones during periods of

prolonged starvation. We believe that increase in the levels of blood ketones may contribute to CR induced metabolic changes (Paoli et al. 2014; Tieu et al. 2003; Veech et al. 2017). We measured blood ketones across the day in mice on all three diets. Lower levels of blood ketones were observed in mice on AL group across the day (Figure 5C). Despite eating lesser amount of food during the light (inactive) phase, we did not observe any increase in blood ketones in AL mice. This suggests that eating less yet continuously, is more than sufficient to prevent activation of ketogenesis in mice on AL group. In case of mice on CR group, levels of blood ketones were relatively high at most time points, with a significant peak at zt14. In case of TR, the induction of blood ketones at zt14 was modest compared to CR, however the ketone levels were similar to AL group at most time points across the day. Similar to blood glucose experiment, we decided to measure blood ketones at increased resolution i.e. between times zt12-18 at one hour resolution (Figure 5D). This increased resolution in time points was able to highlight the difference in kinetics of blood ketones observed between CR and TR groups. In case of CR group, blood ketones were elevated at times zt13-14, whereas in case of TR group, ketones were elevated at times zt12-14 and ketone levels in TR group at zt12 were higher compared to CR group. We also observed that one hour after feeding, blood ketone levels in both CR and TR group returned to levels similar to AL group. Therefore from this experiment, we concluded that reduced calories are the major regulators of CR mediated changes in blood ketones, with minor role of periodic fasting. The difference in levels of ketone induction at zt14 could be explained by duration of fasting; mice on CR group experienced a fasting period of 22 hours, whereas mice on TR group were fasted for 12 hours; however, the difference in blood ketone kinetics cannot be explained by duration

of fasting; at zt12, CR mice experienced a fasting period of 20 hours, whereas TR mice were fasted for 10 hours, yet ketones levels were higher in TR group compared to CR group at zt12.

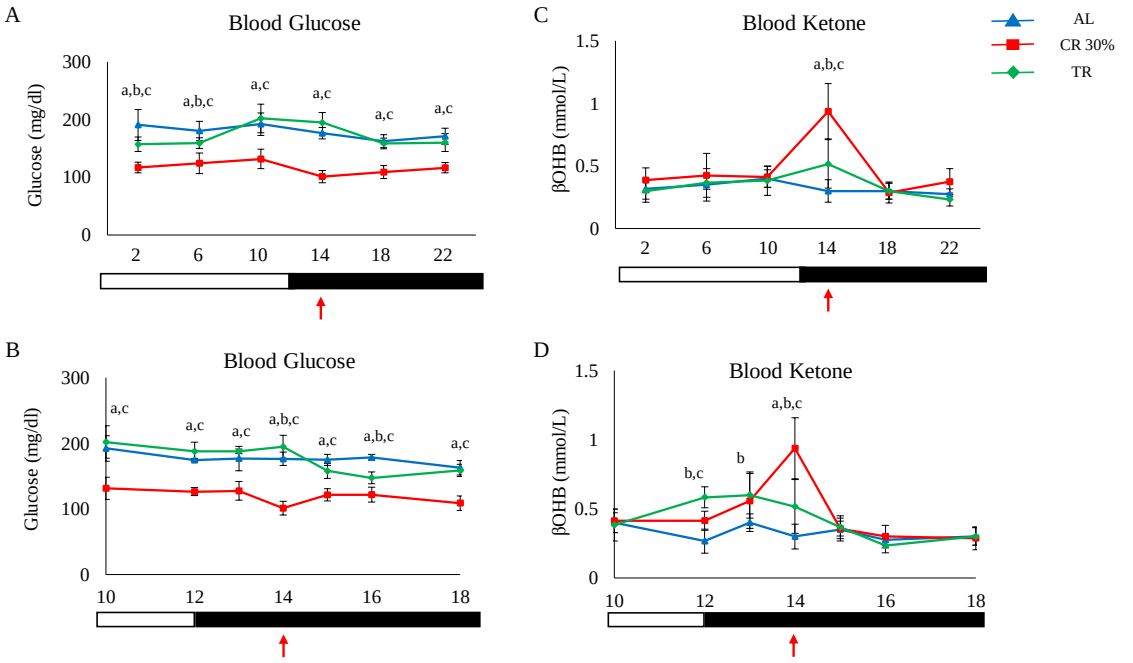

Figure III.5: Blood glucose and ketones are differentially regulated by caloric reduction and periodic fasting

Measurement of glucose (A and B) and ketones (C and D) was performed on blood of mice on AL, CR and TR diets at either 4 hour resolution (A and C) or 1 hour resolution (B and D). Mice were not fasted for this experiment prior to blood collection. AL – blue solid lines, blue triangles; TR – green solid lines, green diamonds, CR – red solid lines, red squares. Letters indicate statistical significant effect of the diets; a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ considered statistically significant.

3.6 Rhythms of blood glucose and ketones are entrained on CR compared to Spontaneous fasting

Increase in blood ketones that we observed upon CR was in accordance with previous studies (Lin et al. 2015; Meidenbauer, Ta & Seyfried 2014), however this increase in ketones was within a narrow time frame, thus indicating that ketone levels are regulated by CR. CR is a form of entrained periodic feeding and hence to dissect the effects of entrained versus non-entrained, we measured blood glucose and ketones in mice subjected to spontaneous acute fasting. Since CR mice consume all their food within 2 hours (zt14-16), we started spontaneous fasting (SF) at zt16 and measured blood glucose and ketones for 24 hours. We noticed that blood glucose levels in SF mice were similar to mice on CR group after 18 hours of fasting, however blood glucose curve at other time points were different for both SF and CR; in case of SF, we see a gradual decrease in blood glucose, as a consequence of duration of fasting, on the other hand, CR group had nearly constant levels of blood glucose, and these levels showed a tendency to increase between times 2 and 18 hours of fasting. In case of blood ketones, we observed a statistically significant increase in blood ketone levels in SF group after 6 hours of fasting, and ketone levels had a tendency to stay higher at remaining time points compared to CR group (Figure 6A and 6B). Thus from this experiment we concluded that changes observed in the levels of blood glucose and ketones in CR group are distinct from non-entrained SF group, thus indicating that levels of blood glucose and ketones are subject to entrainment as a consequence of metabolic adaptation to caloric restriction.

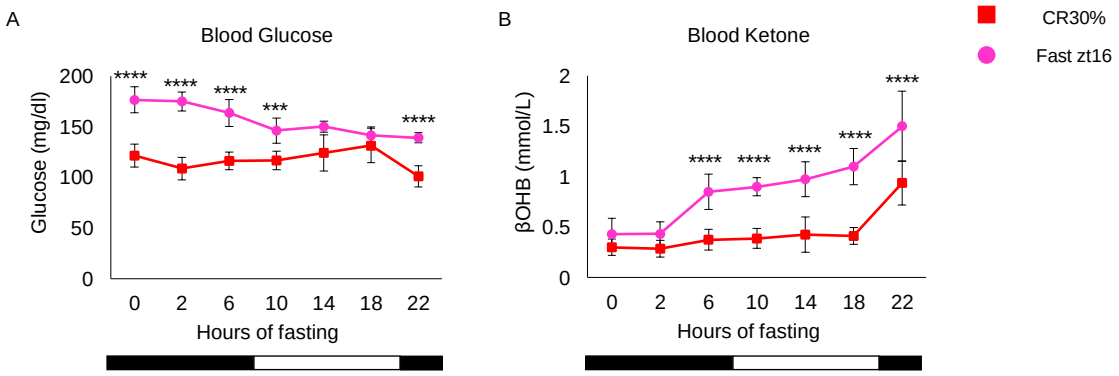

Figure III.6: Blood glucose and ketones daily rhythms are entrained on caloric restriction as opposed to acute spontaneous fasting

Measurement of blood glucose (A) and blood ketones (B) in mice on CR and Spontaneous Fasting (SF). Fasting for SF group was started at zt16 and measured at 4 hour resolution for a period of 24 hours. CR – red solid lines, red squares and SP – pink solid lines, pink circles. Asterisks indicate statistical significant effect of the diets; * - $P \leq 0.05$, ** - $P \leq 0.01$, *** - $P \leq 0.001$ and **** - $P \leq 0.0001$.

3.7 Plasma insulin, Insulin tolerance and Glucose tolerance are differentially regulated by reduced caloric intake and periodic fasting

One of the key hormones involved in regulation of glucose homeostasis is Insulin. High levels of insulin in the blood can lead to development of insulin resistance and contributes to metabolic disorders and aging. Previously it was demonstrated that implementation of CR leads to reduction in the levels of plasma insulin (Larson-Meyer et al. 2006; Masoro et al. 1992; McCarter et al. 2007). To assay the effect of TR on insulin, we measured plasma insulin at six time points across the day in mice on all three diets (Figure 7A). In AL group, levels of plasma insulin were high at all times across the day. In CR and TR group, levels of insulin were higher during the active (dark) phase, which is in agreement with the pattern of food intake in mice. During the inactive (light) phase,

between times zt2-14, insulin levels in mice on CR and TR were not different from each other when compared to mice on AL group. These low levels of insulin during the light phase are expected since mice on CR and TR do not eat food during this phase. However, after feeding, levels of plasma insulin increased in both CR and TR groups with different magnitudes of amplification. In case of CR group, plasma insulin levels increased to 25-fold at zt15 and 15-fold at zt16, whereas in TR group the level of increase was moderate compared to CR group (8-fold increase at zt15 and 3-fold increase at zt16), but, induction of insulin levels in both CR and TR groups is in agreement with the pattern of food intake. Therefore, from this experiment we concluded that time-of-the-day dependent changes that occur in plasma insulin are regulated by both reduction in caloric intake and periodic fasting components of caloric restriction.

Reduction of plasma insulin under CR contributes to improved insulin sensitivity as reported by previous studies (Kalant, Stewart & Kaplan 1988; Masoro et al. 1992; Wang et al. 1997). Therefore we performed intraperitoneal Insulin tolerance test (ip-ITT) on mice on all three diets to gauge the response of TR to exogenous supply of insulin (Figures 7B). In accordance with previously published reports, CR mice had the highest sensitivity to insulin, TR mice too demonstrated improved sensitivity to insulin, and however, the insulin sensitivity was less than CR but better than AL group. The improvement in insulin sensitivity observed in both CR and TR groups is in agreement with reduction in the levels of plasma insulin. We also performed intraperitoneal Glucose tolerance test in mice on all three diets (Figures 7C). CR group demonstrated improved glucose tolerance, which is in agreement with reduced levels of blood glucose, however, we did not observe any difference in glucose tolerance between AL and TR groups.

Therefore, from this experiment we concluded that reduced intake of calories contributed to improved glucose sensitivity, whereas both periodic fasting and reduced caloric intake contributed to improved insulin sensitivity.

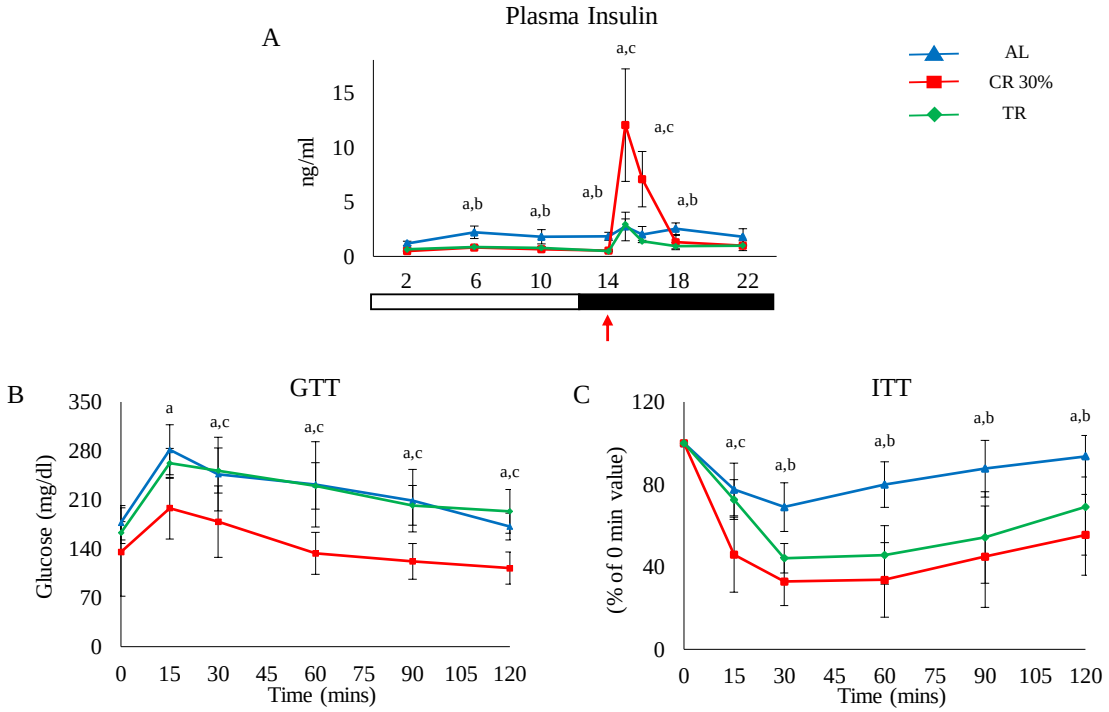

Figure III.7: Distinct effects of reduced calories and periodic fasting on plasma insulin, glucose and insulin sensitivity

(A) Plasma insulin measurements were made in mice on all three diets at time points across the day; intraperitoneal glucose tolerance test (B) and intraperitoneal insulin tolerance test (C) was performed by injecting glucose (0.4 g/kg body weight) and insulin (0.06 U/kg body weight) respectively in mice on all three diets. Both GTT and ITT were performed at zt14 and measured at 15,30,60,90 and 120 minutes. AL - blue solid line, blue triangles; TR -green solid line, green solid diamonds; CR - red solid line, red squares. Letters signify statistical significance between the diets: a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ was considered statistical significance.

3.8 Glucose metabolic gene expression is regulated by caloric reduction and partially by periodic fasting

Glucose is an important source of energy for the cell and regulation of glucose homeostasis is necessary not only to meet the energy demands of the cell but also maintain health of individuals. Liver is a major biochemical organ that plays an important role in maintaining glucose homeostasis by regulating number of glucose metabolic pathways, of which two were investigated in this study, namely, glycolysis and gluconeogenesis. Glycolysis and gluconeogenesis share many steps due to same enzymes catalyzing reactions in both pathways, hence they are reversible reactions. However, there are few enzymes that are unique to both pathways that catalyze irreversible steps and hence termed as regulatory enzymes. In order to understand the mechanism of how glucose homeostasis is improved under CR and TR groups, we measured mRNA expression of few regulatory enzymes at six times across the day in the mouse liver: glycolytic enzymes namely, Glucokinase (*Gck*) and Phosphofructokinase 1 (*Pfk1*); gluconeogenic enzymes namely, Pyruvate carboxylase (*Pcx*), Phosphoenol pyruvate carboxykinase 1 (*Pck1*), Fructose 1,6-bisphosphatase 1 (*Fbp1*) and Glucose 6-phosphatase (*G6pc*). In case of both CR and TR groups, *Gck* mRNA expression was induced at zt18 i.e. 4 hours after feeding. *Pfk1* mRNA expression was induced at all time points across the day on CR but not TR (Figure 8E and 8F). In case of *Pcx* and *Fbp1*, we could not observe any rhythms, but the mRNA expressions for both enzymes were induced at all times across the day on CR diet but not on TR diet. The mRNA expression for *Pck1* was rhythmic in all 3 diet groups, with peak at zt10 in AL and peak at zt14 in CR and TR group. Also the magnitude of induction of *Pck1* mRNA was different in both

CR and TR (about 5-10 fold increase observed at time points zt6, 10 and 14 in CR versus moderate induction of 2.5-fold in case of TR group). mRNA expression of *G6pc* was arrhythmic in both AL and TR groups whereas the expression was rhythmic under CR diet with a peak at zt14 (Figure 8A – D). We also measured the mRNA expression of glycerol kinase (*Gk*), which plays an important role in glycerol metabolism and it was previously demonstrated how CR affects glycerol metabolism (Hagopian, Ramsey & Weindruch 2008). mRNA expression of *Gk* was arrhythmic but induced at all times across the day in CR group, whereas there was no difference in the mRNA expression between AL and TR groups (Figure 8G). Therefore, from this experiment, we concluded that mRNA expressions of majority of glucose metabolism genes were regulated by reduced caloric intake (*Pcx, Fbp1, G6pc, Gk and Pfk1*); *Gck* mRNA expression was regulated by periodic fasting whereas *Pck1* mRNA was regulated by both periodic fasting and reduced caloric intake. The summary of results for mRNA expression of glucose metabolism genes is presented in the figure below.

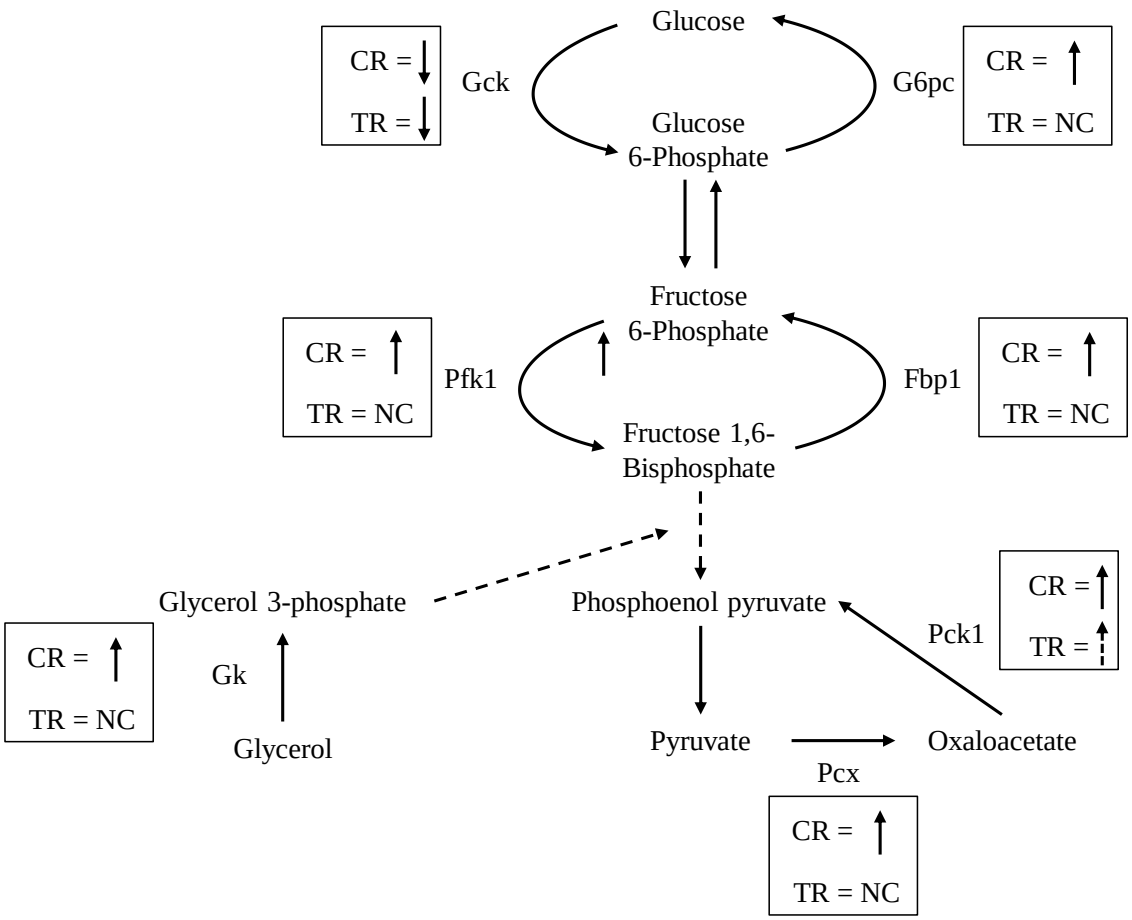

Figure III.8a: Schematic representation of the effect of CR and TR on mRNA expression of key glucose metabolism genes.

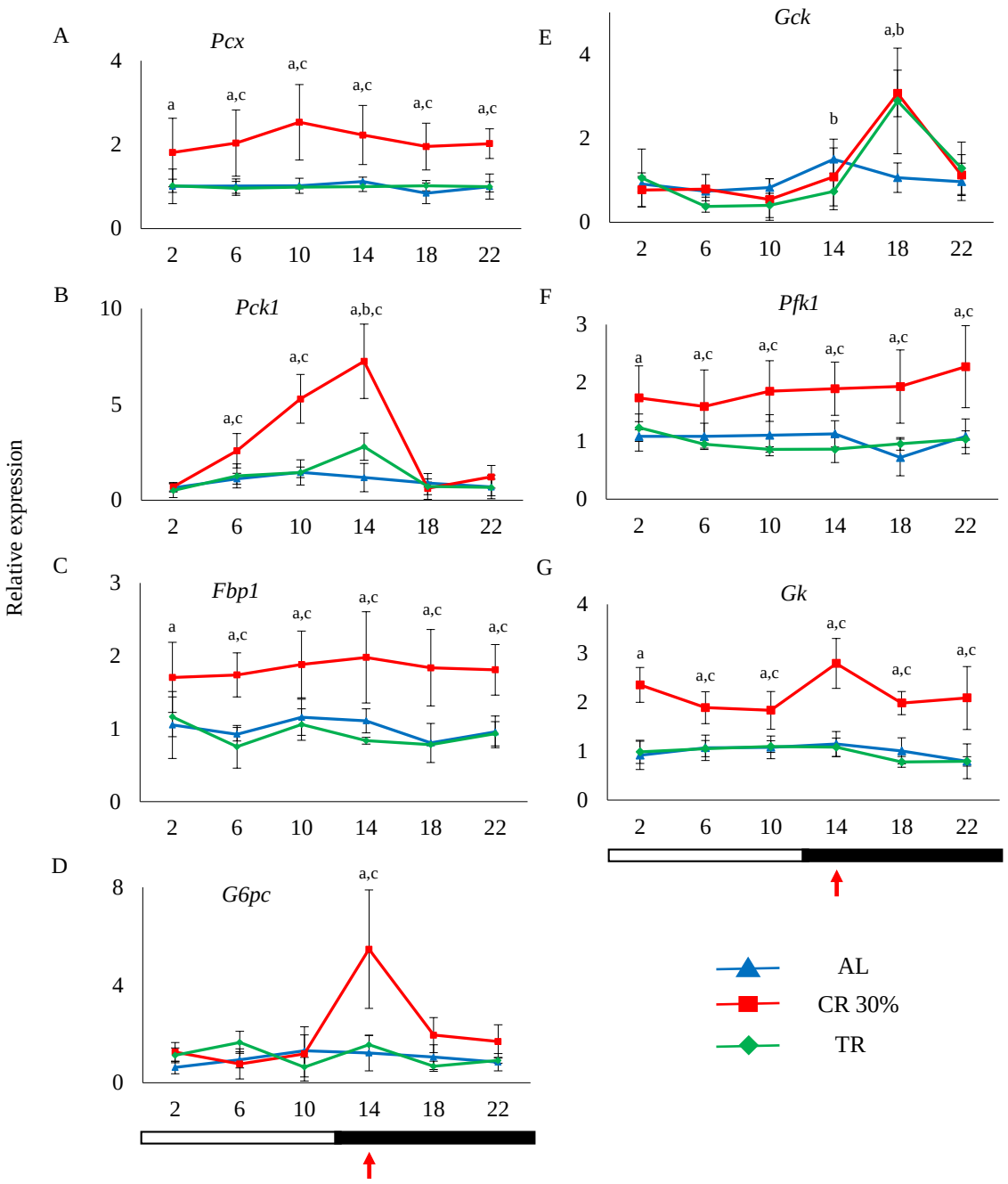

Figure III.8b: Reduced caloric intake contributes to regulation of gluconeogenesis and glycerol metabolism whereas effect on glycolysis is gene specific

mRNA expression of key regulatory enzymes in the process of gluconeogenesis: *Pcx* (A), *Pck1* (B), *Fbp1* (C) and *G6pc* (D); key enzymes in glycolysis: *Gck* (E) and *Pfk1* (F) and glycerol metabolism: *Gk* (G), assayed in the liver of mice on AL, CR and TR. AL – blue

solid line, blue triangles, TR – green solid line, green diamonds and CR – red solid line, red squares. Letters signify statistical significance between the diets: a – AL vs CR, b – AL vs TR and c – CR vs TR. $p \leq 0.05$ was considered statistical significance.

3.9 Periodic fasting regulates mTORC1 activity

Mammalian target of rapamycin (mTOR) signaling pathway has important implications in the process of aging and in caloric restriction. CR has been demonstrated in several studies to decrease the activity of mTORC1 in a number of organisms (Blagosklonny 2010; Kaeberlein et al. 2005; Kapahi et al. 2010; Powers et al. 2006), and we showed that this effect is time-of-the-day dependent in mammals (Tulsian, Velingkaar & Kondratov 2018). Since insulin is one of the major upstream regulators of mTORC1 and we demonstrated earlier the effect of dietary restrictions on levels of plasma insulin across the day, therefore we decided to measure mTORC1 activity in all three diets. We assayed phosphorylation of ribosomal S6 (Serine 235/236), which is commonly used as a candidate marker of mTORC1 activity (Figure 9A and 9B). Phosphorylation of S6 was found to be rhythmic under all three diets; with expression being higher during the active (dark) phase, as opposed to low levels observed during inactive (light) phase. The peaks for phosphorylated S6 were observed at zt14 in AL group and zt18 in CR and TR groups. Therefore, it can be concluded that all three diets did not have any significant effect on the phase of expression of phosphorylated S6. During the light phase, phosphorylation of S6 was higher in AL group compared to CR and TR groups, the probable reason being that mice in AL group still had access to and were eating low amounts of food, whereas in case of CR and TR, mice were fasting. Higher mTORC1 activity was observed at zt18, which coincides with the time when AL and TR groups finish their first major meal,

whereas in case of CR, mice consume the entire food by this time. However, the kinetics of S6 peaks were different in three diets; in CR group, the peak was observed at only zt18, whereas in AL and TR groups, a broader peak from zt14-22 was observed for S6, which is in good agreement with plasma insulin levels. The increase in mTORC1 activity at zt14 observed in AL group is probably due to early feeding habits of mice at zt12-13, however mice on TR diet did not receive food at zt14 prior to collection of liver tissue, yet mTORC1 activity is high, thus suggesting a probable role of mTORC1 in food anticipation in the liver. On the other hand, mice on CR diet too, did not receive food at zt14 prior to collection of liver tissue, but we do not observe increase in mTORC1 activity at this time. Thus, we concluded from this experiment that decrease in mTORC1 activity during the light phase is due to periodic fasting, however reduction in caloric intake contributes to differential kinetics of mTORC1 activity.

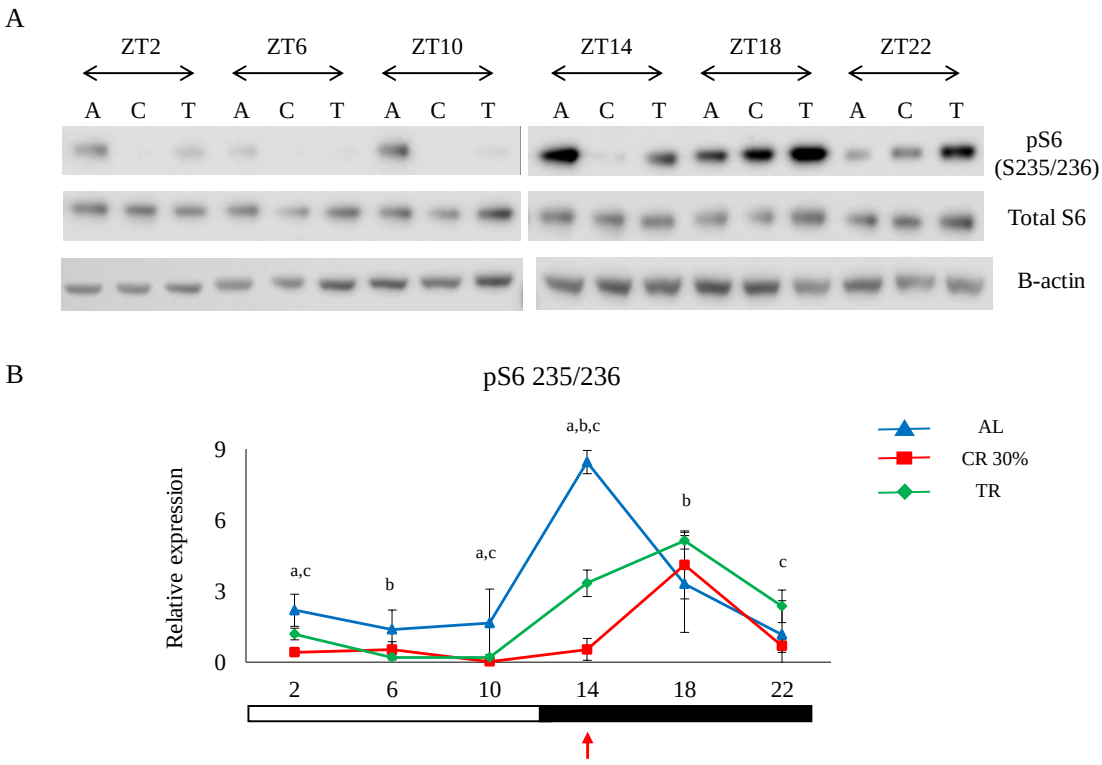

Figure III.9: Circadian rhythms in mTORC1 activity are regulated by periodic fasting.

mTORC1 activity was assayed in the liver of mice subjected to AL, CR and TR diets using ribosomal protein S6 phosphorylated on Serine 235/236. (A) Representative western blot and (B) quantification of phosphorylated S6. B-actin was used as internal control. AL – blue solid line, blue triangles; TR – green solid line, green diamonds and CR – red solid line, red squares. N=4 per time point per group. Time of the day when food was provided to CR and TR are indicated by red arrow. Letters indicate statistically significant effect of the diets ($p \leq 0.05$); a – AL vs CR 30%, b – AL vs TR and c – CR 30% vs TR.

CHAPTER IV

DISCUSSION

Caloric restriction is one of the most widely studied form of dietary restriction, which improves health and increases lifespan in a variety of organisms ranging from nematodes, yeast to mammals (Anderson, Shanmuganayagam & Weindruch 2009; Taormina & Mirisola 2014). When caloric restriction is implemented, mice have a tendency to consume all their food within a couple of hours (Acosta-Rodríguez et al. 2017; Mitchell et al. 2019); thus caloric restriction serves as self-implemented TR, why mice consume all their food in short time frame, is not yet known. Implementation of CR results in increase in the amplitude of circadian clock genes and also resets rhythms of mRNA, protein levels, secretion of hormones and mTOR activity (Makwana et al. 2017; Patel, Velingkaar, et al. 2016; Sato et al. 2017; Solanas et al. 2017; Tulsian, Velingkaar & Kondratov 2018). Similar to CR, there are other diets that emphasize on periodic fasting such as intermittent fasting, and ketogenic diets, that also offer metabolic benefits (Anson et al. 2003; Anton et al. 2018; Newman et al. 2017; Roberts et al. 2017). Time restricted feeding, a form of periodic feeding, which involves providing an unlimited amount of food within a stipulated time frame, also has been demonstrated to provide metabolic benefits through multiple studies (Chaix et al. 2014, 2019; Hatori et al. 2012; Sherman et

al. 2011, 2012). Benefits observed in metabolism under time restricted conditions, were in the form of either an increase or restoration of circadian rhythms of genes and signaling molecules, which highlights the important role of circadian clock in maintenance of health and physiology. However, these benefits of time restricted feeding were reported using a high-fat diet on obese mouse models or mice whose circadian clock was disrupted. Very little is known about the role of TR in mice fed a normal chow diet. A recent study by Mitchell et al in 2019 demonstrated that when mice were subjected to mealtime (MT) feeding, wherein they received 100% of their daily food intake as a single meal once per day, an increase in lifespan was observed in these mice, however the magnitude of increased lifespan was moderate compared to CR in their study, which thus prompted us to investigate the probable mechanisms of lifespan extension and improved health under CR and TR conditions.

TR and body weight

Our current study is an extension of a recently published report by Mitchell et al 2019 wherein we wanted to compare the effects of caloric restriction with time restricted feeding (without reducing the intake of food) to determine which of the components of caloric restriction contribute towards improved health and increased lifespan; was it periodic fasting or reduction in calories or both. One major difference between this published study and our study is the setup of mealtime group. In the published study, authors measured 100% of the daily average food and fed their mice as single meal once per day whereas in our study, we fed our TR mice ad libitum amount of food and 12 hours later food was removed. In both the studies, there was no reduction in caloric intake. Also another difference is that effect on lifespan was the subject of investigation

in the published study, whereas we measured physiological and metabolic parameters. In our study, we observed a reduction in body weight in CR mice, however, we did not observe any decrease in body weight in TR mice. Some published studies do not observe any change in body weight when TR mice fed normal chow (Acosta-Rodríguez et al. 2017; Farooq et al. 2006; Salgado-Delgado et al. 2010; Salim et al. 2007) or high fat diet (Bray et al. 2010; Tsai et al. 2013) and are in agreement with our report. However, there are other studies that observe reduction in body weight when TR mice fed normal chow (Jang et al. 2012) or high fat diet (Arble et al. 2009; Chaix et al. 2014; Jang et al. 2012). Few reasons why these studies disagree with our reports could the design of the study, duration of study, age of mice and diet type. Another possibility could be housing of the mice for the experiment. Acosta-Rodríguez et al. 2017; Jang et al. 2012; Salgado-Delgado et al. 2010 studies housed their mice singly in a cage, however they demonstrated different results; while others have not reported how mice in their studies were caged; in our study, we housed 3-4 mice per cage. We believe that mice when single-caged, tend to lose more heat from their body, which may thus contribute to decreased body weight.

CR, TR and circadian clock genes

Multiple studies have demonstrated the effect of diet on circadian rhythms in peripheral organs, wherein manipulation of the diet, either by varying the amount of food, or essential macronutrients, or restricting the time frame for availability of food, causes circadian rhythms to be either increased, decreased, phase-advanced or phase-delayed (Damiola et al. 2000; Hatori et al. 2012; Potter et al. 2016; Stokkan et al. 2001). This is evident as circadian clock is known to regulate metabolism (Eckel-Mahan & Sassone-

Corsi 2013), however if the diet induced changes in circadian rhythms are analyzed at only one time point across the day, this may cause complications in interpretation of data. Traditionally, multiple studies have reported the effects of CR by collecting data at only one time point in the day, hence our study aimed at minimizing this drawback in design of experiment; therefore we provided our mice with restricted diet at a time when they are active and feeding, collected and analyzed data at multiple time points across the day. We observed that mice in all three diet groups have their first major meal at approximately the same time, and as a result of this feeding pattern, we observed little or no effect on circadian clock gene expression. Implementation of CR and TR results in increase in amplitude of clock genes but do not affect of phase of expression of these genes. CR and TR also have distinct effects on metabolism; we believe that the effect of the diets could be either to supplement or augment the existing rhythms due to clock or may be the effect could be distinct from that of clock. One of the major limitations of this study is in the design of the experimental group. Caloric restriction, as we know, is self-implemented TR, wherein mice consume all food within 2 hours, which suggests that mice are on a stricter time restricted feeding schedule. On this account, metabolic outcomes due to severe fasting on CR will obviously be different from that of TR 12 hour. A better experimental group for comparison with TR 12 hours would be when CR is provided in the form of discrete meals over a period of 12 hours. A study performed by Nelson & Halberg 1986 provided 25% caloric restriction to mice either as single meal in the early light phase, or as single meal in the early dark phase or as 6 discrete meals at 2 hour intervals during the dark phase. The fasting duration for six meals group was 12 hours, similar to 12 hours fasting in our time restricted group. In this study, Nelson and

Halberg observed that despite providing six discreet meals, CR could still extend lifespan in these mice, thus suggesting that lifespan extension can be observed even without severe fasting in CR mice. One drawback of Nelson and Halberg study was that they did not measure metabolic outcome in this experiment, we believe that metabolic profile in CR discreet meal group will definitely be different compared to CR with severe fasting, which needs to be addressed in the future studies. In order to fulfil the criteria of providing CR in the form of discreet meals, automated timed feeders is suggested to be used in future studies.

CR, TR and regulation of glucose homeostasis

To make sure that the body functions normally, maintenance of glucose homeostasis is important, which ensures blood glucose levels are tightly regulated across the day. An imbalance in glucose homeostasis leads to diabetes and neurodegenerative disorders. Insulin is one of the key hormones that helps maintain glucose homeostasis and both glucose and insulin are important in the mechanism of improved health upon CR (Masoro et al. 1992; Mitchell et al. 2015). As expected, CR reduced blood glucose and insulin, and improved glucose and insulin sensitivity. On the other hand, TR reduced levels of blood insulin similar to CR but only moderately improved insulin sensitivity. Also, TR neither changed the levels of blood glucose nor improved glucose tolerance, in comparison to CR. This indicates that there is a different effect on blood glucose and insulin in case of TR. Key factors that regulate the levels of blood glucose include dietary intake, production of glucose by the liver through pathways such as gluconeogenesis and glucose consumption by various tissues such as muscle, adipose tissue and brain. We believe that improved insulin sensitivity might be responsible for improved glucose

homeostasis, however, there are a few factors that need to be considered. One, both CR and TR have different effects on production and secretion of insulin. Glucose tolerance test (GTT) was performed at zt14 and continued for 2 hours until zt16. During this time, we observed an increase in blood insulin levels in CR, but modest in TR, therefore we expect that exogenous supply of glucose during GTT should result in higher secretion of insulin by the β islets of the pancreas, and as a result, levels of blood glucose should reduce and thus lead to better tolerance of glucose for CR mice, although we see an improvement in insulin sensitivity in both CR and TR groups. One limitation in our GTT experiment was that we did not measure insulin during the course of the test, which would have given us a better idea about basal levels and peaks of insulin, thus enabling us to make appropriate comparisons for insulin sensitivity between CR and TR groups, and hence, this needs to be addressed in future studies. Second, both CR and TR have different effects on glucose metabolism in the liver and we have demonstrated this differential effect through mRNA expression of glucose metabolic genes in mouse liver. Key regulatory enzymes in the process of gluconeogenesis: *Pcx*, *Pck1*, *Fbp1* and *G6pc* were induced in CR group but not in TR group. The observed changes in mRNA expression of gluconeogenic genes are in agreement with previous studies, both at the transcription and enzyme levels, however, these studies reported changes in levels of gluconeogenic genes after fasting their mice for 24-48 hours (Dhahbi et al. 1999; Hagopian, Ramsey & Weindruch 2003a, 2003b). Another study by Dhahbi et al. in 2001, demonstrated changes in mRNA expression and enzyme activity of key regulatory gluconeogenic genes, in response to feeding. They also measured plasma insulin and blood glucose levels. Blood glucose regulation is tightly regulated at all time points; both

glucose and insulin levels are reduced upon CR, similar to our observations, however, post prandial plasma insulin levels are much lower, compared to our study. One of the reasons could be the degree of calorie restriction (50%) implemented in their study; second, difference in the use of mouse models for both study; Dhahbi group used C3B10RF1 strain female mice, which is a long lived strain of mouse, as opposed to C57BL/6J male mice; it could be speculated that regulation of insulin maybe gender and species specific; and third, duration of calorie restriction in both studies; Dhahbi group started calorie restriction in their mice after weaning until they were twenty-four months of age, whereas, in our study, when mice were of 12-16 weeks of age, we implemented caloric restriction and maintained them on this diet for 8-10 weeks. The overall conclusion from both studies is that gluconeogenesis is the most preferred metabolic pathway in CR, thus indicating that glucose metabolism is adapted under CR conditions. One study demonstrated that breakdown of glycogen, measured by D-ribonate (by-product of glycogen breakdown) levels decrease upon implementation of CR for 3 months (Green et al. 2017). Breakdown of glycogen feeds into the gluconeogenic pathway to produce more glucose. Since glycogen breakdown decreases, this suggests preference for other non-carbohydrate sources such as glycerol and amino acids for fueling the gluconeogenic pathway. This is evident as studies showed decreased plasma glycerol levels and increased enzyme activities of glycerol kinase and glycerol 3-phosphate dehydrogenase (Hagopian, Ramsey & Weindruch 2008) and increased transamination of amino acids (Hagopian, Ramsey & Weindruch 2003a). Thus this justifies the statement that glucose metabolism is adapted under CR conditions. With respect to TR, we do not see any effect on gluconeogenic genes. One interpretation of

this result could be that 8-10 weeks is not sufficient time to see improvement on glucose homeostasis under TR conditions, in which case mice may be subjected to extended periods of restricted feeding in the future. Another interpretation could be that since TR involves an eating window of 12 hours, hence sufficient amount of energy is stored in the form of glycogen, and during the fasting period, breakdown of glycogen serves as alternate source of energy, whereas gluconeogenesis is kept to a minimum. Hatori et al. 2012 and Chaix et al. 2014 studies measured the mRNA expression of gluconeogenic genes under high-fat fed TR diet and they observed not only a decrease in gluconeogenic gene expression but also gene rhythms were restored, which is in contrast to ad libitum high fat feeding conditions, wherein they observed a constitutively high expression of *Pcx* and dampening of *G6pc*, thus denoting disruption of glucose homeostasis. However, in our study, we observed a minor peak of *Pck1* gene at zt14 upon TR whereas we do not see any effect for other gluconeogenic genes, which justifies our conclusion that TR has modest effect on glucose homeostasis. This effect is different from that observed on high fat diet, hence future studies must be aimed at investigating the interaction between diet composition and TR. Another important factor that needs to be considered in future studies is the role of transcriptional regulators of gluconeogenic genes. Several studies have independently demonstrated that some of the transcriptional factors/co-activators such as FOXO, PGC1α, CREB and HNFs are involved in transcriptional regulation of gluconeogenic genes (Chang, Jun & Park 2016; Hirota et al. 2008; Oh et al. 2013; Puigserver et al. 2003). This will help provide some information regarding regulation of gluconeogenic genes under TR.

During insulin tolerance test, when we inject insulin, we expect that gluconeogenesis will be inhibited and this will lead to decrease in blood glucose levels in both CR and TR, but the decrease in glucose levels will be much stronger in case of CR. During the light (fasting) phase, it is expected that gluconeogenesis will result in production of glucose, and therefore we expect blood glucose levels to be high during this phase in CR mice, however, we did not observe any increase in blood glucose levels, in fact, the levels were much lower compared to TR and AL groups. Thus, this suggests that there may be increase in uptake of glucose by extrahepatic tissues such as skeletal muscle, which may also be another contributing factor for differential regulation of glucose homeostasis between CR and TR groups. This theory helps justify our observation why despite constitutive high expression of gluconeogenic genes, we see reduced blood glucose levels in CR mice. In TR, we observe high levels of blood glucose across the day and is not different from AL group, whereas there is a significant decrease in insulin, compared to AL, at several times across the day. How this uncoupling of glucose and insulin occurs in TR is not known, it may be hypothesized that either duration of 8-10 weeks was not sufficient enough to see a coordinated effect of insulin and glucose, or, the amount of insulin produced by β-cells is not sufficient to lower blood glucose in TR, which will need to be investigated in future studies. Lastly, blood ketone levels are differentially affected by both CR and TR. During periods of prolonged fasting, ketone bodies serve as alternate sources of energy for extrahepatic tissues such as brain and skeletal muscle. How production and uptake of ketones and glucose are regulated, is not well known. Ketones not only serve as alternate source of energy, but they were recently demonstrated to act as signaling molecules involved in the process of food

anticipation (Chavan et al. 2016). We observed a sharp increase in blood ketones only at one time (zt14) in case of CR, whereas in TR, ketones were higher than CR at zt12, but the magnitude of induction at zt14 was several fold less than CR. We believe that this difference in blood ketone kinetics may potentially contribute to difference in GTT we observed in CR and TR. Measurement of ketone bodies can be sometimes challenging due to the reversible kinetics of the reaction that results in the interconversion of acetoacetate to β- hydroxybutyrate. Hence we are not sure if current equipments such as ketone meters can accurately detect specific ketone bodies that are being measured. One way to counteract this issue is to employ mass spectrometry methods in the future to accurately detect specific ketone bodies such as β-hydroxybutyrate.

Future directions of current study

From our study we observed that glucose homeostasis is differentially regulated in CR and TR. Since our entire study was based on studying glucose homeostasis in the liver of mice, extrahepatic tissues such as skeletal muscle and adipose tissue are also contributors of glucose homeostasis. Therefore, we will plan to measure mRNA expression of glucose metabolic genes and mTOR signaling in skeletal muscle and adipose tissue to determine the effects of CR and TR on extrahepatic tissues. Glucose tolerance and Insulin tolerance tests were performed at one time point only, we would want to investigate what would be the sensitivity of glucose and insulin at other time points, especially during the fasting (light) phase and how this would impact uptake of glucose by tissues. Our entire study was conducted using male mice, hence in the future we will plan to perform same experiments using female mice; we believe that effects on glucose homeostasis will be different in females. Also our study focused on investigating

the effects on glucose homeostasis, however there are other parameters and signaling pathways that are affected by caloric restriction such as Sirtuins, Insulin signaling and Igf-1 signaling, these pathways are involved in aging mechanisms and interlinked with CR and circadian clock. We will also investigate CR and TR mechanisms in lipid homeostasis as fatty acid signaling is also implicated to play a role in mechanisms of CR. Since circadian clock and dietary restrictions are interlinked, we would want to investigate the role of reduced calorie intake and periodic fasting components of CR in circadian mutant mice.

CHAPTER V

CONCLUSION

From our study, we can conclude that some of the benefits observed on metabolic parameters in caloric restriction can be partially explained through periodic fasting. This in agreement with recently published report by Mitchell et al in 2019, wherein they observed a moderate increase in lifespan (11-14%) in mealtime feeding, in contrast to caloric restriction (28% increase in lifespan). However, for reasons not known, mice subjected to mealtime feeding in this study had a tendency to consume the entire amount of food within a short period of time, resulting in fasting period equivalent to 12 hours, indicating that mealtime feeding is similar to self-implemented TR, thus allowing us to draw parallels, with respect to TR, in our study. Better insulin sensitivity correlates with increased lifespan, as indicated by CR studies, therefore, the moderate improvement in insulin sensitivity upon TR correlates with modest increase in lifespan. This is important, since TR implemented in our study, did not involve any reduction in food intake and did not reduce body weight in mice, but still contributed partially to improvement of metabolic factors as depicted in the graphical summary below. Hence our study helps provide a potential explanation why mealtime feeding results in increase in lifespan.

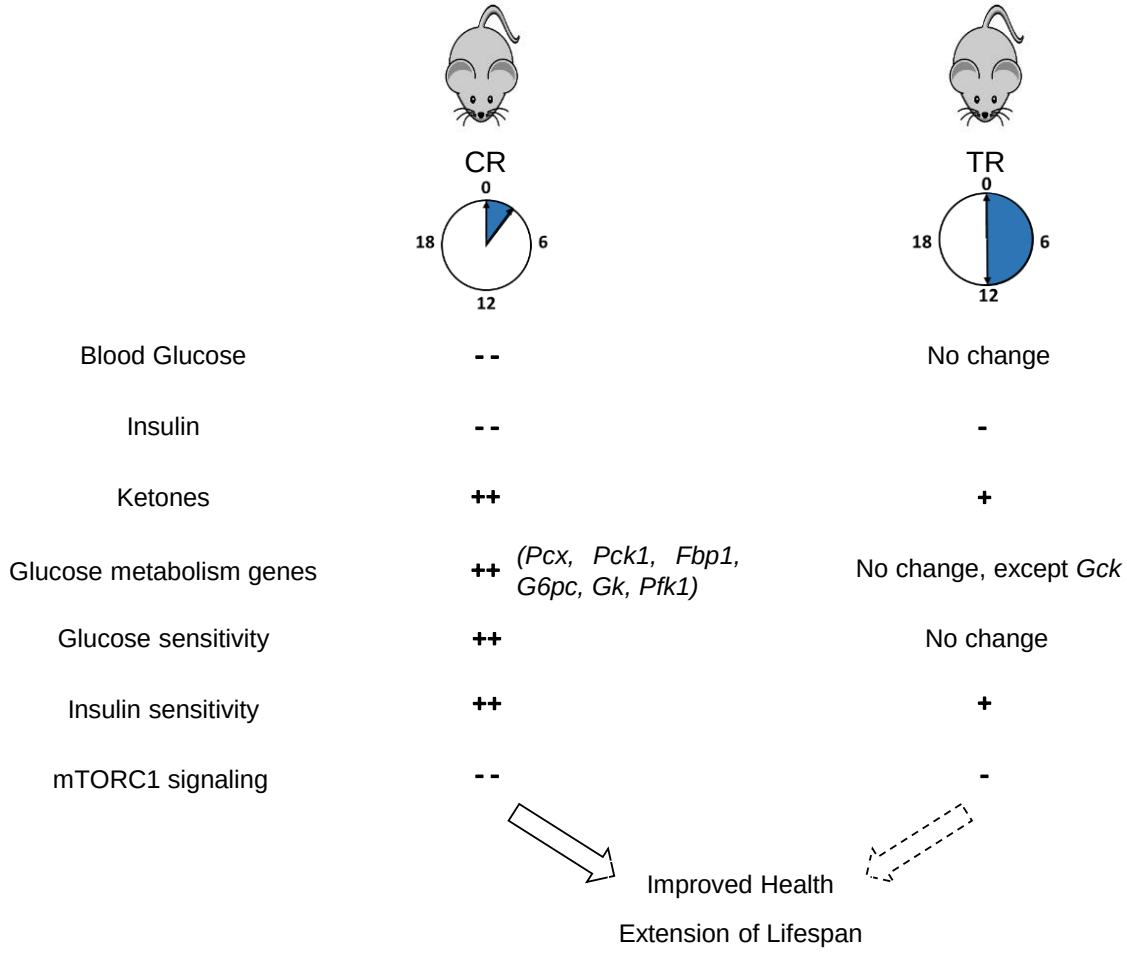

Figure V.1 – Graphical Summary of physiological and metabolic processes affected by CR and TR, which in turn contribute to improved health and increase in longevity

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