

INVESTIGATION OF THE EFFECTS OF LONG-TERM NOCTURNAL  
FEEDING ON BLOOD LEPTIN AND LIPIDS VALUES, WEIGHT  
MANAGEMENT, AND BEHAVIOR, IN ELDERLY WISTAR RATS

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## **ABSTRACT**

### **INVESTIGATION OF THE EFFECTS OF LONG-TERM NOCTURNAL FEEDING ON BLOOD LEPTIN AND LIPIDS VALUES, WEIGHT MANAGEMENT, AND BEHAVIOR, IN ELDERLY WISTAR RATS**

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Today, obesity is one of the most important health problems. For long, obesity has been linked to the diet type and daily caloric intake. The effect of time restricted feeding (TRF) on the weight management is less documented. The aim of this study was to investigate whether long-term night-time restricted feeding affects body weight gain, metabolic parameters, blood leptin levels, and behavioral performance in elderly overweight male Wistar rats. For 3 months (10 years in human), 14-months old, overweight male Wistar rats (n=6) were subjected to a time-restricted diet (TRF group) with food (standard lab chow) provided during 8 h of the dark phase of the diurnal cycle. Control group (n=6), was maintained on ad libitum diet (ADLIB group). Blood levels of triglycerides, HDL, LDL and leptin were measured and a battery of behavioral tests was performed before and after the diet implementation. Longitudinal (pre-post treatment) and cross-sectional (TRF versus ADLIB diet) comparisons revealed: no between-group differences in food intake and body weight gain, locomotor activity (Open Field), anxiety levels (OF & Elevated Plus Maze), and short- or long-term memory retention (Water Maze); positive effect of TRF on

motor performance in Accelerod task; trend towards slower place learning in TRF group during post-treatment test; increase in the blood LDL and leptin levels in ADLIB but not TRF group. In conclusion, prolonged time-restriction feeding even when overlapping with diurnal phase of increased activity does not significantly affect metabolic and behavioral parameters in elderly overweight male rats.

**Keywords:** time-restricted feeding, rat, blood biochemistry, behavior

## ÖZ

### YAŞLI WISTAR SIÇANLARDA UZUN SÜRELİ GECE DÖNEMİ BESLENMENİN KAN LEPTİN VE LİPİD SEVİYELERİ, VÜCÜT AĞIRLIĞI VE DAVRANIŞ ÜZERİNDEKİ ETKİLERİNİN ARAŞTIRILMASI

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Obezite günümüzde diabet ve kardiyovasküler hastalıklar yanında yaşlılık ve bilişsel bozuklukları da tetikleyebilen önemli sağlık sorunları arasında yer almaktadır. Şimdiye kadar yapılan çalışmalarda diyetle ilgili obezite ağırlıklı olarak beslenme tipi ve günlük kalori alımıyla ilişkilendiriliyordu. Zaman kısıtlamalı beslenme tarzının (ZKB) kilo yönetimine etkisi daha az araştırılmıştır. Bu çalışmanın amacı, yaşlı, kilolu Wistar sıçanlarda yüksek aktivite dönemine (karanlık dönemi) sınırlı, uzun süreli ZKB'nin, kilo alma, metabolik parametreler ve kandaki leptin seviyesi ile davranışsal performans üzerindeki etkilerini incelenmektir. 3 ay süresince (insan ömründe 10 yıl), 13 aylık kilolu Wistar erkek sıçanlar standart laboratuvar yemiyle günlük döngünün karanlık döneminde 8 saat boyunca zaman kısıtlamalı beslenmiştir. Deneyle boyunca, kontrol ad libitum grubu (ADLIB, n=6) kısıtlamasız beslenmiştir. Trigliserit, HDL, LDL, V-LDL ve leptinin kan seviyeleri ölçülmüş ve Açık Alan (AA), Yükseltilmiş Artı Labirent (YAL), Accelerod ve Morris Su Labirenti (MSL) gibi farklı davranış testleri, diyet uygulamasından önce ve sonra uygulanmıştır. Yatay (diyetten önce ve sonra) ve dikey (ZKB ve ADLIB) kıyaslamaları ile ortaya çıkan sonuçlar: Besin ve kilo alımında, lokomotor aktivitede (AA), anksiyete

seviyesinde (AA ve YAL), kısa ve uzun dönem hafıza gücünde (MSL) gruplar arasında fark yokken, Accelerod testinde diyetin motor performansa olumlu etkisi bulunmuştur. Diyet sonrası dönemde ZKB grubunda daha yavaş yer öğrenme eğilimi gözlenmiştir ve ayrıca LDL ve leptin kan seviyelerinde ADLIB grupta diyet süresince artış, ZKB grubunda ise azalma tespit edilmiştir. Sonuç olarak, uzun süreli ZKB günlük döngüde aktivitenin yüksek olduğu karanlık dönemde uygulansa bile yaşlı erkek sıçanlarda metabolik ve davranışsal parametreler üzerinde çok belirgin etkisi bulunmamıştır.

**Anahtar Kelimeler:** Zaman Kısıtlı Beslenme, Sıçan, Kan Biyokimyası, Davranış



**To My Mother...**

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## LIST OF ABBREVIATIONS

ADLIB	Ad Libitum
ANOVA	Analysis of Variance
AgRP	Agouti-related protein
BMI	Body Mass Index
CART	Cocaine and amphetamine-regulated transcript
CNS	Central Nervous System
CR	Caloric Restriction
EPM	Elevated Plus Maze
GABA	Gamma-aminobutyric acid
HDL	High Density Lipoprotein
LDL	Low Density Lipoprotein
g	gram
min	Minute
mL	Milliliter
MWM	Morris Water Maze
NE	North-East
NPY	Neuropeptide Y
NW	North-West
OFT	Open Field Testing
POMC	Proopiomelanocortin
rpm	Revolutions per minute
SE	South-East

s	Second
SEM	Standard Error of Mean
SW	South-West
V-LDL	Very Low Density Lipoprotein
TRF	Time-Restricted Feeding

## **CHAPTER 1**

### **INTRODUCTION**

Cell-autonomous circadian clocks found in the brain and most peripheral tissues control behaviour and physiology of all mammals. These clocks are synchronized to local environmental time by two primary cues, the daily light-dark (LD) cycle, and the timing of food intake (Patton, 2010). LD cues act on the suprachiasmatic nucleus (SCN). These cues are related to food intake and they are thought to act on many other circadian oscillators independently of the SCN. In humans, the effects of meal timing on circadian rhythms have not been well characterized, but there is increasing evidence that the timing of food intake affects metabolism and body weight regulation, and that disrupted daily rhythms of food intake, as in shiftwork, may predispose to metabolic disorder and other negative health effects (Green et al., 2008). Studying these mechanisms provide insights into how the circadian system in humans may be affected by altered mealtimes, as occurs in shift workers, in various disease states, in frequent travelers, and in accordance with some social customs (e.g., Ramadan).

SCN, the master pacemaker, controls activity and resting periods consolidating them into distinct phases of diurnal cycle. Typically, animals engage in their largest feeding bouts during periods of activity. As a consequence the SCN controls the phase when animals eat by controlling when they are actively foraging for food. In nocturnal animals such as rats, the period of increased activity overlaps with the dark phase of diurnal cycle (Rosenwasser et al., 1981). When food access is restricted to a few hours at the same time each day (restricted feeding, RF), daily behavioural and

physiological rhythms are altered. These changes are mediated by food entrainable circadian oscillators (FEO) independent of the SCN.

The study objectives were to investigate potential changes in the body weight gain, leptin blood levels, lipid metabolism and behavior in elderly laboratory rats subjected to nocturnal restricted feeding confined to 8 hrs of the dark phase of day/night cycle but before having free access to food through all 24 hrs.

Leptin has natural rhythmicity and it is linked with meal time in healthy humans and also rodents. Meal time can cooperate with the endogenous rhythm of leptin hormone to effect metabolic signals, especially leading to excessive body weight gains during 'wrongly' timed feeding (Arble et al., 2011). In addition to fat storage information, the strong connection of the leptin hormones with feeding suggests that leptin may be able to be in contact with meal timing information to the brain. Moreover, in general, what we know about the leptin effect on brain development and plasticity comes from rodent models, predominantly rats and mice. However, with increasing knowledge about that leptin may also influence neuroplastic events in the human brain (Bouret, 2010). Leptin improves learning and memory by enhancing hippocampal synaptic plasticity. (Harvey, 2007). This suggests that alterations in sensitivity to leptin could be mediating cognitive and synaptic deficits in rats maintained on a high calorie diet (Strahanan et al., 2008). Besides, the hypothesis by Strahanan and colleagues (2008) suggest that differences in the balance of proteins, carbohydrates, and fats may influence hippocampal plasticity directly, and thus learning and memory, independently of peripheral metabolic alterations. An alternative hypothesis would suggest that time-restricted diet type has an effect on learning and memory depending on leptin as an inducer.

## **1.1.General view on obesity and related health problems in human populations in industrialized countries**

In 2008, it was estimated that obesity is a world-wide problem with the number of 508 million obese people and 1,46 billion overweight people and many more currently (Imes and Burke, 2014).

In the past, obesity was a rare condition but it is growing day by day. Therefore, innate mechanisms to control obesity have not evolved yet. People have tendency to gain weight and associated health problems because of the new lifestyle in the modern world. New lifestyle is associated with consumption of larger amounts of palatable and energy dense fast foods and sugar-sweetened beverages (Lafontan et al., 2015) and often life is more sedentary with less physical activities. On the other hand, our body regulates its energy balance according to homeostatic regulatory system which is important for survival and favors energy savings rather than energy spendings (Berthoud, 2004).

To diagnose obesity, World Health Organization reference standarts for BMI (Body Mass Index) can be used. BMI is a major measurement based on the body fat percent (BF%). For instance, obesity standarts are  $BF\% > 25\%$  in men and  $BF\% > 35\%$  in women (Romero-Corral et al., 2008).

Obesity and metabolic syndrome caused by obesity are global problems today. Metabolic syndrome is the name of risk factors (high triglyceride level, high blood pressure, low HDL cholesterol level, excess abdominal fat, , high fasting blood sugar) that raise the risk of several other health problems (National Hearth, Lung, and Blood Institute, <http://www.nhlbi.nih.gov>). Obesity causes higher morbidity observed as hypertension, stroke, coronary heart disease, sleep apnea and respiratory problems, gallbladder disease, some types of cancer (gallbladder cancer, colon cancer, endometrial cancer, breast cancer) and osteoarthritis (National Heart, Lung, and Blood Institute, 1998).

According to INTERSALT (International Study of Salt), hypertension is more frequently seen in overweight and obese patients. It has been reported that 10 kg higher body weight cause 3.0 mm Hg higher systolic and 2.3 mm Hg higher diastolic blood pressure (Dyer and Elliott, 1989).

According to National Heart, Lung, and Blood Institute report in 1998; type 2 diabetes is also related with weight gain in both men and women and specifically, abdominal obesity causes high risk of type 2 diabetes.

Moreover, it is reported in this publication that in women obesity may have negative impact on reproductive performance and cause menstrual irregularity.

According to this report, obesity may also lead to mood disorders because of negative attitudes of people to an obese individual, activity limitations at work and the stress related with concerns about self body image.

#### **1.1.1. Biological predispositions to obesity**

Degree of the risk can vary depending on such factors as ethnicity/race, societal conditions, cultural food context, social status and economic disadvantages), age and gender. It is known that the risk of morbidity in coronary heart disease is the highest in aged population. From National Health and Nutrition Examination Survey, it is also seen that there are differences between men and women, white non-hispanic, black non-hispanic and hispanic groups (National Heart, Lung, and Blood Institute, 1998). Race/ethnicity may have underlying genetic components; however, race/ethnicity with self-identification is complicated. Whether genetic differences across populations are linked with obesity development remains unclear. It is related with energy-dense environment in a population because it would tend to increased in accumulation of adipose tissue. In addition to this, obesity associated with race/ethnicity differences may be related to different patterns of fat distribution (Caprio et al., 2008). Besides, there is a relation between race/ethnicity and the differences in lipoproteins and lipids (Lee et al., 2006). The relation between societal



conditions and obesity exists due to the fact that the lower-cost but high energy foods constitute a greater part of the diet of lower-income individuals (Darmon and Drewnowski, 2008).

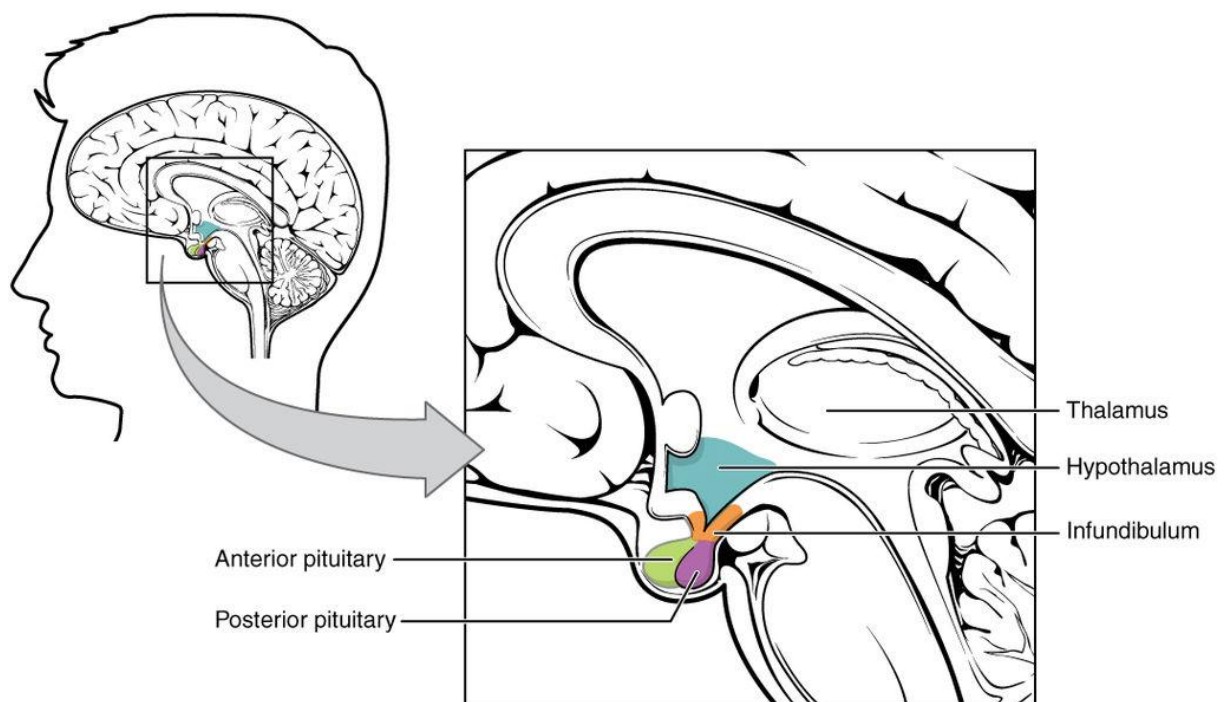
Personal genetic profile is important for predisposition to weight gain. For instance, we know that parental obesity is the major factor for childhood obesity (National Heart, Lung, and Blood Institute, 1998). In some patients, obesity is linked to mutations in genes involved in regulation of food intake, energy metabolism and weight management such as leptin or its receptors. Today, more than 200 single gene mutation were shown to be linked to obesity in human populations. These mutations are found in 10 genes and 8 of them are well known for their role in leptin-melanocortin signaling pathway in hypothalamus (González-Jiménez et al., 2012). However, obesity, as many other diseases, is usually a product of interplay between a gene defect and adverse environment factors (Berthoud, 2002).

## **1.2. Control-regulatory mechanisms of food intake, energy metabolism and weight management in mammals**

### **1.2.1. Neural Substrates**

Feeding behaviour is an example of ultradian biorhythms repeating every few hours. Food intake is directly related to energy metabolism and energy homeostasis in the living body and it is controlled and regulated by neuroendocrine mechanisms. In mammals, the neural network which controls food intake and is responsible for energy homeostasis is hierarchially organized and includes the brain-stem, hypothalamus, and corticolimbic structures as the major building blocks. Forebrain and hindbrain circuits are responsible for the neural mechanisms of food intake and body weight regulation (Berthoud, 2002). The main brain center implemented in the control over feeding behavior is hypothalamus. Caudal brainstem circuits are more basic in both phylogenetic and ontogenetic sense (Berthoud, 2004).

The caudal brainstem receives the information from taste buds and gastrointestinal track then passes this information to the autonomic motor neurons to start food processing and handle the nutrients. On the other hand, in all vertebrates, the hypothalamus, structure located in diencephalon below the thalamus, just above the brainstem (**Fig.1.1**), part of the limbic system (“emotional brain”), exerts control over feeding behavior and food intake.



**Figure.1.1.** Location of the hypothalamus, in relation to the pituitary and to the rest of the brain (Illustration adapted from Anatomy and Physiology, OpenStax College, 2013).

Hypothalamus is composed of several small nuclei among which lateral nucleus, primary source of hormone called orexin, is known as hunger center while ventromedial nucleus as satiety center. Bilateral lesion of lateral nucleus results in cessation of food intake. In contrast to this, bilateral lesion of the medial part of the ventromedial nucleus triggers hyperphagia leading to obesity. There is a crosstalk

between these two nuclei. The third hypothalamic nucleus important for the control and regulation of appetite and thus food intake is the hypothalamic arcuate nucleus with central projections releasing neuropeptide Y (NPY), agouti-related protein (AGRP), and GABA, the inhibitory neurotransmitter. Some of its neurons project to lateral nucleus. These neurons can produce repeated eating when they are activated. Insulin, peptide YY, and leptin inhibit these neurons but ghrelin activate them.

There are four different hypotheses related to this regulation (Theologides, 1976):

1. Lipostatic hypothesis: This hypothesis suggests that adipose tissue produces a humoral signal that acts on the hypothalamus to decrease food intake and increase energy output while it is also proportional to the amount of fat and. It has been evident that a hormone leptin, released from adipose tissue, acts on the hypothalamus to decrease food intake and increase energy output.
2. Gut-peptide hypothesis: Gastrointestinal hormones like cholecystokinin (CCK), glucagon, gastrointestinal releasing peptide (GRP), and the assertion for others is that they inhibit food intake. The entering food to the gastrointestinal tract has influence for increasing of the release of these gastrointestinal hormones, which producing satiety by acting on the brain. The brain contains two types of cholecystokinin receptors and they are CCK-A and CCK-B.
3. Glucostatic hypothesis: The ventromedial nuclei which contains the satiety center and its activity is probably governed by the glucose utilization by the neurons. It has been postulated that when the arterio-venous blood glucose difference is low, and when neuronal glucose utilization is low, the activity of the satiety center decreases. The individual feels hungry when the activity of the feeding center escapes from inhibition by the satiety center, under these conditions. Intraventricular administration of 2-deoxyglucose which is taken up but not metabolized by the nervous tissue increase rapidly the food intake.

4. Thermostatic hypothesis: Body temperature decrease to the below a given set-point stimulates appetite, whereas an increase above the set-point inhibits appetite, according to this hypothesis.

Nutritional information is collected firstly and then it is integrated with other internal and external signals. Cortico-limbic systems manage human and animal interactions with food-providing environment in foraging behavior basing on such factors as food availability, cost-benefit relations and experience (Zheng et al.,2009). Cortico-limbic structures are responsible for motivational and rewarding aspects of feeding behavior (Lenard and Berthoud, 2008). During famine periods energy deposition as body fat is favored as an advantage. This mechanisms are still protected in hibernating mammals. Hibernating animals such as hamsters, chipmunks and bears deposit fat in white adipose tissue (WAT) such that prior to hibernation periods WAT mass increases with body weight becoming near double and metabolic rate highly decreasing. Therefore, energy cost is reduced in these hibernating animals. Before this period, they become resistant to increased leptin levels and gain a lot of weight in a short time. Then, they display progressive weight decline during hibernation (Carey et al., 2003).

### **1.2.2. Endocrine Systems**

The discovery of leptin (from the Greek root “leptos”, meaning “thin”) was an important factor to better understand mechanisms of feeding behavior. Leptin,167-amino-acid peptide, is secreted from adipose tissue and inhibits food intake. Thus, leptin levels circulating in the body change according to the periods of food intake and fasting. For instance, it decreases during starvation while overfeeding increases blood leptin levels (**Figs. 1.2. and 1.3**). Recent studies show that there is a relationship between secretion of leptin and feeding rhythms with the lowest level at mid-afternoon and the highest level at midnight (Bodosi et al., 2004). Leptin receptors (LepRs) are located throughout the central nervous system (CNS). Long isoforms of this receptors are responsible for regulation of energy homeostasis and

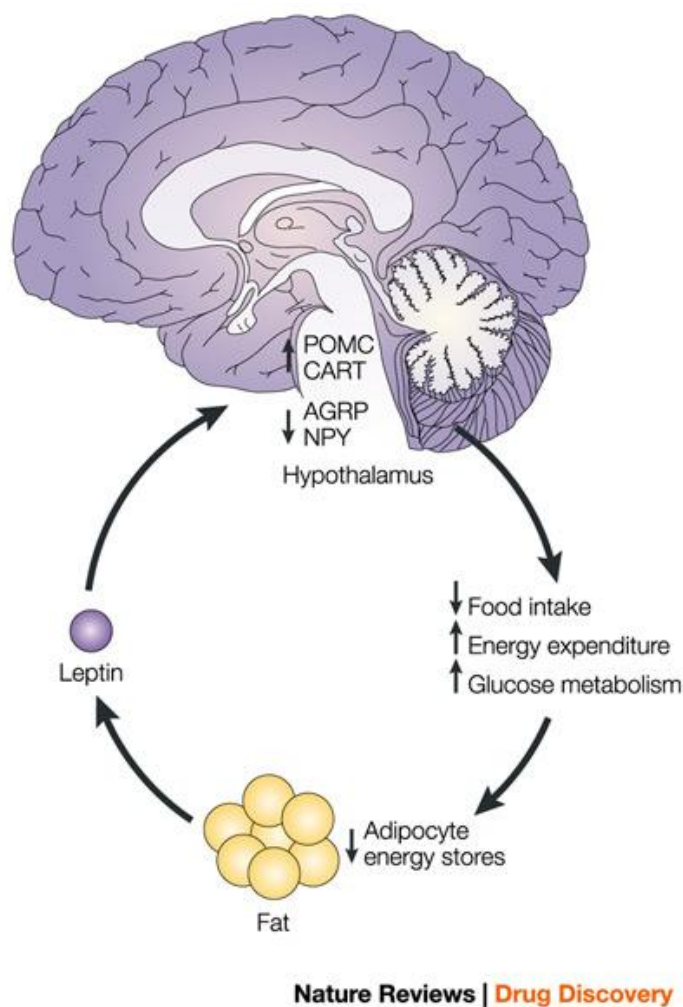
short isoforms provide transportation of leptins across blood-brain barrier. High density of leptin receptors is found in hypothalamus, especially in arcuate nucleus. In obesity, circulating leptin level are high, however, its receptors become resistant to leptin. Because of leptin signaling disruption and impaired leptin transport, circulating leptin fails to reduce adiposity (Park and Ahima, 2015). Leptin secretion is not a typical endocrine secretion because it is a transcriptional procedure occurring by increased amount of adipocyte induction (Lee and Fried, 2006). Since the leptin discovery, we know that evolutionarily, low leptin levels indicate “hungry brain” and stimulate the food search in the environment increasing energy intake and reducing energy expenditure (Heiman et al., 1997). The deficiency of leptin creates increased anabolic efficiency. The body conserves energy for thermogenesis and metabolic processes. It is thought that this effect is stronger in rodents than in humans (Berthoud, 2004).

In addition to leptin there are several other endogenic agents affecting feeding process. Neurons responsible for their secretion play crucial role in feeding mechanisms translating the metabolic action to endocrine, autonomic, and behavioral responses (Berthoud, 2004). Some of these neurons are proopiomelanocortin (POMC) neurons releasing melanocortins. POMC mutations cause obesity just like leptin knockout (Millington, 2007). Some other neurons are secreting agouti-related protein (AgRP) and neuropeptide-Y (NPY) all implemented in food intake regulation. There is also a cross-talk between these hormones.

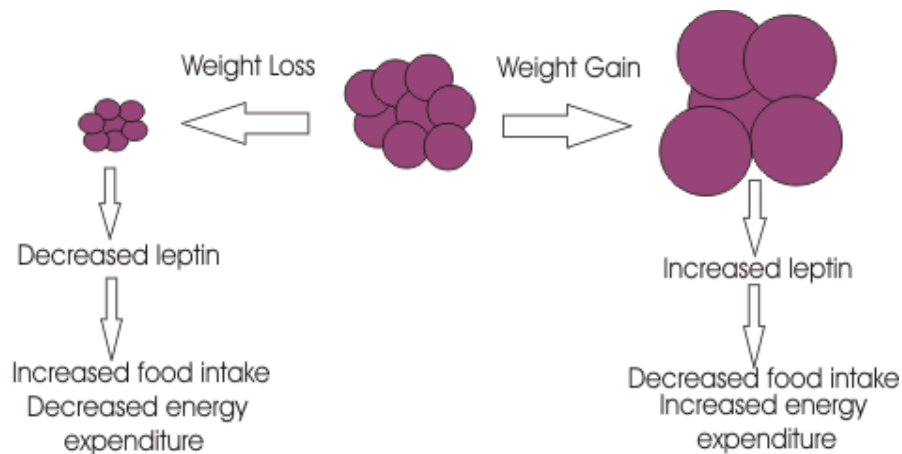
With the high levels of leptin, agouti-related protein (AgRP), and neuropeptide-Y (NPY) is inhibited, while POMC as well as cocaine and amphetamine-regulated transcript (CART) are activated. Leptin directly activates POMC neurons and deactivates AgRP/NPY neurons. Fasting stimulates NPY expression and NPY stimulates feeding by inhibiting POMC expression (Schwartz et al., 2000).

The roles of the regulatory leptin molecule on biochemical blood parameters and weight management were discussed earlier. Recent studies show that at the same

time, leptin is a potential cognitive inducer that modulates cellular processes underlying learning and memory. For instance, it has been demonstrated that activity dependent synaptic plasticity is regulated by this hormone (Harvey, 2013). Also impairments in hippocampus-dependent spatial learning have been reported in rodent species non-sensitive to leptin (Li et al., 2002; Farr et al., 2006).



**Figure.1.2.** Leptin and the regulation of adipose-tissue mass (adapted from Crowley et al., 2002).



**Figure.1.3.** Secretion of leptin proportional to adipocyte lipid volume (adapted from <http://www.diabetesity.eu/Leptin.htm>).

### 1.3. Studies on the genetic and molecular mechanisms of obesity

Such studies are carried on genetically modified animals susceptible to developing obesity such as A/J and C57/BL mice and the Osborne–Mendel rat and the other strains of mice (SWR) and rats (S5B/PI) which are resistant against obesity development (Bray et al., 2004). As a result of these studies, fat mass and obesity associated (FTO) DNA region was reported to encode a 2-oxoglutarate-dependent nucleic acid demethylase and to be highly expressed in hypothalamic nuclei controlling energy balance (Gerken et al., 2007). Moreover, a strong relation was detected between BMI and SNPs located 188 kilobases (kb) downstream from the melanocortin 4 receptor gene (MC4R) (Loos and Lindgren, 2008). Several of related genes are expressed highly or known to act in CNS, suggesting, as monogenic forms of obesity rarely, the role of CNS pathways in tendency to overall obesity (Thorleifsson et al., 2009).

### 1.4.Strategies for obesity treatment and prevention

In 1972, to fight with the obesity problems, North Karelia Project has been launched in Finland and with this study it was revealed that an integrated community-based intervention on diet and life-style can reduce the risk of coronary heart diseases and

mortality by 80% (Pekka et al., 2002). There is another example for the community-based intervention programs; EPODE (Ensemble, Prévenons l'Obésité Des Enfants) which has been initiated in France. This program aimed at the reduction of childhood obesity country-wide and now it has spread to 293 towns in Europe ([www.epode-european-network.com](http://www.epode-european-network.com)). The conclusion arising from these programs is that in order to fight out obesity, one should modify his/her lifestyle according to physical activity and nutrition intake, and if necessary undergo cognitive therapy. Nutrition intake should be limited with 500-1000 kcal per day. Physical activity takes a big part in the daily life with initially 30 min and then 60 min or more, 3-5 times in a week. Cognitive therapy is related with restructuring thoughts from overeating and inactivity to healthy behaviors (Dietz et al., 2015).

On the other hand, the obesity caused by lack of leptin or impaired leptin signaling can be treated by administration of exogenous leptin (Pellemounter et al., 1995).

#### **1.4.1. Physical exercise as a tool for increase energy spendings**

##### **1.4.1.1. Human studies**

Physical activities and exercise are the components of the energy spending and energy balance of the body. Body weight increases with age normally because of decreased metabolic rate and physical abilities but doing exercise for lifetime can prevent weight gain, and also reduce the obesity risk (Owen et al., 2010). It is generally accepted that sedentary people gain more weight than moderately active people. There is a recent study showing that short-term implementation of overfeeding and lack of activity resulted in alterations in the expression of several genes related with metabolism, nutritional balance and insulin action within adipose tissue and impairments of the metabolic outcomes.. After all, these changes are prevented by the addition of daily exercise. In this study, 7 days (short-term) overfeeding with enforced physical inactivity caused impaired insulin sensitivity and resulted in alterations in SREBP-1c, FAS, GLUT4, IRS1, IRS2, visfatin, PDK4,



HSL, adiponectin, leptin, PPAR $\gamma$ , IL-6, AMPK, apelin, TNF $\alpha$ , IL-18 and LPL expression in adipose tissue (Walhin et al., 2013).

Generally, in European countries health in older adults is better because they choose walking and cycling for transportation in contrast with the countries of North America and populations from Australia which are highly car-dependent populations (Huy et al., 2008).

Regular physical exercise even without caloric restrictions reduces fat mass in the body including abdominal fat and, have benefits on vascular function in the periphery and cerebral circulation, and also is associated with higher cognitive function scores (Barnes and Joyner, 2012). However, to lose 1 pound of body weight during one week from exercise alone without dietary restrictions, a person should walk 5 miles per day, (Artal, 2015).

It has been also reported that daily physical activities also decrease insulin resistance in high-risk subjects (Lafontan et al., 2015; Herzig et al., 2014, Ross et al., 2000). In contrast to this, Stafne et al. (2012) showed that insulin resistance in normal BMI (Body Mass Index) subjects with gestational diabetes (especially pregnant women during 3rd trimester) did not change significantly with exercise programs. Besides, glucose regulation between exercise (home-based aerobic exercise program) and control groups did not show significant differences with normal BMI or overweight subjects in this study groups with gestational diabetes.

It was suggested that physical exercise training may modify glucose metabolism during pregnancy (Hopkins, 2010). In obese pregnant women, additional weight gain can cause gestational Diabetes Mellitus but even for this group, daily moderate exercise programs at least 30 min/day can prevent hyperglycemia. According to Artal (2015), pregnancy period can be a motivational phase for changing lifestyle and initiating lifelong healthy behavior with daily exercise habit for obese women.

As it can be seen from these reports, exercise is an important factor to control body mass and energy metabolism but it is not the only solution for obesity.

### **1.3.1.2. Animal Studies**

Most researchers studying effects of physical exercise in rodents have used wheel-running or treadmill tasks, rotarod/accelerod task and sometimes implemented rewarded activities such as lever pressing or maze tasks (Dishman et al., 2006). Exercise reduces adiposity and body weight in obese rodents (Mayer et al., 1954). Chronic exercise also provides protection against harmful effects of diet rich in saturated fat (Molteni et al., 2004). It is also suggested that chronic wheel running in rats has stress protective effects and prevents learned helplessness (Greenwood et al., 2003).

### **1.4.2. Caloric-restriction (CR) diets as a tool for decreased energy intake**

#### **1.4.2.2. Animal studies on the effects of caloric-restriction diet**

##### **A. The role in weight control**

It is known that caloric-restriction cause weight loss and has an important role in weight management in non-obese and also obese and overweight humans. It is suggested that obese and overweight people should make a life style intervention by dietary restriction. For instance, 5% weight loss is a healthy recommendation (Dietz et al., 2015). In a review study, Dietz et al., in 2015 reported that different types of caloric restrictions provide different amounts of weight loss. When it is applied for 12 months in human subjects, low carbohydrate/high protein diet reduced body weight by 5.5 kg while low fat/high protein diet reduced the patients' weight by 4.1 kg. Low-carbohydrate diet as a kind of CR seems useful as a diet procedure when we analyze patient weight after short-term application (in human, 90 days) because low-carbohydrate diet provides significant weight loss according to Dena et al. (2003). However, this weight loss is associated with decreased caloric intake but there is no relation between low-carbohydrate diet content and patients chosen older than age

50 years (Dena et al., 2003). Moreover, Frederick et al. in 2003 showed that low-carbohydrate diet in severe obesity provides more weight loss than calorie and fat-restricted diet. Also in children aged 2-7, prevention of sugar-sweetened beverages consumption may prevent 0,43 kg weight gain in a year by preventing 131 kcal daily intake (Wang et al., 2008).

## **B. Physiological correlates**

Caloric-restriction as an alternative technique to improve health conditions and prolonge life-span was first presented by McCay et al. in 1930s (McCay et al., 1935). Since then, calorie restricted diet was applied in a variety of species such as; mice, rats, worms, yeast, fish, and flies(Weindruch et al., 1988).

In 1935, McCay and colleagues reported that in rats, reducing the amount of food (percent diet content: starch 22, cellulose 2, lard 10, sucrose 10, salt mixture 6, dried yeast 5, cod liver oil 5 and casein 40) resulted in extended life-span but also in some retardation of growth. They retarded the growth by restriction of calories but with the diet was designed to provide adequate levels of all necessary constituents. However, CR was not widely studied in these years. Later in 20th century, CR studies were performed on various model organisms such as budding yeast, flies, spiders, worms, fish and monkeys (Lin et al., 2002; Lin et al., 2004; Jiang et al., 2000; Chapman and Partridge, 1996; Chippindale et al., 1993; Austad, 1989; Houthoofd et al., 2003; Klass, 1977; Novak et al., 2005; Colman et al., 2009). In 1987, a study on aged mice showed that motor performance and learning was increased in CR mice group (Ingram et al., 1987). In 1997, CR in mice and rats proved to extend life-span and to protect tissue structure and function against the age-dependent damage (Sohal and Weindruch, 1996, Flier et al., 1997). Glucose and insulin levels were reported to be decreased in both rodents and monkeys maintained on CR diet. Calorie restricted monkeys showed also lower body temperatures than control group, and these two parameters, blood insuline levels and body temperature, were accepted as biomarkers for longevity in model animals (Roth et al., 2002). It was also shown that

implementation of CR diet in non-obese humans and animals improves insulin sensitivity, secretion of many hormones, and sympathetic nervous system activity (Gresl et al., 2001, Heilbronn and Ravussin, 2015). Improved insulin sensitivity stimulates decrease in circulating fatty acid concentrations in obese individuals (Boden, 2001). However, when comparing human and animal data one should not forget about differences in the metabolic rate and energy expenditure between humans and rodents used as animal models in such studies (Heilbronn and Ravussin, 2003).

In additions to mammals such as small rodents and monkeys, another interesting model organism used recently in the studies on the effect of CR in feeding is Zebrafish. It shows many similarities with mammals. For instance, it lives on average 3 years like rats and mice, ages gradually and shows similar features in the aging process. Moreover, zebrafish and mammalian gene maps are similar with about the same number of chromosomes (Postlethwait et al., 2000). Zebrafish has also an integrated nervous system and shows behavioral similarities such as social behavior (Kishi et al., 2003; Gerhard and Cheng, 2002; Lieschke and Currie, 2007). Zebrafish, as a model organism, because of these similarities with mammals, has an advantage to study effects of lifetime CR feeding (Arslan-Ergul et al., 2013). In this review study, Arslan-Ergul et al. reported that Zebrafish is an excellent model organism for studying molecular mechanisms underlying CR because it is more applicable than human studies due to moral issues and also Zebrafish has similar responses for dietary restrictions.

The duration of CR diet is also important factor. It is suggested that short-term CR is not effective as a prevention against high serum levels of LDL and total cholesterol ROS production, and thus cardiovascular diseases while long-term CR application can reduce endothelial dysfunction by decreasing LDL and cholesterol levels in both obese and non-obese people. Endothelial dysfunction is the main cause of cardiovascular disease. It is because of the secretion of procoagulant instead of anticoagulant. (Heilbronn and Ravussin, 2003).

### **C. Effects on behavior**

The results of long-term caloric restrictions on behavior are contradictory. In a study by Yanai et al., (2004)., starting from 2.5 months of age, lifelong, CR rats were fed 10 g standard laboratory chow while control group was fed with the same chow ad libitum. After this long-term dietary application rats were tested for their cognitive performance when they were over 20 months old. CR rats showed poorer performance in MWW task but longer life span (24-27 months) as compared to ad libitum group (Yanai et al., 2004). However, in a study on C3B10RF1 mice (long-lived F1 hybrid strain) the group maintained on restricted feeding demonstrated better learning skills in psychomotor and spatial memory tasks and also increased locomotor activity in the runwheel at middle and elderly ages (from 11 month to 15 and 31 month to 35.) (Ingram et al., 1987).

Studies by Arslan-Ergul et al. in 2013 showed that, ad libitum fed and CR fed animals both exhibit decline in performance when they tested in Morris water maze. However, ad libitum group continued to decline in old ages while the performance of CR group was stabilized. This results showed that CR does not prevent age related declines but help to stabilize change which occurs during aging process. Moreover, earlier studies indicate that it can help to stabilize decline in learning and memory performance (Adams et al., 2008).

### **D. Effects on aging and longevity**

Until the late 1900s, CR was not widely discussed in the context of its potential effects on aging process as a scientific model (Roth et al., 2001).

Up-to-date literature suggests that CR increases the life span according to studies because reduction in energy intake provides fewer free radicals produced in mitochondria of cells, thus, cells are exposed to less oxidative damage. Moreover, the organisms show resistance to stress and thus lower plasma norepinephrine and blood pressure. All these improves cardiovascular functions especially when caloric

restriction and intermittent fasting (IF, reduced meal frequency) are applied together (Mattson and Wan, 2005). Therefore, CR is highly related with increased life span (Heilbronn and Ravussin, 2003).

Lipofuscin is a lysosomal waste which has high lipid, sugar and metal content and is known as a brownish pigment accumulating in tissues including skin. The accumulation of the lipofuscin is increased with aging and it is suggested that it may contribute to the impairment of cognitive functions (Flood et al., 1995). In some studies it was reported that long-term restricted feeding reduced lipofuscin accumulation in tissues including brain (hippocampus and frontal cortex) parallel to extending life span and increasing quality of life. (Moore et al. ,1995, , Idrobo et al., 1987). Dietary restriction reduces the energy intake and body temperature, thus, metabolic rate of the body is reduced. As it was discussed previously, reduced metabolic rate and ROS production can provide extended life span as a beneficial result of dietary restriction in rodents. However, we can not definitely state that in mammals there is a causal relationships between slower metabolic rate and extended life span. (McCarter et al., 1985, Piper and Bartke, 2008).

#### **1.4.3.Changing diet composition**

When the rats can choose the diet, they especially prefer high-carbohydrate diets based on sucrose or polycose (Glass et al., 1997). However, rats can maintain their diet as balanced meal. For instance, active rats prefer to consume more carbohydrate and fat and less protein than sedentary rats (Collier et al., 1969).

Macronutrient composition of the diet does not only influence the amount of energy intake of the subject but it also influences palatability of the food by affecting hedonic/reward aspects of the feeding behavior. NPY is linked to these aspects of feeding behavior and modulates the motivation of food ingestion so it is thus sensitive to diet composition (Beck, 2006). A negative correlation between hypothalamic NPY concentration levels and carbohydrate-to-fat ratio in the diet has been reported (Beck et al., 1992). According to this study, when a rat fed with

obesogenic diet from weaning to 2 months, in arcuate nucleus NPY level was decreased associated with hyperphagia (abnormally increased appetite). When rats have choice between high-fat and high-carbohydrate diet, their choice is reflected in paraventricular nucleus NPY concentration. Beck et al. suggest that these changes can be a regulatory mechanism against over-consumption of food as well as fat accumulation. The feeding period at the beginning of the dark period, rats which preferred carbohydrate have higher hypothalamic NPY concentrations (Jyanwar-Uniyal et al., 1993).

Up-to-date studies have shown that high-carbohydrate and high-fat diets have negative peripheral effects. These effects can be prevented by that consuming carbohydrates riched in fibers and daily exercise (Grimm, 1999).

Roberts et al. (2006) reported that when exercise intervention was applied together with a diet containing 12–15% of calories from fat (polyunsaturated-to-saturated fatty acid ratio 2.4:1), 15–20% of calories from protein, and 65–70% of calories from primarily unrefined carbohydrate, high in dietary fiber (>40 g/day) to the overweight or obese men, significant reduction was found in total cholesterol (total-C), LDL-C, TG, LDL-C-to-HDL-C ratio and both HDL-C and total-C-to-HDL-C . With the combined effect of the 21-day diet and exercise intervention, the body weight in subjects significantly reduced (BMI;  $P < 0.01$ ). In this study, researchers did not investigate independent effects of diet and exercise intervention which is supervised during the experimental period.

#### **1.4.3.1. The role in weight control**

Studies show that the consumption of low fat diet with the total reduction of energy intake provides more weight loss. This method is successful in overweight subjects (Bray et al., 2004). When it was analyzed for the rapid weight loss, it was shown that ad libitum, low fat, high carbohydrate diet may not be so effective as energy reduced (calorie counting) diet. However, this kind of diet provides more satiety (Shah et al., 1994).

Consumption of sweeteners (high fructose corn syrup and sucrose) in many populations increased day by day. This increased fructose consumption causes high triglyceride in blood and induces peripheral leptin resistance in less than 4 weeks. In a study, rats fed with fructose diet (60% kcal fructose and 10% fat) gained less weight than control group and decreased their food intake with leptin injection. Thus, fructose diet can prevent leptin resistance in high fat fed rats in this study. Therefore, they showed that dietary fructose has beneficial effects on metabolism (Haring and Harris, 2011). They tested whether leptin resistance was because of the relatively short period of consumption of a high-fructose diet in rats and whether both fat and fructose concentration increase in the diet would accelerate the onset of leptin resistance. Moreover, after consuming a low-fat/high-fructose diet for 3 weeks, rats in this experiments were resistant to peripheral injections of leptin.

#### **1.4.3.2. Effects on physiology and behavior**

Stark et al. (2000) reported that Sprague Dawley rats were fed with control diet (53% cornstarch, 10% sucrose, and 7% soybean oil), high fat diet (25% soybean oil, 35% cornstarch) or high carbohydrate (CH) diet (53% fructose, 10% sucrose) for 3 month period. Glucose tolerance tests were carried out on 3. and 9. weeks and triglycerol, glucose, serum insulin, and cholesterol levels were measured. Also Huang et al. (2004) carried out a similar experiment but during 2 month with high CH diet changed as 60% fructose and 20% lard. At the end of the 8th week, peritoneal fat tissue and liver were weighted and also plasma cholesterol, triglyceride, insulin and leptin levels were measured. These experiments suggested that both, high CH and high fat diet can result in elevated plasma glucose and also can cause impairment in pancreatic functions and glucose intolerance.

Many studies showed that high caloric diets inducing hyperglycemia and insulin resistance, at the same time, may cause damage to the hippocampal function and structure (Greenwood and Winocur, 2005; Kanoski et al., 2007). Hippocampus, a critical brain structure for learning and memory, was also reported to gate the input



from the prefrontal cortex or amygdala to nucleus accumbens. The latter reports also suggest a relationship between hippocampus and the the reward system (French and Totterdell, 2002; Kanoski et al., 2007). The potential adverse impact of high caloric diet on hippocampus was proved by Valladolid-Acebes et al. (2011) who reported that C57BL/6J mice fed with high fat diet (45% kcal fat) for 8 weeks manifested significantly poorer learning and memory than the control group maintained on a low-fat diet (10% kcal fat).

On the other hand, the role of insulin in neuroplasticity was demonstrated in hamsters maintained on high fructose diet (Mielke et al., 2005). In this study, in hamsters, fed high fructose diet, a significant reduction in insulin-mediated phosphorylation of two important proteins (threonine 308 and serine 473) and deficit in synaptic plasticity was observed. In another experiment carried out by the same group (Mielke et al., 2006), this time on C57BL/6 mice maintained on high fat diet (45% kcal fat) compared to control diet (5% kcal fat), a significant impairment of glucose regulation due to modified insulin-mediated signaling was found in the hippocampus. However, in this study, despite of profound peripheral changes, the high-fat diet had no significant effect on learning and memory.

Another study with different diet compositions (standart chow and water; high fat diet and water; high fat diet and fructose solution; standart diet and access to fructose solution as the only intake of water) applied to the mice for 3 months also showed that although saturated fat diet had wide peripheral effects, it had a relatively small effect on learning and memory when fructose solution was used as a motivational reward.(Messier et al., 2007). Thus, the results of high fat diet on hippocampus-dependent cognitive performance are inconsistent.

#### **1.4.3.3. Effects of diet composition on aging and longevity**

It is discussed that early studies with rodents showed that reduced food intake (Caloric Restriction) increases longevity. However, an interesting study done by Orentreich et al. in 1993 reported that when rats are deprived one of the essential

aminoacids: methionine, this can mimic the dietary restriction effects, including increased longevity. Miller et al. in 2005 supported this idea by applying methionine-deficient diet to mice. Interestingly, reduced protein diet (but not carbohydrates or fat) and thus reduced methionine intake can also suppress mitochondrial production of reactive oxygen species (ROS), reduce oxidative damage and increase longevity (Ayala et al., 2007).

### **1.5. Sleep and Circadian Rhythms**

Evidence for the relationship between sleep and metabolic and endocrine functions was reported more than four decades ago. It is clear that good sleep is necessary for metabolic health in the light of the up-to-date findings.

Sleep duration is important for reducing adiposity and highly related with weight loss from adipose tissue as a response to temporal caloric restriction. Sleep modulates 24 pattern of secretion of two key hormones; ghrelin with its orectic and leptin with its anorectic signalling (Ahima et al., 2000; van Dijk, 2001). Shorter sleep causes increased hunger because of the changes in neuroendocrine response in human; higher circulating concentrations of the orexigenic hormone, ghrelin; and reduced concentrations of the anorexigenic hormone, leptin especially when the subjects are on the calorie restricted diet. Thus, although the organisms who sleep less tend to lose weight almost at the same ratio with the organisms who sleep more, losing weight from adipose tissue is more difficult in sleep curtailment during calorie restricted diet (Nedeltcheva et al., 2010). They indicated that in the preservation of human fat-free body mass, sleep has a really important role during caloric intake reducing periods. However, when energy and sleep restriction applied together in overweight adults it is resulted in a modified state of negative energy balance characterized by decreased loss of fat and considerably increased loss of fat-free body mass. This was accompanied by markers of enhanced neuroendocrine adaptation to increased hunger, caloric restriction, and a shift in relative substrate utilization toward oxidation of less fat.

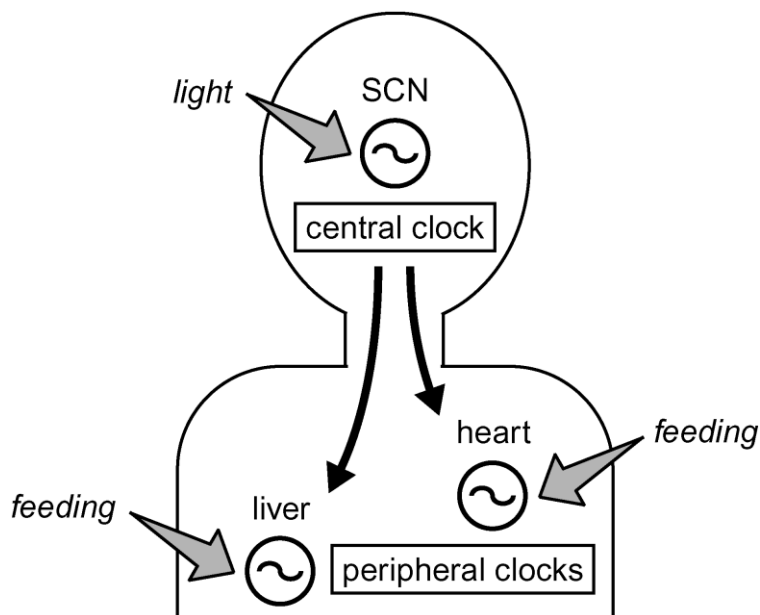
There is one important question to clear up: Is there a necessity of peripheral clocks for a proper alignment of behavioral states (sleep/wakefulness, fasting/feeding) with the peripheral metabolism in liver, muscle, adipose tissue and pancreas? (Huang et al., 2011). The circadian rhythmicity and sleep/wake homeostasis interaction is the reason of regulation of energy homeostasis as well as the influence of environmental factors such as stress, exercise, food intake, and postural changes are the other reasons that result from this interaction. On the other hand, it has been well documented that food intake regulation involves “hunger” and “satiety” signals sent by hypothalamus as well as by peripheral organs (Morselli et al., 2012).

As mentioned earlier, sleep is an important modulator for neuroendocrine system and because of that sleep loss causes many problems and increases the obesity risk. Moreover, short sleep duration causes increased level of ghrelin and decreased level of leptin, decreased insulin sensitivity and glucose tolerance, increased concentrations of cortisol on evenings (Beccuti and Pannain, 2011). Delayed feeding occurs during prolonged night-time wakefulness and it leads to desynchrony between peripheral clocks and central circadian (Bass and Takahashi, 2010). There is a link between circadian desynchrony and obesity, thus, obesity can represent a chronobiological disease (Garaulet et al., 2010). It has been demonstrated that sleep deprivation changes eating behavior by increasing caloric intake 14%, particularly for carbohydrate-rich nutrients (Tasali et al., 2009). On the other hand, sleep loss also affects energy expenditure and energy balance because sleepiness and fatigue increase sedentary behavior but decrease exercise-related energy expenditure (Schmid et al., 2009).

### **1.6. Time-Restricted Feeding as a Novel Approach**

The third dietary approach is the time-restricted feeding (TRF), however, until now, this issue has not been thoroughly investigated. Time restricted feeding limits the time and food duration and availability with no calorie reduction (Schibler et al., 2003; Hirota and Fukada, 2004; Cassone and Stephan, 2002). However, the effects of

TRF may be different depending on the timing for food availability and light conditions (Froy and Miskin, 2010). Several physiological activities (locomotor activity, hormonal secretions, body temperature, heart rate etc.) show biological rhythms controlled by a “master clock”. The central clock (“master clock”) being reset by light captured by retinal ganglion cells is localized in hypothalamic suprachiasmatic nucleus (SCN) and is controlling activity of multiple “peripheral clocks” (**Fig.1.4**).



**Figure 1.4.** Central and peripheral clocks in mammals (adapted from Hirota and Fukada, 2004)

It is suggested that biorhythms controlled by central and peripheral clocks may change with time-restricted feeding (Mistlberger, 1994; Hirao et al., 2006). Entrainment stimuli (zeitgebers) control the phase and period of circadian clocks in the brain and in peripheral organs and food intake provides cues and reveals physiological responses that can act as these stimuli (Mistlberger, 2011). In laboratory rodents, feeding time is a strong and dominant zeitgeber for peripheral circadian clocks. The reason of this is related with organism’s physiology. Organisms must adapt their physiology to the water and food absorption. Thus, the gastrointestinal tract absorbs

food metabolites, the pancreas secretes digestive enzymes, skeletal muscles must regulate glycogen synthesis and utilization, and the kidney controls glomerular filtration and urine production, depending on food and beverage intake. If nocturnal rats and mice are allowed to eat only during the day the phase of gene expression of all of these organs is inverted (Schibler et al., 2003). With forced change in feeding time, master and peripheral clocks may desynchronize because feeding is a dominant zeitgeber (Hirato and Fukada, 2004).

Thus, it has proven to be a strong entraining signal for peripheral oscillators controlling metabolism and behavior when restricting food intake to a few hours daily (Damiola et al., 2000; Díaz-Munoz et al., 2000; Stephan and Becker, 1989).

Other important thing is that rats and other species show food anticipatory activity (FAA) in daily mealtime under circadian (24h) schedule (Mistlberger, 1994). The presence of palatable foods causes the anticipatory behavior expression even if standard rodent chow is available ad libitum and nutritional value of the meal does not affect this food anticipatory behavior. If rats are restricted to some meals containing just two of three main nutrient groups (protein, fat, or carbohydrate), they will anticipate those macronutrients that presented in that regulated time (Mistlberger and Rusak, 1987; Mistlberger et al., 1990; Abe and Rusak, 1992). As it is known light is a quantifiable Zeitgeber, but there is a complication in the presence or absence of a meal, both in the nature of the food substance itself and in the behavior it elicits (Cassone and Stephan, 2002).

In the Minana-Solis (2009) study, the daily feeding was 2 hrs long. This is at the low end of the usual meal duration for a restricted feeding protocol. Some of the studies that show no influence of restricted feeding on the SCN used much longer feeding windows, up to 12 hours in the light phase. Also, depending on species and the starting weight of the animal, behavioral anticipation emerges after a variable number of days on restricted feeding.

There are some experimental studies demonstrating how changing time of feeding affects physiology and behavior. Comparisons between two nocturnal mice groups according to their sleep/wake cycle show that the group fed with high-fat diet during 12 hours dark phase gained less weight than the group fed with the same diet but only during 12 hours light phase (Arble et al., 2009). Similarly, mice (wild type) fed ad-libitum only during light phase of the sleep-wake cycle showed more weight gain than the mice fed during dark period only. It was also revealed that genetically obese mice with impaired diurnal feeding rhythm can be self-recovered for obesity and metabolic problems when they are fed only during dark phase (Masaki et al., 2004). These observations show that the timing of feeding and physical activities during 24-hr diurnal cycle is very important for body weight management..

In one of the studies, two diet types: ad-libitum feeding and caloric restriction with intermittent fasting (IF, alternate day fasting), were compared. In the intermittent fasting group, food was available during every other day but then they were getting twice more food than ad-libitum feeding group. The mice maintained on intermittent fasting diet showed extended life span as compared to the ad libitum-fed control even so there was no between-group difference in the overall calorie intake (Froy and Miskin, 2010). This diet type alike caloric restriction diet was reported to reduce insulin levels and serum glucose, protect cardiovascular system and increased resistance of neurons in the brain to excitotoxic stress. It was suggested that TRF by increasing neurotrophic factors may have neuroprotective effects against ischemic injury of the brain and neurodegenerative disorders. Its potential cardioprotective effects arise from decreased body weight, heart rate, and blood pressure (Anson et al., 2003; Ismayil et al., 2005; Donald et al., 2006).

It has been suggested that TRF can be used as a non-pharmacological prevention of obesity. According to this idea, it has been reported that high-fat diet (HFD) with TRF is less harmful than with ad libitum feeding (Hatori et al., 2012). In this study, HFD group was fed with 21% carbohydrates, 18% protein, 61% fat, and time-restriction was applied according to Zeitgeber times (Z13-Z21). The results of this

study showed that time-restriction with exercise has beneficial effects for preventing obesity because when HFD was applied with time restriction, rats did not gain as much weight as HFD ad libitum group.

### **1.7.Aim of the Study**

Today Body Mass Index data show an increasing tendency world wide . On the other hand an obese society means less healthy society with higher spendings on medical care and thus higher economic burden. Thus, new prevention strategies are needed. Time restricted feeding is a novel nonpharmacological approach to reduce obesity development. Up-to-date studies suggest that time restricted feeding without caloric restrictions can also regulate physiological parameters and control body weight. The aim of the present study was to test the effects of TRF correlated with circadian activity rhythm not only on weight management and metabolic/ endocrine indices such as blood glucose, lipids and leptin levels but also on a wide scope of behaviors including locomotor performance, anxiety levels and learning skills in the same middle-age overweight male Wistar rats.





## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Subjects

Twelve 13 months elderly male Wistar rats were used in the present study. Rats were divided into 2 groups: time restricted feeding (TRF) group (n=6) and control, ad-libitum (ADLIB) group (n=6) subjected to a standard diet. The attention was paid to have similar group mean body weights at the beginning of experiments.

During the experiments, rats were kept in the animal house, in the Department of Biological Sciences at the Middle East Technical University (METU), under 12 h/12 h light/dark cycle (lights on at 07:00 p.m., lights off at 07:00 a.m.), stable temperature ( $22 \pm 1^{\circ}\text{C}$ ) and controlled humidity. All animals had free access to water. The access to food (a standard lab chow) was free in ADLIB group and restricted to 8 h of the dark phase of the diurnal cycle in TRF group.

It has been found that a potentially addictive drug before the exposition of 8 hours in a daily period animals do not develop addiction. Because of this situation chronic overeating has also similar physiological pathways with drug addiction (Johnson and Kenny, 2010) it may be that the restriction period is more important limiting effects of food restriction in the weight gain than the part of the day that the eating is restricted to. Therefore, 8 hours restriction in the active phase (food seeking period) was applied in our study.

One month before starting the tests, light/dark cycle was reversed (lights off at 07:00 a.m., lights on at 07:00 p.m.) and the tests were carried out during the dark phase of the cycle using red light for illumination. Throughout the experiments, rats were housed individually in transparent plexiglass cages so they were able to see and hear

each other but at the same time it was possible to measure the exact amount of food consumed each day by the individual subjects. To control the body weight gain, rats were weighted at the same time of the day in everyday (between 16.00 and 17.00 p.m.).

### **2.1.1. Diets and Feeding Schedule**

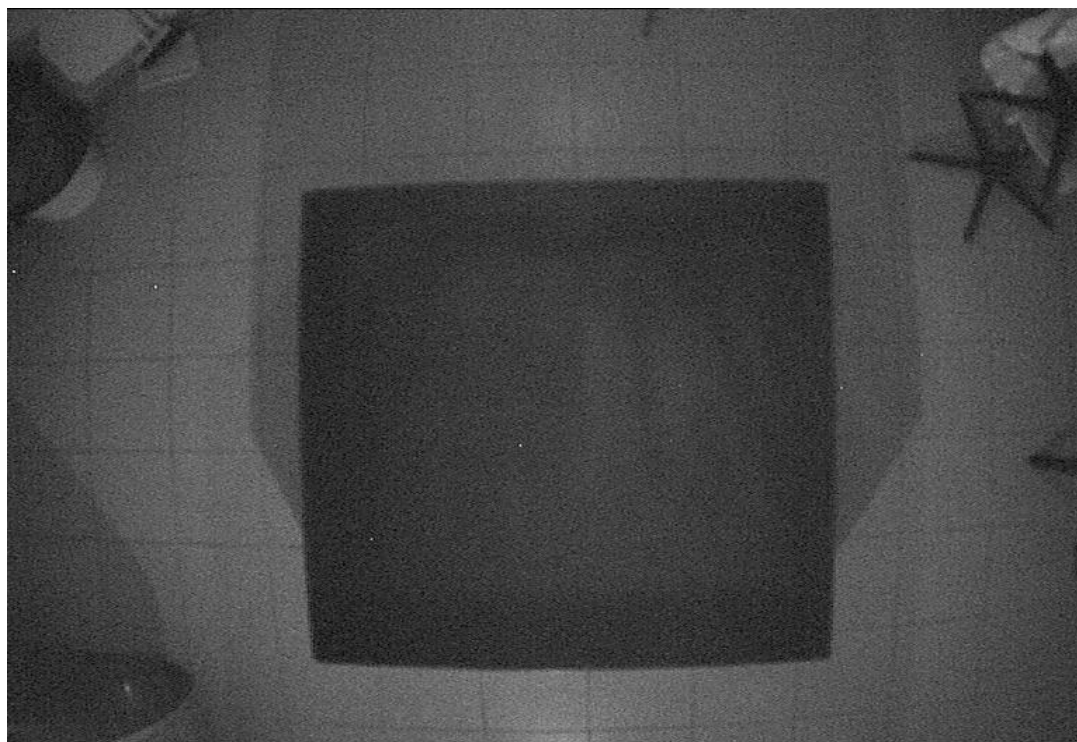
Diet content was the same for all rats (8.81 % raw cellulose, 24 % raw protein, 1.20 % calcium, 0.80 % phosphor, 1.50 % lysine, 0.57 % methionine and %15-20 corn, %10-30 wheat, %10-15 barley, %25-35 soybean pulp, %20-30 clover flour, %20-30 boncalite).. ADLIB group had continuous access to lab chow while in the TRF group the access to food was limited to 8 hours during the dark phase of the diurnal cycle between 8.00 a.m. and 4.00 p.m. (Panda et.al., 2012). . Time restricted feeding was applied for 90 days, a time period approximately corresponding to 10 years of life in human. Amount of food consumed daily by a single rat from the TRF group was calculated by subtracting the amount of food (g) still found at 4.00 p.m. from the amount of food provided by the experimenter at 8.00 a.m. In the ADLIB group, the amount of food left at 8.00 a.m. was subtracted from the amount of food delivered at 8.00 a.m. on the previous day. Rats were weighted daily at 4.00 p.m.

## **2.2. Apparatus used in behavioral testings**

### **2.2.1. Open Field Box**

Open Field (OF) apparatus is routinely used for measuring locomotor activity and anxiety in small rodents (Hall et.al., 1932). Open Field Box was made from the plain wood painted black in the METU wood atelier (Fig. 2.1). It had a square shape with side walls 120 cm long and 50 cm high. A computerized video-tracking system (EthoVision System 3.1 by Noldus Information Technology, Holland) was used to monitor animals' movements. The OF was divided by virtual lines into 2 equal squares, the biggest square that covers the all area was the peripheral zone, and the small one which placed inner part of the big square was the central zone of the arena.

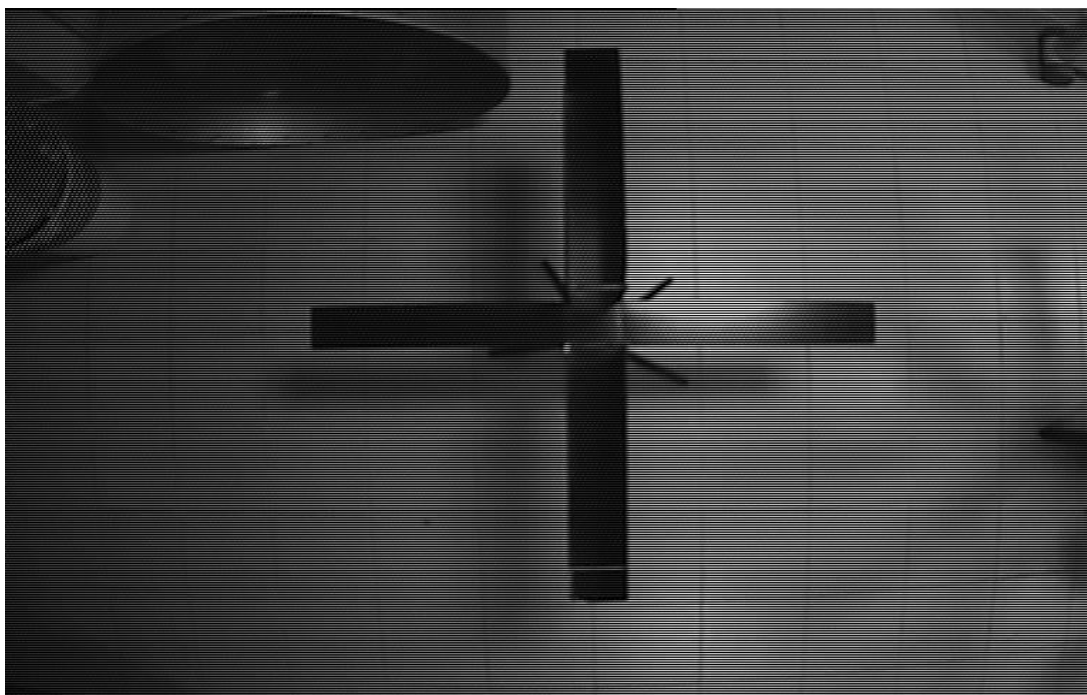
The records of the system were time spent and distance moved (ambulation) in each of the zones for 10 min. in 5 min. intervals.



**Figure.2.1.** Open Field Apparatus

### **2.2.2. Elevated Plus Maze**

The elevated plus maze (EPM) is designed for measuring anxiety levels in small rodents. The EPM elevated 80 cm above the floor was made of polyester and consisted of a central platform (10 × 10 cm), two open arms (50 × 10 cm) and two closed arms (50 × 10 cm) with the latter arms surrounded by dark, 30 cm high Plexiglas walls with no ceiling. The arms were arranged in a cross shape with the two open arms and two closed arms facing each other (Fig. 2.2).



**Figure 2.2.** Elevated Plus Maze Apparatus

### **2.2.3. Accelerod**

Rotarod/accelerod apparatus (Commat Ltd, TR) was used to measure sensorimotor coordination, muscle strength, , and motor learning as previously defined (Dursun et al., 2006). The apparatus had a rod of 6.5 cm in diameter rotating with pre-selected speed. In this apparatus. Four animals could be tested at the same time (Fig. 2.3). The floor of the apparatus was made of a metal grid. On the first, shaping session, it was kept under the mild electrical voltage ( 1 mA) to motivate the animals to staying on the rod as long as possible.



**Figure 2.3.** Accelerod Apparatus

#### **2.2.4. Morris Water Maze (MWM)**

The water maze constituted of a circular metal tank 60 cm high and 150 cm in diameter . The tank was filled by water up to 45 cm (Fig. 4). The water was made opaque with a non-toxic food paint. The temperature of the water was maintained at 22°C ( $\pm 1$ ) by an automatic heater. A movable transparent plexiglas platform (10x10 cm) was placed in the tank 2-3 cm below the surface of the water such that the animal could not see it but could easily climb on it for escaping from the water. The pool was virtually divided into four quadrants (NE, NW, SE, and SW) and the plexiglass platform was placed in the center of one of these four quadrants. Computerized video tracking system (EthoVision System 3.1) was used to track the animal in the pool and to record Distance Moved, Escape Latency, and Swim Velocity. The recording was stopped by the system whenever the animal found the

platform. The experiments were carried out in the room which is furnished with several immobile extra-maze cues such as sink, posters, window that could be used as a spatial reference frame in place learning (an allothetic paradigm defined as object-centered strategy of pathfinding). The room was illuminated with a diffused red light from two sides of the pool.



**Figure 2.4.** Morris Water Maze Apparatus

### 2.3. Experimental Procedure

**Table 2.1.** Experimental Design and Time Schedule

<b>Experiments</b>	<b>Days</b>
Handling and Habituation	7
Open Field Testing	1
Rotarod	3
Morris Water Maze	7
Blood Collection	1
Diet (TR and ADLIB)	90
Open Field Testing	1
Elevated Plus Maze	1
Rotarod	3
Morris Water Maze	6
Blood Collection	1

#### 2.3.1. Handling and Habituation

Habituation to the new environment and reversed light/dark cycle lasted 1 month. Before starting experiments. Additionally, prior to the experiments, rats were handled daily (1 min each) for 7 consecutive days.

#### 2.3.2. Behavioral Tests

In this study, behavioral tests lasted 11 days and were done twice; before and after the diet implementation, when the animals were 14 and 18 months old, respectively.

Each time, behavioral tests were run in the same order and included Open Field Testing (OFT) for measuring the general locomotor activity/ anxiety, Elevated Plus Maze (EPM) for measuring the anxiety, Rotarod/Accelerod for measuring the muscle strength/sensorimotor coordination and motor learning, and Morris Water Maze (MWM) for measuring spatial learning and memory.

### **2.3.2.1. Open Field Testing**

Open Field Testing is used to measure locomotor activity and anxiety. In this apparatus, the behavior of an animal is dependent on three factors: its spontaneous locomotor activity, internal urge to explore a new environment (exploratory drive) and anxiety caused by the exposure to a large, illuminated open field. Before the testing, the arena was divided on the computer screen into two regions by imaginary squares of which central square assigned as “Central Zone” and the one placed next to the walls as “Peripheral Zone”. Each animal stayed in the OF for 10 min and the following measures were automatically taken for the consecutive 5 min time intervals by the computerized video tracking system (EthoVision System 3.1):

1. Distance moved in Peripheral Zone (cm)
2. Distance moved in Central Zone (cm)
3. Total distance moved in the whole Arena (cm)

### **2.3.2.2. Elevated Plus Maze**

On the following day, rats were tested in the EPM. On a single day testing session, each animal was placed in the center of the maze while they are facing an open arm. Rats were allowed to explore the maze for 5 min. During this time, the total time spent in closed and open arms separately, and total time spent on the central platform were recorded by the computerized video tracking system (EthoVision System 3.1). The EPM tests were carried out as previously described (Elibol-Can et al., 2013).



### **2.3.2.3. Accelerod Test**

The test was repeated during 3 consecutive days. On the first day of this test (the shaping training), the rod rotation was increased from 0 to 40 rpm linearly (revolution per min) with acceleration of 2.0 m/s<sup>2</sup> (meter/second square). Animals stayed at the rod until they touch down to the accelerod floor or maximum for 2 min. When they fell down onto the grid floor they received for 3 s an electric shock of 1 mA (miliampere) intensity. On the second day of the test, no electrical shock was applied and the rod rotation rate was stepwise increased from 0 to 60 within 2 min. The conditions on the last, third day of the test were the same as on the second day.

On each training day, animals were given two trials 10 minutes apart. The built-in timer on the accelerod measured automatically the time that rats stayed on the rod..

### **2.3.2.4. Morris Water Maze**

Before the diet, after the end of the Open Field Testing, and after the diet, on the completion of the Elevated Plus Maze, the Morris Water Maze testing began.

#### **2.3.2.4.1. Shaping Training**

On the first two days of the water maze procedure, shaping training took place to get the animals used to a novel environment and teach them to climb the platform. A single training session was composed of 4 trials with 8-10 min inter-trial intervals and rats were taken to the test in the same order and at the same time of the day. On the consecutive shaping trials, the platform which was raised and placed 30 cm from the edge of the pool in a position randomly. Animals were released into the water from different starting points: first in the closest point to the platform, then from a distance that is gradually increased from the platform. An animal swam in the water until it found the platform in each time of the test. If an animal failed to find the platform within this time it was gently guided to it by the experimenter and allowed to stay on the platform for 10s. On the shaping sessions, the curtains were drawn

around the circular pool not to let the animals see the distant visuo-spatial cues belonging to the experimental room (Dursun et al., 2006, Elibol–Can et al. ).

#### **2.3.2.4.2. Place Learning (Acquisition Training)**

After the shaping training, curtains were removed and animals were exposed to the distal cues in the room. This training was continued for four days with four trials per day . Inter-trial intervals were 8-10 min. On four consecutive trials, animals were released to the pool according to the four different starting points (NE, SE, NW, SW), each time facing the side wall of the pool. They were given 60 s to find the hidden platform which was moved to a new quadrant after the shaping training and remained in the same position throughout the acquisition training. During the training trials, the experimenter was standing near the computer always in the same place.

On each trial, the video-tracking Noldus EthoVision 3.1 system recorded the following measures:

1. Swim distance moved (cm),
2. Swim velocity (cm/min),
3. Mean escape latency (sec).

When dividing the animals into ADLIB and TRF groups attention was paid to have similar learning scores in both subgroups.

#### **2.3.2.4.3. Probe Trial**

After the completion of the acquisition training, on the following day, a probe trial was applied to test whether the animals has learned the place of the hidden platform. The real platform was removed and an imaginary circle measuring 40 cm in diameter and called “Annulus 40” was drawn on the computer screen around the previous platform position . Only one 60 s probe trial was applied and the video-tracking system automatically recorded the time spent and the distance swam in A. the platform quadrant and B. Annulus 40. The frequency of entering the platform quadrant and the distance moved in the opposite quadrant were also registered

#### **2.3.2.4.4. Memory Retention Test**

Memory retention test was applied 3 months after the acquisition training, upon the completion of the diet period, in order to test the long-term place memory. After three months, animals were released to the pool with the same platform position as before the treatment.

On the following 4 days, the acquisition training with a new platform position was carried out observing the same training rules as before.

#### **2.3.3. Blood Sampling and Biochemistry**

Animals were taken to the operation room for blood sampling under the red light. An anesthetic cream was applied to the tail and blood samples were taken from the tail vein. The samples were collected into centrifuge tubes and then centrifugated at 4000 rpm for 8 min. After the centrifugation, supernatants were taken into serum tubes with red caps and stored at +4°C for 3-4 days until sent for analysis.

Blood samples were tested by Duzen Laboratory Group, Ankara. HDL (High Density Lipoprotein), LDL (Low Density Lipoprotein), V-LDL (Very Low Density Lipoprotein), Total Cholesterol, Triglyceride and Leptin levels were identified. Leptin concentrations were measured by using BioVendor Leptin Detection Kit and the other biochemistry parameters were measured by autoanalyzer in Duzen Laboratory.

Blood samples were taken from the rats before and after the diet implementation at the same time of the day.

#### **2.3.4. Data Analyses**

In the behavioral data analyses, group means  $\pm$ SEM were calculated and two-way repeated measure ANOVA was applied to assess the effects of time and treatment. The statistical analyses and the graphics were made using Graft Pad Prism 5 program

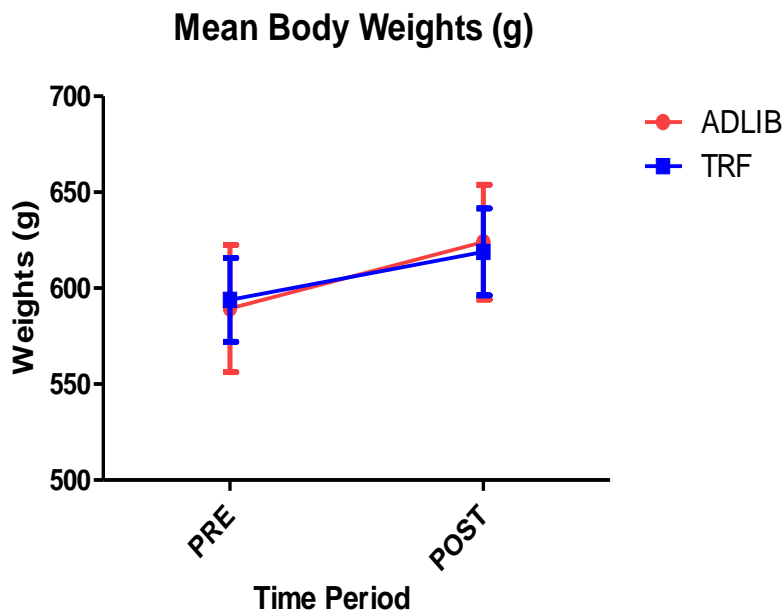
For the evaluation of blood biochemistry parameters, HDL, LDL, V-LDL, Total Cholesterol, Triglyceride and Leptin Wilcoxon test for paired comparisons was used. The degree of significance in the data determined by the “p” value. If the “p” value was less than or equal to 0.05, the difference was accepted as significant statistically. The criterion of statistical significance was  $p=0.05$ .

## CHAPTER 3

### RESULTS

#### 3.1. Changes in the Body Weights

The body weight of the rats was controlled daily throughout the whole experiment. Fig. 3.1. presents mean body weights (g) calculated for the five days before the diet and the last five days of the diet in the experimental group and for the corresponding time periods in control (ADLIB) group. As seen from the graphic, in both groups, with age, an increase in animals' body weight was observed. Two-way repeated measures ANOVA applied to these data revealed the time effect significant ( $F_{(1,10)}=19.49$ ,  $P=0.0013$ ) while treatment effect insignificant. The time x treatment interaction was also insignificant.

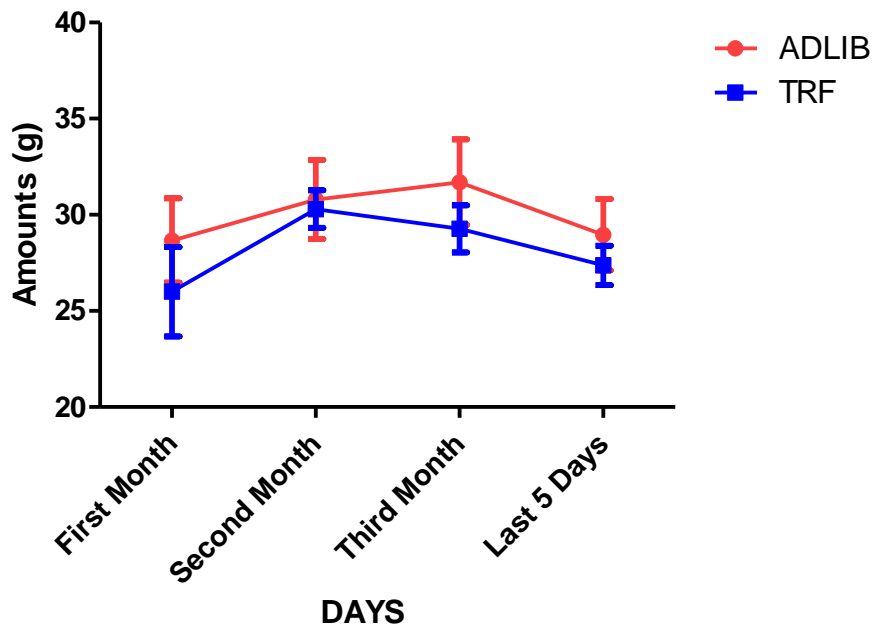


**Figure 3.1.** The mean body weights ( $\pm$ SEM) calculated for the five days prior to the diet and the last five days of the diet period in the ad-libitum (ADLIB) rat group and for the corresponding time periods in control, time-restricted feeding (TRF) group. Error bars denote SEM.

### 3.2. Changes in the Amount of Daily Consumed Food

Amount of food daily consumed by the rats was estimated throughout the diet period. Fig. 3.2. presents mean amount of food consumed daily by each group during the first five days of the 1st, 2nd, and 3rd diet month and the last five days of the diet period. Two-way repeated measures ANOVA with days as repeated measure and group as independent factors revealed the days effect significant ( $F_{(3,30)}=7.239$ ,  $P=0.009$ ) but the main group effect and the day x group interaction insignificant. In both groups, the daily food consumption increased during first two months and then showed decreasing tendency.

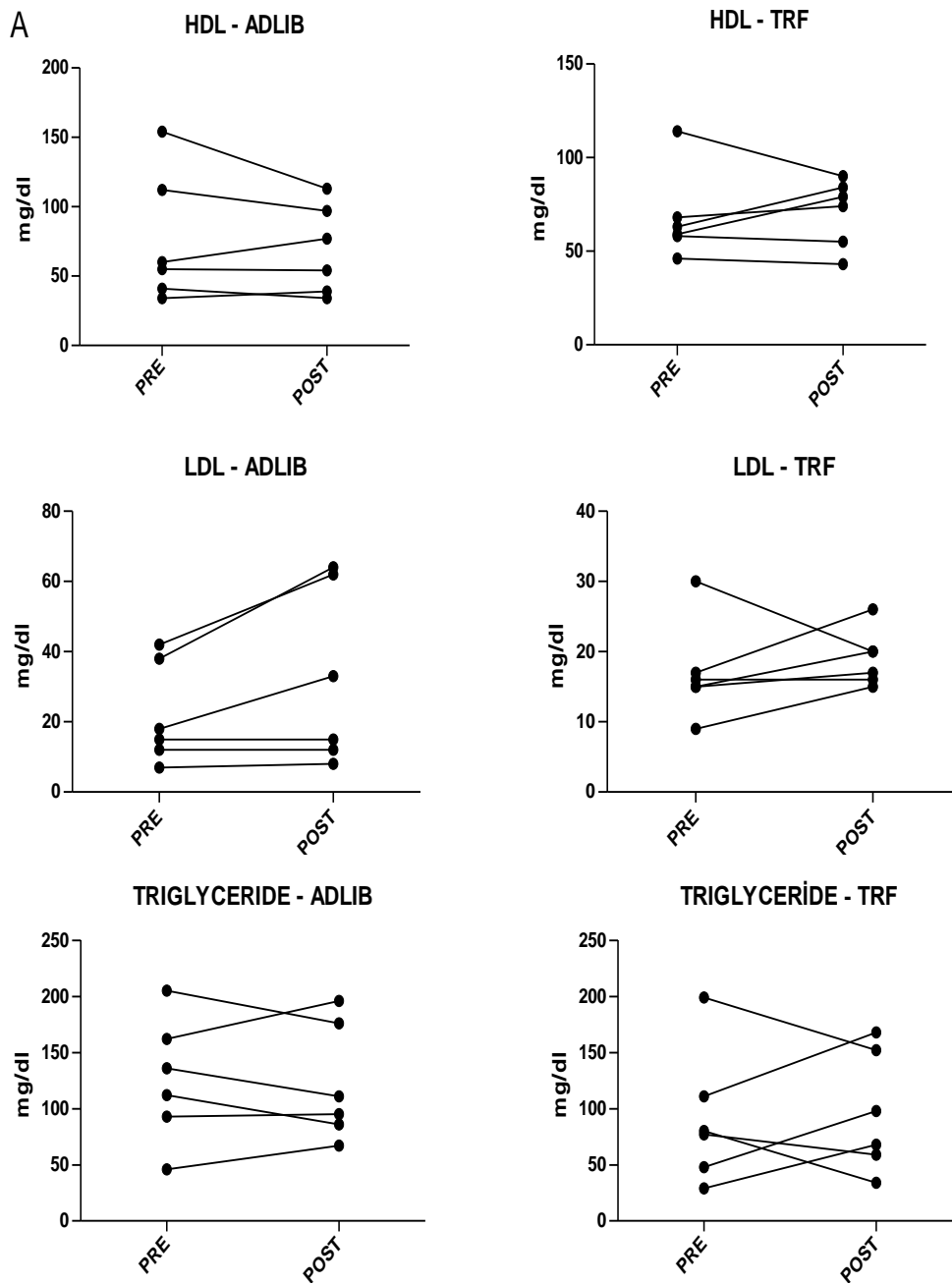
### Mean Amounts of Daily Consumed Food (g)



**Figure 3.2.** Mean amount of daily consumed food ( $\pm$ SEM) in ad-libitum (ADLIB) and time-restricted feeding (TRF) rat groups for the first five days of the 1st, 2nd and 3rd diet month, and for the last five days of the diet period. Error bars denote SEM.

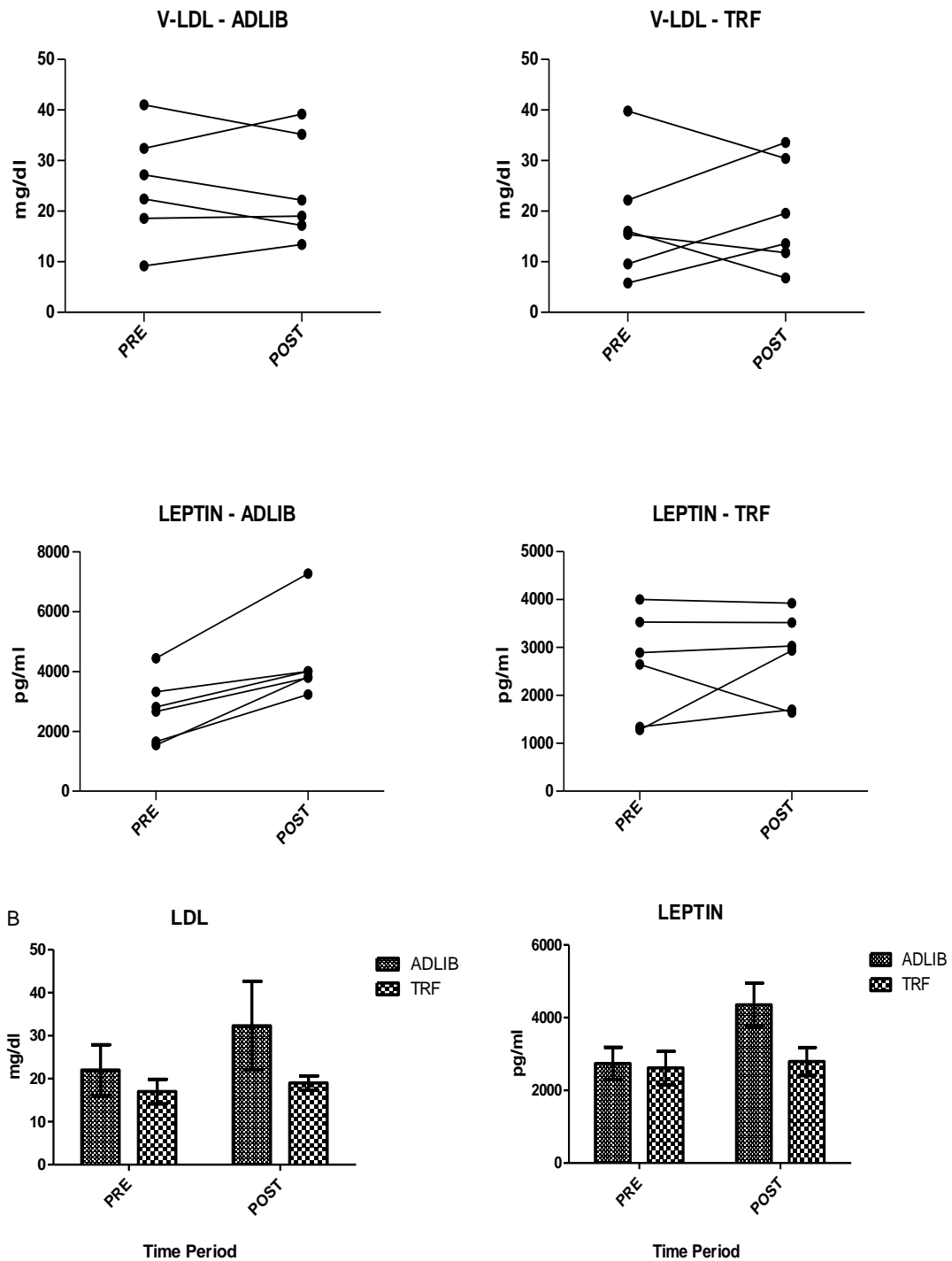
### 3.3. Values of selected biochemical parameters of bood serum

Before beginning of the diet and after 3 month of the diet period, upon the completion of behavioral tests, blood samples from the rats were collected and the concentrations (mg/dl) of LDL (Low Density Lipoprotein), HDL (High Density Lipoprotein), V-LDL (Very Low Density Lipoprotein), Triglyceride and and the concentration (pg/ml) of Leptin were estimated in 1 ml serum samples. Leptin levels were measured using BioVendor Leptin Detection Kit with 400-4000 mg/dl sensitivity range.



**Figure 3.3.** Individual estimates of lipids and leptin (A) and group means of LDL and leptin serum concentrations (B) in ADLIB and TRF rat groups prio and after the diet period.





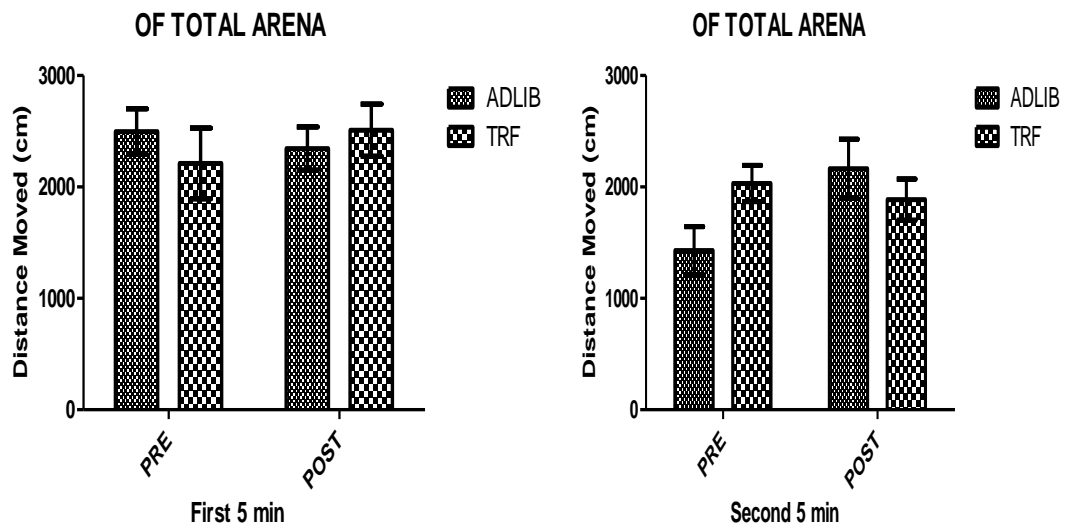
**Figure 3.3 (Continued).** Individual estimates of lipids and leptin (A) and group means of LDL and leptin serum concentrations (B) in ADLIB and TRF rat groups prio and after the diet period.

Wilcoxon test for paired comparisons was performed on LDL, HDL, VLDL, triglyceride and leptin data. According to the test results, only the serum levels of leptin in ADLIB group showed significant increase over 3 months corresponding to the diet period in TRF group ( $P=0.0313$ , two tailed). The rise in LDL levels observed in ADLIB but not TRF group across 3 months period was marginally significant ( $P=0.1250$ ) (see Fig 3.3.B). Two-way repeated measures ANOVA performed on the LDL and leptin data revealed insignificant group effect ( $F_{(1,10)}=1.225$ ,  $P=0.2943$ , and  $F_{(1,10)}=1.798$ ,  $P=0.2096$ , respectively) and significant time factor for both LDL and leptin levels ( $F_{(1,10)}=5.166$ ,  $P=0.0463$  and  $F_{(1,10)}=13.81$ ,  $P=0.004$ , respectively). These results indicate tendency towards time- dependent increase in both LDL and leptin levels in ADLIB but not so much in TRF group.

### **3.4. Results of Behavioral Tests**

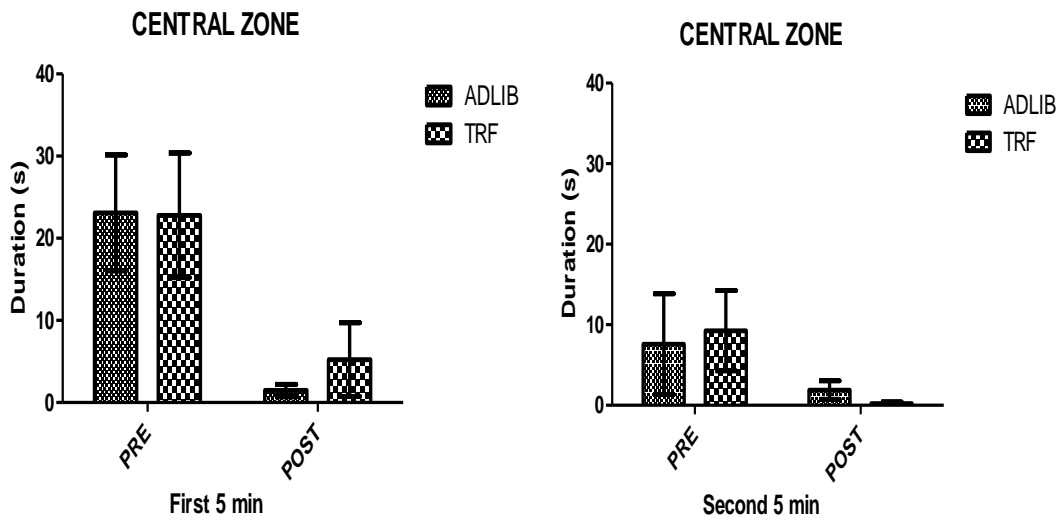
#### **3.4.1. Open Field Test**

The open field test was carried out twice: once prior to, and the second time after the diet period. The animals locomotor activity (distance moved) and total time spent in zone were recorded for the whole arena and for two imaginary zones of the OF: central and peripheral, for two consecutive 5 min time intervals, independently. The distance moved was accepted as an index of animals' locomotor activity. Two-way repeated measures ANOVA (time x diet) was performed on the distance moved in the whole arena for the two 5 min intervals, independently. According to the results of statistical analyses, neither the time factor nor the diet type had significant effect on the animals' overall locomotor activity (Fig.3.4.).



**Figure 3.4.** The mean distance ( $\pm$ SEM) moved in the total arena, during the consecutive 5-min time intervals prior to and after the diet period in ad-libitum (ADLIB) and time-restricted feeding (TRF) rats. Error bars denote SEM.

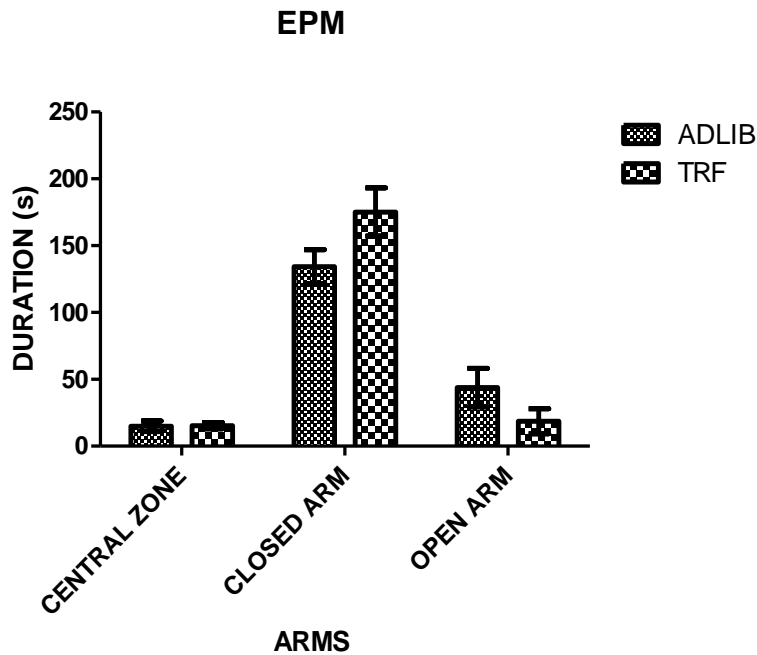
The time spent in central zone was accepted as the index of anxiety. As it can be seen from the Fig.3.5., in both groups, the time spent in the central zone prior to diet was much longer than three months later. Two-way repeated measures ANOVA (time x diet) performed for the time spent in the central zone during the first 5 min interval revealed time effect significant ( $F_{(1,10)}=16.85$ ,  $P=0.0021$ ) pointing towards a decrease in exploratory behavior upon the second exposure to the OF. The effect of type of the diet remained insignificant. The same analysis done for the 2<sup>nd</sup> 5 min interval in the central zone revealed neither the effect of diet nor the effect of time..



**Figure 3.5.** The mean time ( $\pm$ SEM) spent in central zone of the OF, during the consecutive 5-min time intervals prior to and after the diet period in ad-libitum (ADLIB) and time-restricted feeding (TRF) rats. Error bars denote SEM.

### 3.4.2. Elevated Plus Maze Test

EPM test was run only once, after the 3 months of differential diet applied to ADLIB and TRF rat groups. In this test, as expected, all animals spent more time in closed arms (Fig.3.6.) with TRF group spending relatively less time in the open arms compared to ADLIB group. Two-way ANOVA (arm of the plus maze x diet) revealed the effect of arm significant ( $F_{(2,29)}=91.47$ ,  $P<0.0001$ ) while the effect of group remained insignificant.



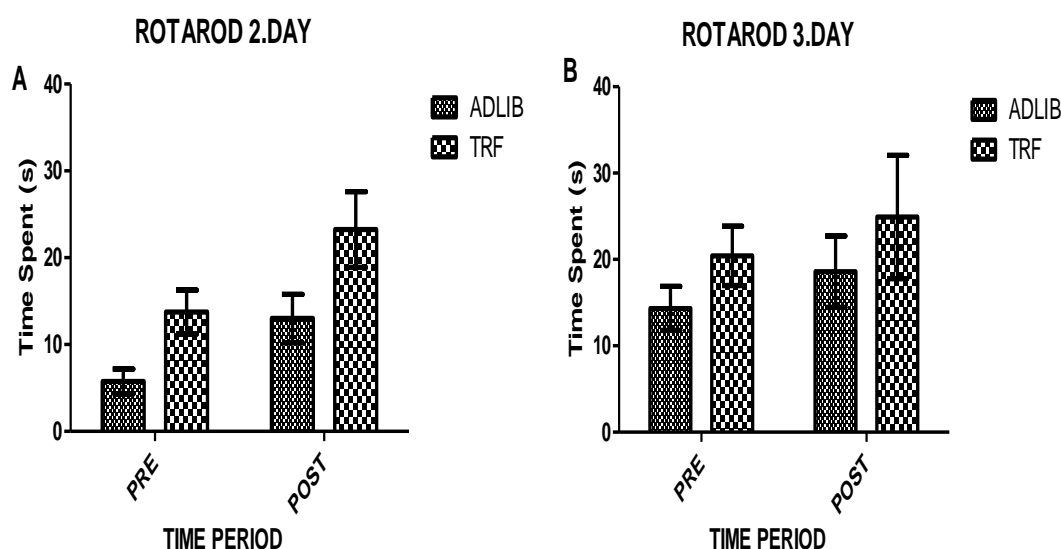
**Figure 3.6.** Comparison of the animal’s behavior in the elevated plus maze test as a function of the diet type (ADLIB vs TRF). The bars represent mean time spent in open arms, closed arms, and central zone of the EPM. Error bars denote SEM.

### 3.4.3. Sensorimotor Performance on the Accelerod

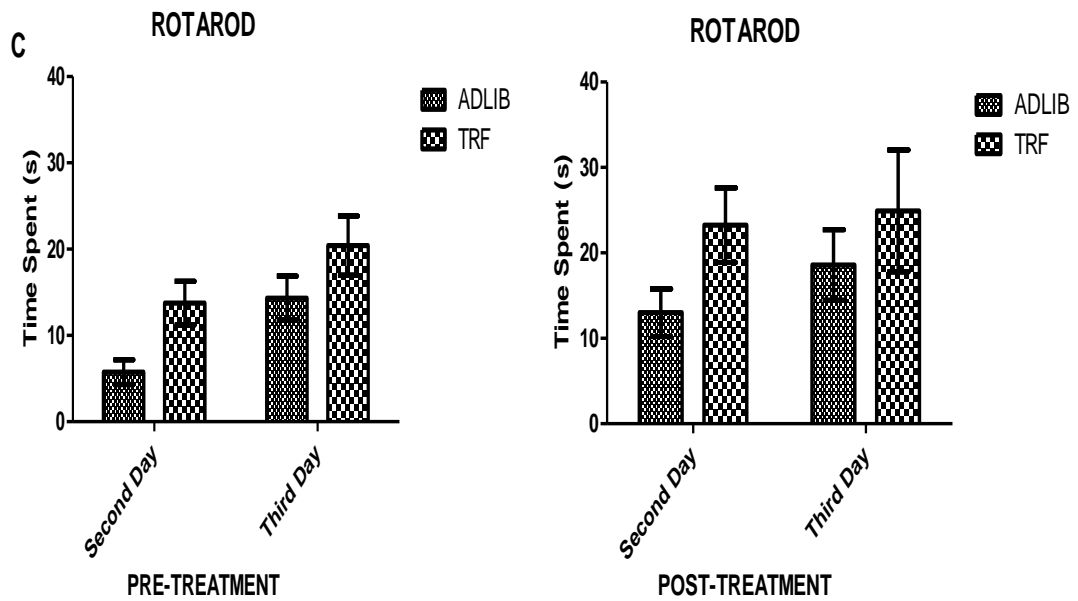
Accelerod test was applied before and after the diet, each time for three consecutive days, two trials each day. The first day on accelerod was considered a shaping training with a low electrical shock applied each time the animal fell from the rotating rod onto the grid floor. This training aimed to motivate the animal to make an effort not to fall from the rod. The data collected during the shaping training was not used in the behavioral analyses.

As seen from Fig.3.7, in both groups, the mean time spent on the accelerator increased on the 3rd testing day compared to the 2nd day, and on the 2nd day of testing, it was higher during post-diet period comparing to the period prior to the diet. However, despite of semi-random selection of animals for the ADLIB and

TRF groups, the rotarod performance of TRF group was from the beginning of experiments better than that in ADLIB group which was masking the potential effect of diet on animals motor skills and motor learning. Two-way repeated measures ANOVA performed for each testing day independently, confirmed a significant group effect and significant time effect on the 2nd testing day ( $F_{(1,10)}=7.79$ ,  $P=0.0191$ , and  $F_{(1,10)}=10.12$ ,  $P=0.0098$ ). In contrast to this, the group and time effect were insignificant for the 3rd day of testing. Two-way repeated measures ANOVA was also performed to compare the 2nd and the 3rd day performances during pre- and post-diet periods independently (Fig.3.7.C). In this analysis, group factor was yielded insignificant and the time factor was shown to be significant for the pre-diet period only ( $F_{(1,10)}=37.00$ ,  $P=0.0001$ ).



**Figure 3.7.** Mean time ( $\pm$ SEM) spent on the rotating rod until falling down with accelerated speed gradually increases from 0 to 60 rpm in 2 min, for pre- and post-diet period and each diet group, independently. A: pre- post comparison for the 2nd day of testing; B: pre- post comparison for the 3rd day of testing; C: day 2 versus day 3 comparison for pre- and post-diet testings, independently. Error bars denote SEM.

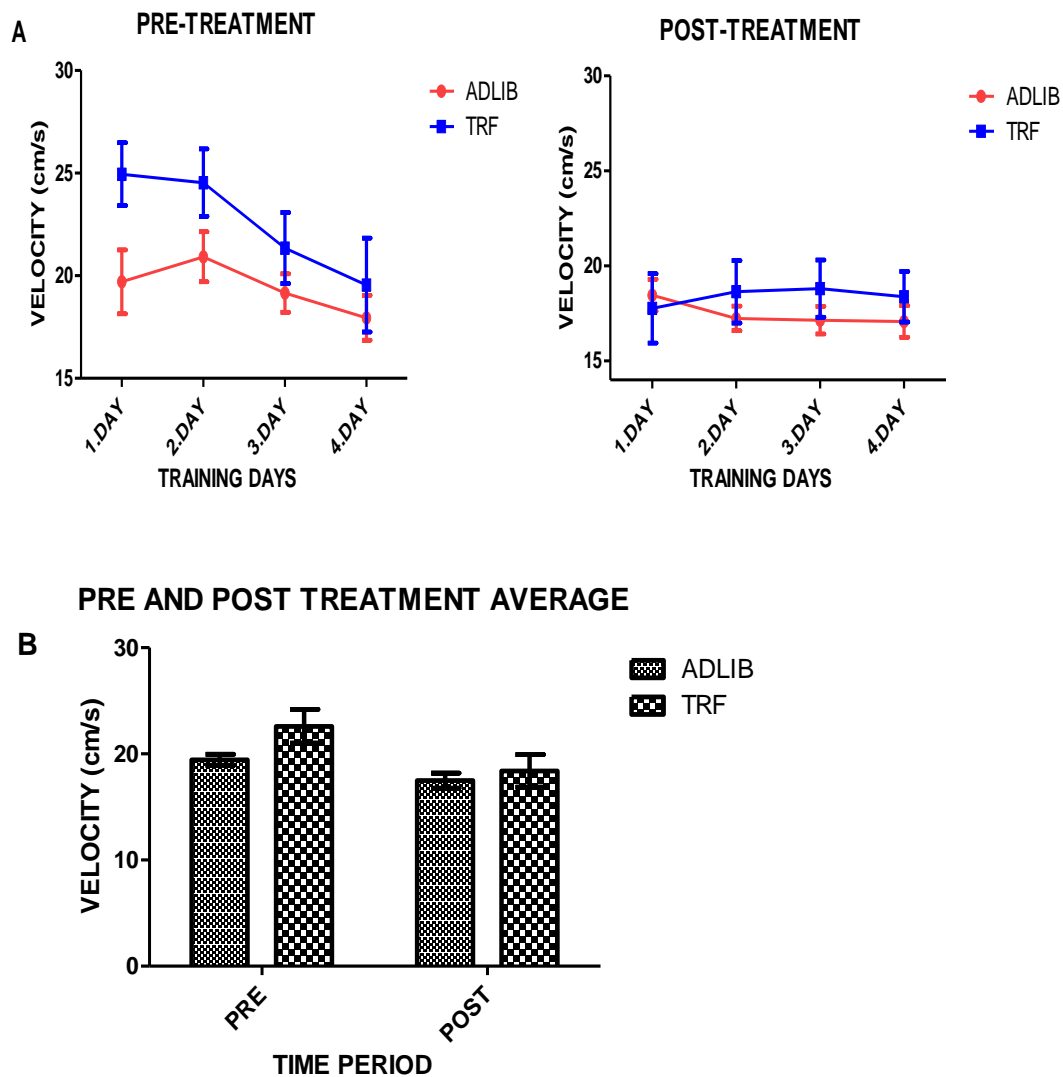


**Figure 3.7 (Continued).** Mean time ( $\pm$ SEM) spent on the rotating rod until falling down with accelerod speed gradually increases from 0 to 60 rpm in 2 min, for pre- and post-diet period and each diet group, independently. A: pre- post comparison for the 2nd day of testing; B: pre- post comparison for the 3rd day of testing; C: day 2 versus day 3 comparison for pre- and post-diet testings, independently. Error bars denote SEM.

### 3.4.4. Learning Tests

#### 3.4.4.1. Classical MWM Training

##### 3.4.4.1. 1. Swim Velocity



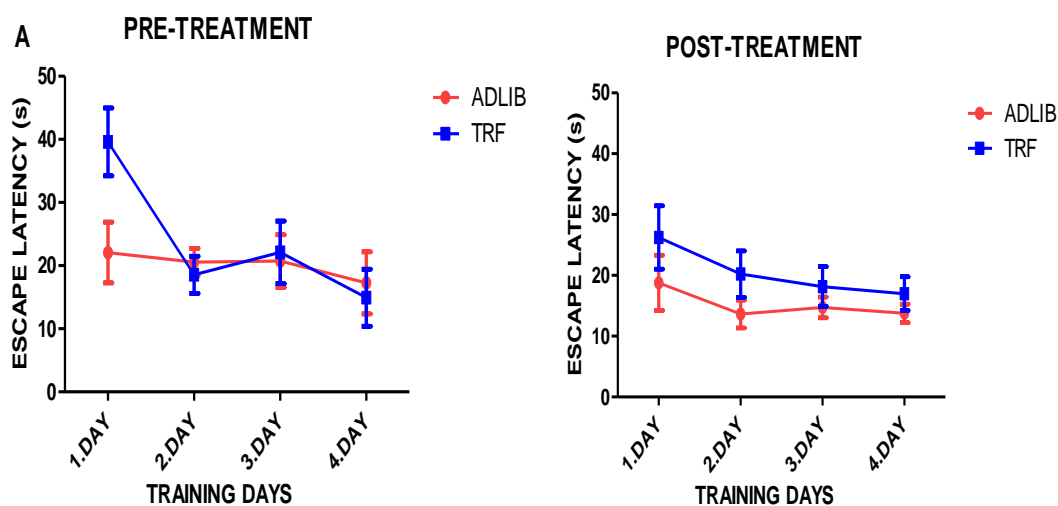
**Figure 3.8.** A. Mean swim velocity ( $\pm$ SEM) in the total arena calculated for each training day and each diet group, independently, during pre- and post-treatment training. B. Comparison of the group mean velocities calculated for the whole training period before and after the diet, independently. Error bars denote SEM.



Two-way repeated measure ANOVAs performed for pre- and post-diet training independently, confirmed significant effect of day on the swim velocity during the MWM training prior to the diet ( $F_{(3,30)}=5.169$ ,  $P=0.0053$ ) but not during the post-diet MWM training (see Fig.3.8 A.) Two-way repeated measures ANOVA performed on individual swim velocity means calculated for the whole pre- and post-diet training periods, independently, did not reveal significant group difference in swim velocity (cm/min) with significant time effect ( $F_{(1,10)}=41.29$ ,  $P<0.0001$ ) only (see Fig.3.8 B.).

### 3.4.4.1. 2. Escape Latency

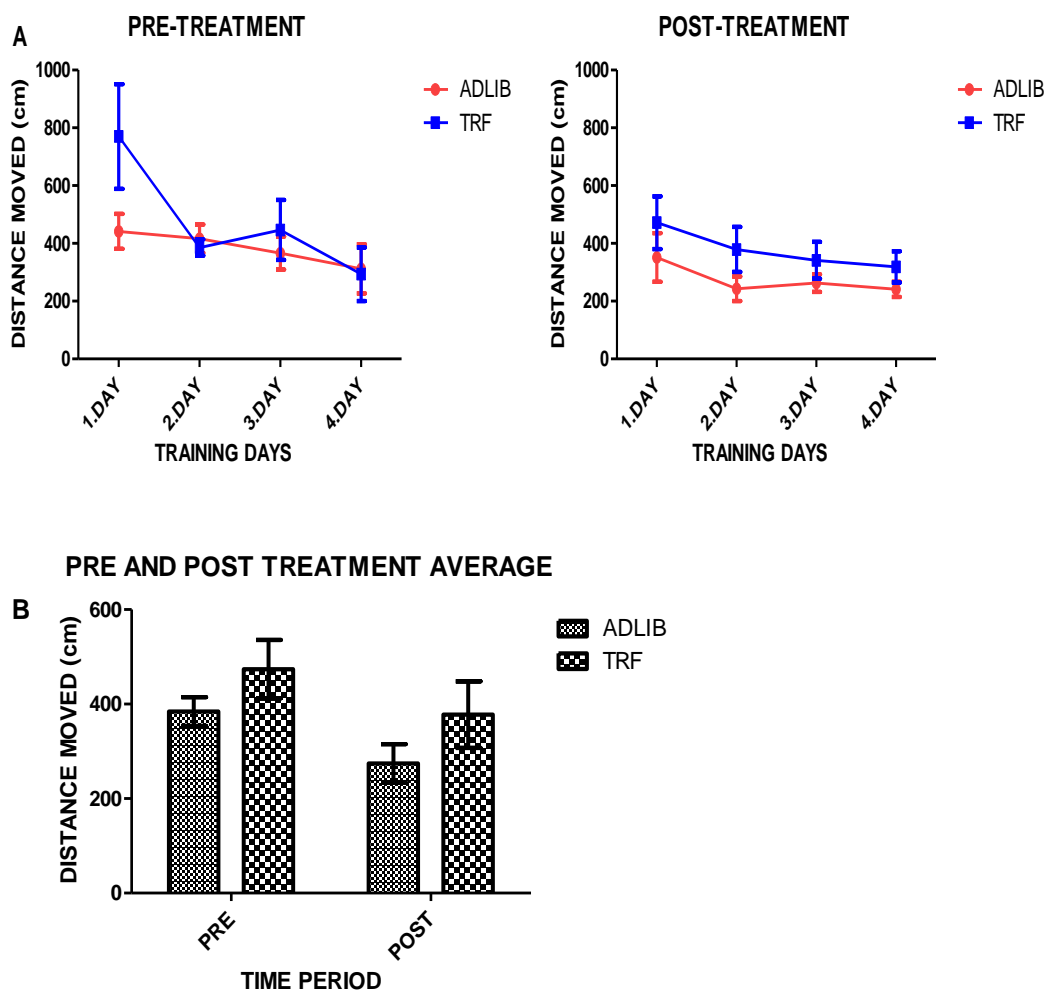
As seen from the Fig.3.9.A., in both groups, a decrease in the swim latency to reach the hidden platform (escape latency) was observed across the days of training, both before and after the diet period. Repeated measures ANOVA performed for pre- and post-diet training, independently, confirmed the significance of the day factor ( $F_{(3,30)}=5.891$ ,  $P=0.0028$  and  $F_{(3,30)}=7.849$ ,  $P=0.0005$ , respectively) with group (diet) effect insignificant.



**Figure 3.9.** Mean escape latency ( $\pm$ SEM) to reach the invisible platform calculated for each training day and each diet group, independently, during pre- and post-treatment training. Error bars denote SEM.

### 3.4.4.1. 3. Swim Distance To The Hidden Platform

As seen from the Fig.3.10., in both groups, a decrease in the swim distance to the hidden platform was observed across the days of training, both before and after the diet period.



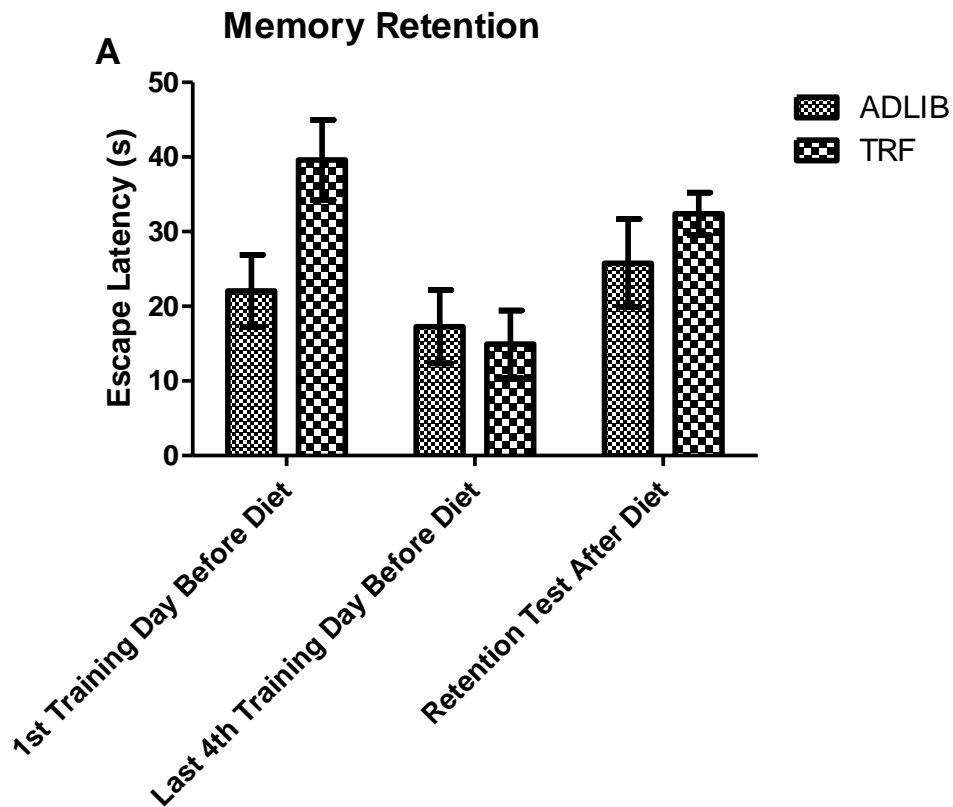
**Figure 3.10.** A. Mean swim distance ( $\pm$ SEM) to the hidden platform calculated for each training day and each diet group, independently, during pre- and post-treatment training. B. Comparison of the group mean swim distance calculated for the whole training periods before and after the diet. Error bars denote SEM.

Repeated measures ANOVAs (group x training day) run on the distance values calculated for pre- and post-diet training independently (see Fig.3.10.), yielded the effect of day significant during both pre- and post-diet training ( $F_{(3,30)}=3.839$ ,  $P=0.0194$ , and  $F_{(3,30)}=7.454$ ,  $P=0.0007$ , respectively) with group effect insignificant. Two-way repeated measures ANOVA with (group x training period as independent factors) also confirmed the significance of the training period effect only ( $F_{(1,10)}=23.60$ ,  $P=0.0007$ ) only (see Fig. 3.10.B).

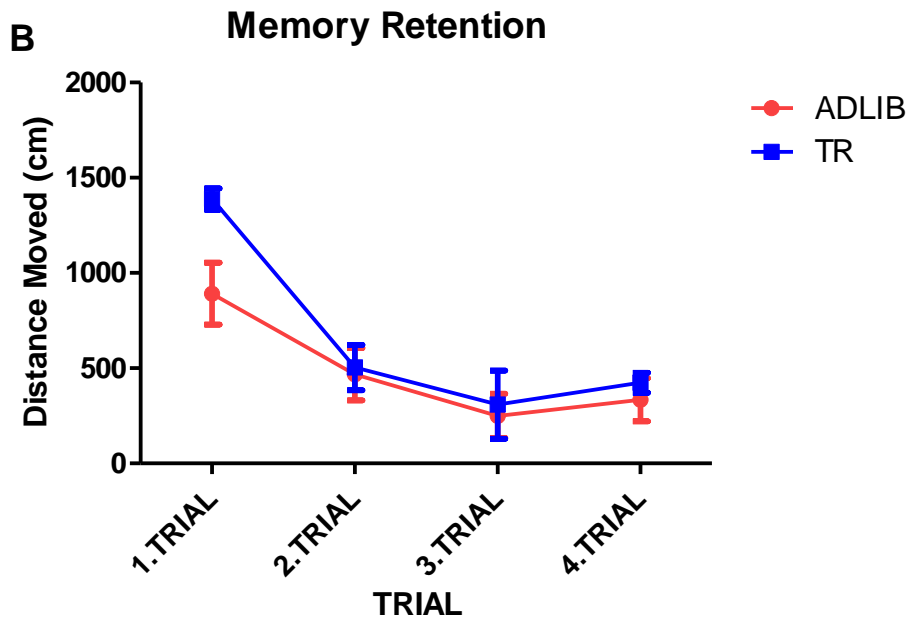
However, as seen from the figures, the TRF group showed tendency towards slower task acquisition during post-diet period compared to ADLIB group, therefore one-way ANOVA with group as independent variable was performed on swim latency data for each post-diet training day, independently.

#### **3.4.4.2. Memory Retention Test**

This test was used to compare the long term memory between ADLIB and TRF rats. On the first day of the water maze testing after the diet period, platform position was the same as during the MWM training before the diet and both, escape latency and swim distance to hidden platform were recorded on the four consecutive trials.



**Figure 3.11.** Comparison of A. mean escape latencies ( $\pm$ SEM) between the first and the last training day before the diet, and the testing day (retention) after the diet, and also B. mean distance moved ( $\pm$ SEM) between trials on the retention day. Error bars denote SEM.

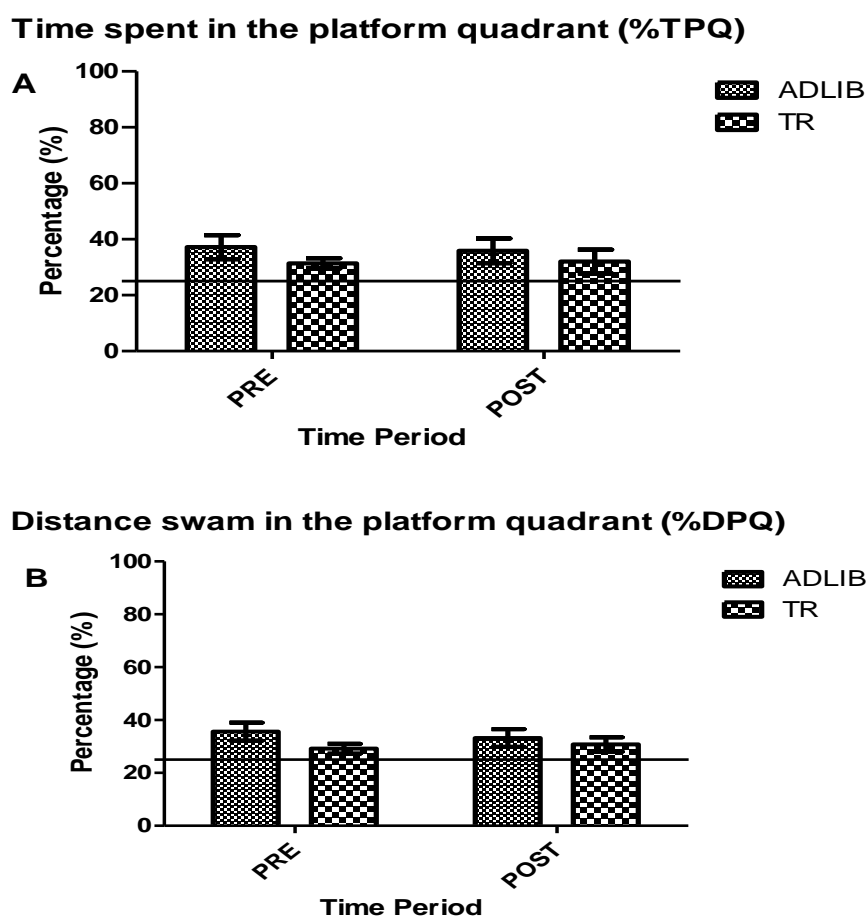


**Figure 3.11 (Continued).** Comparison of A. mean escape latencies ( $\pm$ SEM) between the first and the last training day before the diet, and the testing day (retention) after the diet, and also B. mean distance moved ( $\pm$ SEM) between trials on the retention day. Error bars denote SEM.

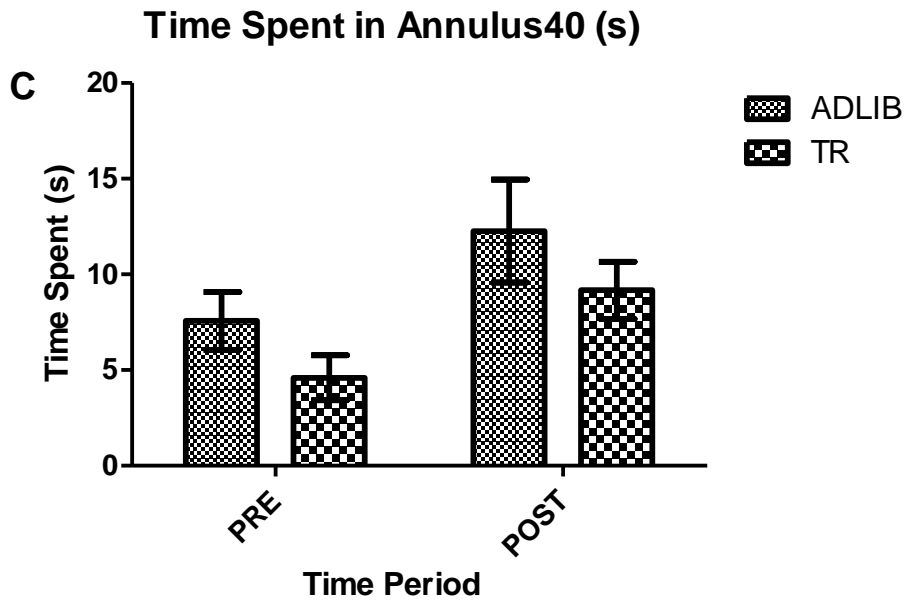
As seen from Figs.3.11 A & B ., during the pre-diet training, in both groups, swim distance and escape latencies highly decreased but after the 3-month delay, their values again increased to the level noted on the 1<sup>st</sup> pre-diet training day. Two-way repeated measure ANOVA (group x time of testing) done on escape latencies yielded significant the time effect only ( $F_{(2,20)}=6.466$ ,  $P=0.0068$ ). Besides, two-way repeated measure ANOVA was performed for distance moved on the retention day for comparing the difference between trials with trial and diet type as independent variables. In this test trial effect was significant ( $F_{(3,30)}=33.31$ ,  $P<0.0001$ ) but group effect remained insignificant.

### 3.4.4.3. Probe Trial

Two 60s probe trials were done after the pre- and the post-diet training in the MWM to assess the preference of place for the platform quadrant and thus, to measure the strength of the habit. As seen from the Fig. 3.12 (A,B,C), three different measures of the habit strength were used: percent time spent in the platform quadrant (%TPQ), percentage of distance swam in the platform quadrant (%DPQ), and time in the Annulus 40.



**Figure 3.12.** Mean percent time (+SEM) spent in the platform quadrant (A), mean percentage of distance swam ( $\pm$ SEM) in platform quadrant (B) and mean time ( $\pm$  SEM) spent in Annulus 40 (C) and on 60 s probe trials during pre- and post-diet testing in TRF and ADLIB groups. Error bars denote SEM. Line at 25% represents chance level.



**Figure 3.12 (Continued).** Mean percent time (+SEM) spent in the platform quadrant (A), mean percentage of distance swam ( $\pm$ SEM) in platform quadrant (B) and mean time ( $\pm$  SEM) spent in Annulus 40 (C) and on 60 s probe trials during pre- and post-diet testing in TRF and ADLIB groups. Error bars denote SEM. Line at 25% represents chance level.

Two-way repeated measure ANOVA (group x time spent) performed for Annulus 40, yielded significant time effect only ( $F_{(1,10)}=6.318$ ,  $P=0.0307$ ) with longer time spent in A40 by both groups during the post-diet testing. The same analysis run for percent time spent in platform quadrant %TPQ, and percentage of distance swam in the platform quadrant (%DPO) showed both group and time effect insignificant.





## CHAPTER 4

### DISCUSSION

#### **4.1. Weight changes and daily calorie intake observed in time-restricted and ad libitum rats**

Night time-restricted rats and ad libitum rats showed almost the same weight gain within 3 months diet period (equivalent of approximately 10 years in human). In previous studies by other authors (Arble et al., 2009; Masaki et al., 2004), mice fed with high-fat diet or normal chow during their dark phase period showed less weight gain in contrast to the group of mice fed during light phase. Moreover, in a study on a rat model of shift-working, rats subjected to TRF during the day gained more body weight than the controls fed ad libitum and the groups fed during the night (Salgado-Delgado et al., 2010). Previous other studies on rodents and humans also indicated that eating during the normal resting period leads to a loss of blood glucose rhythm and overweight (Van Cauter et al., 2007; Liu et al., 2007; Tore'n et al., 2009). These results are in line with a report that nighttime-restricted feeding normalized clock gene expression in diabetic mice with initial dampened locomotor activity and shifted clock gene daily rhythms (Kudo et al., 2004). The discrepancy between ours and the other results may be due to the differences in the diet content, duration of TRF period, age of the animals. The increase in body weight observed equally in both groups could be related to the decreased metabolic rate and physical activity occurring with age (Owen et al., 2010).

Interestingly, average amount of consumed food and thus daily calorie intake by both groups were also the same. Similar results were also earlier reported by other authors (Froy et al., 2008; Honma et al., 1983; Grasl-Kraupp et al., 1994; Arble et al., 2011;

Hatori et al., 2012). It shows that animals used to receive ad libitum food within only a few hours every day adjust feeding behavior to the feeding protocol very quickly and consume their daily food amount during given limited time.

#### **4.2. Changes in values of selected biochemical parameters of blood serum during pre and post-diet period in time-restricted and ad libitum rats**

Blood samples were collected from rats before the introduction of the diet and after three months of diet application and within-group (longitudinal) and between-group (cross-sectional). Comparisons were done. The concentrations (mg/dl) of HDL (High Density Lipoprotein), VLDL (Very Low Density Lipoprotein), and Triglyceride showed no significant change that could be related to the differences in feeding conditions. Thus, in our study, TR feeding did not affect much the lipid profiles. LDL and leptin levels only showed tendency towards time-dependent increase in ad libitum but not in time-restricted group. Similar results were reported by Hatori et al. (2012) who also found higher leptin levels in ad libitum groups compared to time-restricted groups maintained either on standard or high fat diet. Similarly, Sherman and colleagues (2012) showed that the ADLIB groups had higher levels of the adiposity hormone leptin as compared with the TRF groups. The lower leptin levels in TRF group in our study are, however, inconsistent with some other previous reports that fasting during the dark phase and feeding during the light phase leads to a greater drop in leptin than fasting during the light and feeding during the dark (Arble et al., 2011).

The higher leptin levels in ADLIB group can be due to the fact that ADLIB group was allowed to eat in both biologically active and resting phases of daily cycle while TRF group was allowed to eat only in the active phase related with circadian leptin rhythm. However, in this study, higher leptin levels in ADLIB group compared to TRF group did not affect daily calorie intake and weight management.

The physiological range for blood lipids in human and rodents are similar: LDL; less than 100 mg/dL; total cholesterol; less than 200 mg/dL, HDL; less than 40 mg/dL,

and fasting triglyceride; less than 150 mg/dL. In young adults total cholesterol can be greater than or equal to 225 mg/dl for human subjects and it is also similar in rats (labtestsonline.org, last access September 5, 2015). In obesity, dyslipidemia manifested as high triglyceride, low HDL, however, normal LDL is often reported (Poirier et al., 2006; Isomaa et al., 2001; Lakka et al., 2002; López-Candales, 2001). In the present study, only LDL were significantly higher in ADLIB group compared to TRF group. Also some other authors (Sherman et al., 2012), have not found significant differences in glucose and triglycerides levels between ADLIB and TRF groups but in the latter study, total cholesterol and HDL levels were lower in TRF groups for both high-fat and low-fat fed rats.

#### **4.3. Longitudinal and cross-sectional comparison of behavioral indices.**

Alike in several previous studies (Schmitt and Hiemke, 1998; Walsh and Cummins, 1976; Dursun et al., 2006; Liebsch et al., 1998), in this study too, locomotor activity was measured by the distance moved in the whole OF during fixed (10 min) time interval. In previous studies by other authors (Arble et al., 2009), ad libitum light- or dark-phase feeding had no effect on locomotor activity, while TR dark-phase feeding caused increase in animal motor activity. However, in our study, no between-group differences in locomotor activity were found. An overall decrease in locomotor activity observed after the diet period can be related to aging and an overall increase in the body weight, but also to a decrease in exploratory drive on the second exposure to the familiar environment.

Index of anxiety was the percent time spent in the central zone of the OF which is shorter with higher anxiety levels (Liebsch et al., 1998). In our study, time spent in the central zone did not show diet-related between-group differences but also showed a general decrease over time. This indicates no effect of night-phase-locked RF on anxiety. The decrease in time spent in the center of arena upon the second exposure to the OF may be also related with a decrease in exploratory behavior upon the 2nd exposure to the OF. However, Halagappa et al., (2007) reports that calorie-restriction

(40%) and intermittent-fasting applied to 3xTgAD mice between 3-17 months of age increased exploratory behavior in the open field in these animals, with significantly lower body weights in the CR group.

Animals' behavior in the Elevated Plus Maze was consistent with the results obtained in the OF. In the EPM, no difference was found between the two groups in the time spent in open or closed arms. However, it is reported that in rats subjected to chronic food-restriction with 2 hours feeding after 22 hours fasting, anxiety-like behavior was reduced (Inoue et al., 2004). In this TRF model, observed marked changes in meal size and eating rate, but not meal frequency, are explained by desensitization of satiation-like processes, which would be a consequence of decreased serotonergic activity (Halford and Blundell 2000; Inoue et al 1997). Thus, according to this model, chronic food restriction might reduce serotonin transmission and as a result of that also reduce anxiogenic-like behavior. Nevertheless, temporal pattern of TRF applied in this study did not affect rats' anxiety levels.

In the test on sensorimotor coordination and motor learning (rotarod/accelerod) run at the end of the 3 months diet period, TRF group demonstrated significantly better performance than ADLIB group. This result is in line with previous reports that time or dietary restrictions in feeding have positive effect on the animals' performance in motor tasks (Hatori et al., 2012; Ingram et al., 1987; Forster and Lal, 1991). However, in this study, unfortunately, time-restricted group showed better performance also at the beginning of the diet so our result despite of being consistent with observations by other authors can not be very convincing.

In the place learning test (Morris Water Maze task) time-restricted group showed tendency towards slower task acquisition after the diet period compared to ad libitum group, however, between-group differences in either escape latency or swum distance were yielded insignificant. Similarly, no between-group differences were noted in any of three measures of memory retention after 3-month diet period (% time spent/distance swum in the platform quadrant and time in the Annulus 40

measured during probe trial). On the other hand, suprisingly, an impairment in the MWM performance was previously reported by some authors (Ingram et al., 1987; Yanai et al., 2004). Also, in the rat and mice aging models diet calorie restrictions in the diet (60% of ad libitum calories) failed to provide protection against aging-related cognitive deficits (Stewart and Kalant, 1989, Bellush et al., 1996).

Only life-long restricted feeding was reported to lead to small but significant improvements in performance in the water maze in aged animals (Stewart et al., 1989, Markowska, 1999, Adams et al., 2008). Also, in Alzheimer's disease mice model, calorie-restricted and intermittent fasting groups showed better performance in the MWM probe trial (Halagappa et al., 2007).



## **CHAPTER 5**

### **CONCLUSION**

This study carried out on the rodent model evidently shows that long-term (3 months equivalent to 10 years in human) time restricted feeding even when it is locked to the diurnal activity phase does not significantly has no impact on daily calorie intake, weight management, physiological parameters such as lipid and leptin levels, and behavioral parameters (locomotor activity, sensorimotor coordination and motor learning, emotionality, and cognitive performance) when applied to elderly subjects. Additionally, it was shown that the amount of food consumed daily was the same for ADLIB and TRF groups demonstrating that under time restricted feeding animals accordingly adjust their feeding behavior retaining calory intake at the same level as during ad libitum feeding. Thus, it is still an open question whether time-restricted feeding without reducing calorie intake prevents metabolic diseases.





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