

FOOD DEMAND AND ENERGY BALANCE: A NEUROECONOMIC ANALYSIS OF
MELANOCORTIN SYSTEM AND EXERCISE

By

DENIZ ATALAYER

A DISSERTATION PRESENTED HERE TO THE GRADUATE SCHOOL OF THE
UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2010

© 2010 Deniz Atalayer

To my mom, dad and my mentors: Dr. Neil E. Rowland and Dr. Philip Teitelbaum

ACKNOWLEDGMENTS

My dissertation is a product of 'ad libitum' encouragement and support by my parents, my advisor Dr. Neil E. Rowland and my mentor Dr. Philip Teitelbaum. Unlike the 'non-fan' crowd that I unintentionally created around myself in the lab, they believed in me at all times. Today with this accomplishment, I promise to make those who believed in me be proud. Today with this accomplishment, not only I got one step closer to what I would like to pursue in the future but also to whom I would like to become: Dr. Rowland; the most humble scientist I have yet to meet, and, who is my role model as a scientist, as a teacher, and as a person. I would like to end this dedication by quoting from my mentor, Dr. Philip Teitelbaum, as an advice to any young scientist and for my own remembrance forever: "Do not keep your knowledge in isolation!"

TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS.....	4
LIST OF TABLES.....	7
LIST OF FIGURES.....	8
ABSTRACT	10
CHAPTER	
1 GENERAL INTRODUCTION	12
Economic Structure of the Food Environment	12
Obesity/Positive Energy Balance on Food Demand	15
Melanocortin System and Obesity	16
Diet-Induced Obesity.....	19
Melanocortin System and Cholecystokinin	20
Negative Energy Balance and Food Demand.....	21
Summary	23
2 GENERAL METHODS.....	29
Animals.....	29
Diets.....	30
Apparatuses.....	30
3 POSITIVE ENERGY BALANCE AND FOOD DEMAND	34
Introduction	34
Melanocortin 3 and 4 Receptor Knock Out Mice	34
Diet-Induced Obese Mice	37
Effects of CCK.....	38
Subjects and Housing.....	38
General Methods	39
Procedures	40
Experiment 1: Food Demand Analysis in MCR Knockout Mice	40
Experiment 2: Food Demand analysis in Diet-Induced Obese Mice.....	41
Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice	41
Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice	43
Data Analyses.....	44
Results.....	44
Experiment 1: Food Demand Analysis in MCR Knockout Mice	44
Experiment 2: Food Demand analysis in Diet-Induced Obese Mice.....	46

Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice	46
Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice	48
Discussion	48
Experiment 1: Food Demand Analysis in MCR Knockout Mice	48
Experiment 2: Food Demand analysis in Diet-Induced Obese Mice.....	49
Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice	50
Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice	52
Chapter Discussion	52
4 NEGATIVE ENERGY BALANCE AND FOOD DEMAND	66
Introduction	66
Subjects and Housing	67
General Methods	67
Procedure	67
Experiment 5: Voluntary Running Wheel Activity and Food Demand.....	67
Experiment 6: Running Wheel Activity as Food Foraging.....	68
Data Analysis.....	70
Results.....	70
Experiment 5: Voluntary Running Wheel Activity and Food Demand.....	70
Experiment 6: Running Wheel Activity as Food Foraging.....	72
Experiment 5 and 6: Voluntary Running versus Running as Food Foraging Effort	75
Discussion	76
Experiment 5: Voluntary Running Wheel Activity and Food Demand.....	76
Experiment 6: Running Wheel Activity as Food Foraging.....	79
Chapter Discussion	80
5 GENERAL DISCUSSION	99
LIST OF REFERENCES	106
BIOGRAPHICAL SKETCH	115

LIST OF TABLES

<u>Table</u>		<u>page</u>
1-1	Appetitive costs, alternative terms, and definitions	28
3-1	Fat content of mice from experiment 1	62
3-2	Effect of CCK-8 (6 µg/kg) on intake of crunchies by undeprived mice.....	63
3-3	Effect of CCK-8 on chow intake by deprived mice.....	64
3-4	Fos-positive cells in paraventricular hypothalamus following CCK-8 (6 µg/kg)...	65
4-1	Total caloric energy intake at each FUP schedule in experiment 5	96
4-2	Running distance (m) of mice in experiments 5 and 6 as a function of the fixed unit price (FUP) for food.....	97
4-3	Total caloric energy intake at each schedule in experiment 6	98

LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1 The demand function.....	25
1-2 Concurrent approach costs and unit prices imposed.....	26
1-3 The comparison between nose poke and lever press operants.....	27
3-1 Daily (23 h) food intake.....	56
3-2 Intake analyzed for number of meals and mean meal size.....	57
3-3 Weight changes of mice during the demand series.....	58
3-4 Scatterplots showing fat pad amounts.....	59
3-5 Daily food intake, number of meals and mean meal size in DIO (HF) and LF mice.....	60
3-6 Effect of CCK-8 on intakes of chow in 30 min tests after 18 h food deprivation.....	61
4-1 Individual day-to-day data from a typical mouse.....	83
4-2 Daily food intake, number of meals taken, and meal size of sedentary and runner mice.....	84
4-3 Nose poke responses.....	85
4-4 The mean body weight and dissected fat pads sedentary and wheel running mice.....	86
4-5 Individual day-to-day data of a typical mouse.....	87
4-6 Bar plots for mean number of responses (\pm SEM) for both groups.....	88
4-7 The number of responses emitted for both groups plotted against the pellets consumed.....	89
4-8 Lever press responses (FUP responses) for runGR and pokeGR.....	90
4-9 Demand functions for runGR and pokeGR.....	91
4-10 Mean number of meals (meal frequency) taken per day for runGR and pokeGR.....	92
4-11 Mean daily meal sizes for runGR and pokeGR.....	93

4-12	Body weight change.	94
4-13	Voluntary and foraging running wheel activity from both experiments.....	95

Abstract of Dissertation Presented to the Graduate School
of the University of Florida in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy

FOOD DEMAND AND ENERGY BALANCE: A NEUROECONOMIC ANALYSIS OF
MELANOCORTIN SYSTEM AND EXERCISE

By

Deniz Atalayer

August 2010

Chair: Neil E. Rowland

Major: Psychology

One of the main factors contributing to the obesity epidemic is the obesity favorable food environment of today's modern societies that promotes excessive energy intake while minimizing the energy expenditure. The purpose of the present work is to combine the analyses of the effects of both positive and negative energy balance on food demand and meal size by exploring the some of the physiological and neuronal mechanisms of the melanocortin system and the effects of physical activity.

Homozygous deletion of the melanocortin-4 receptors (MC4R^{-/-}) is known to result in positive energy balance as MC4R^{-/-} mice and humans develop obesity, while those with deletion of the melanocortin-3 receptors (MC3R^{-/-}) do not become markedly obese. In the first part of this dissertation, the effects of positive energy demand on food demand were examined. MC3R^{-/-}, MC4R^{-/-} mice with wild type (WT) and mice lacking both of these receptors (double knockout, DKO) were compared on select feeding and neuroanatomical dimensions. In a food demand protocol, DKO and MC4R^{-/-} were hyperphagic at low unit costs for food, due primarily to increased meal size. However, at higher costs, their intake dropped below that of WT and MC3R^{-/-} indicating increased elasticity of food demand. To determine whether this higher elasticity was due either to

the genotype or to the positive energy balance per se, the same food demand protocol was conducted in dietary obese mice. The difference between the demand functions of WT and dietary obese mice was smaller compared to that of WT and genetically obese mice. To assess a mechanism for the larger meal size in MC4R^{-/-} and DKO, we examined the acute anorectic effect of peripherally-administered cholecystokinin (CCK) and subsequently the induction of Fos-immunoreactivity in select brain regions. The anorectic effect of CCK was comparable in MC4R^{-/-}, DKO, and WT, but was unexpectedly absent in MC3R^{-/-}. CCK-8 induced c-Fos was lower in PVN in MC3R^{-/-} than the other genotypes.

The second part of this dissertation examines the effects of negative energy balance on food demand. Physical activity has long been suggested as a method to better maintain weight loss and that obesity induced by a high fat diet can be treated without energy restriction by exercise. The effects of running wheel activity on food intake and meal patterns were measured under several economic conditions in CD 1 mice. Voluntary wheel running activity increased daily food intake and running animals consumed bigger but fewer meals compared to the sedentary. Although they ate more, running mice had significantly lower body fat, especially in subcutaneous depots. In the foraging protocol, when food intake was contingent on the running wheel activity, mice were able to emit more responses compared to when food is contingent upon a nose poke response. In both voluntary and foraging running protocols mice had inelastic demand functions compared to the non-running groups indicating that negative energy balance affects how much mice are willing to work for food.

CHAPTER 1 GENERAL INTRODUCTION

Economic Structure of the Food Environment

From an evolutionary perspective, foraging for food is essential for an animal. In order to survive it has to remember food sources, prepare for consumption, ingest sufficient amounts of food and digest them efficiently (Adan *et al.*, 2006). The decision to eat is controlled both by endogenous factors (hunger and satiety), and also by environmental cues such as availability of palatable or novel foods and predator exposure when searching for food. Animals searching for food often perform a series of behaviors; traveling, catching and consuming with the inherent strategy of optimizing and allocation of scarce resources. These behaviors constitute a form of cost for the animal in the nature. The field of economics is defined as the computational analysis of anticipated cost-benefit balance and considered a science of highly organized human behavior (Hursh, 1984). One of the main principles of the theory of economics states that if the price increases the consumption of most goods decreases (Hursh *et al.*, 1988). Recently, these principles, which relate the commodity to its price, have been applied to the field of behavior analysis (Lea 1978; Hursh 1980; Collier *et al.*, 1986; Killeen, 1995). Earlier studies on behavioral economics found that feeding patterns vary according to this cost of access to the food (Collier, 1985; Collier *et al.*, 1986, Hursh, 1980). From this perspective, feeding behavior can be considered as being highly influenced by the economic structure of the current environment in which the individual lives, because whether in the natural habitat of animals or the very industrialized lifestyle of humans, the demand for food is always in an exchange for some type of

cost. For animals, at least three different types of costs related to food consumption have been defined in the literature (Morato *et al.*, 1995):

- Cost of procuring access to food -procurement cost (travel effort and/or time)
- Cost of consuming food –consummatory cost (the cost within the patch such as digging or climbing for food items, sucking, catching, holding etc., which are the equivalent of operant schedules in an experimental setting in most studies)
- Cost of processing food (physiologic consequences/digestion)

Collier *et al.* (1972) showed that number of meals eaten daily and associated meal sizes could be altered profoundly by relatively small costs imposed during the consummatory phase. They also showed that meal structure can be differentially affected by the changes in the former two costs categorized above. In a previous study in our lab, we analyzed the meal structure based on the systematic changes in both types of costs in mice (Atalayer and Rowland, 2009). In addition, in order to better capture the functional difference between the two types of cost, we introduced a different terminology for these two costs (Table 1-1). We proposed that approach cost (procurement cost as previously termed) is analogous to travel or effort that occurs while food is still spatially and/or temporally distant, and may be associated with a lower probability of success. In contrast, unit price (consummatory cost as previously termed) is the effort expended once the food item or patch is reached, such as digging in a substrate or hulling seeds; it occurs in proximity to the food and is usually associated with a higher (but not necessarily fixed) probability of success.

In most research protocols with animal models investigating eating in terms of economics of behavior, demand functions have been most usually determined in which the price is defined as time or effort to obtain uniform morsels of food such as small, nutritionally complete pellets. Effort is usually assessed as the number of behavioral

responses required per reinforcement or reward (e.g., food, water). Thus, from the economics of behavior point of view, the apparent question is how the animals will change the rate or amount of responding as the required effort (cost) to access to the commodity, the food, is increased (Hursh, 1980). It is recognized that the answer is a product of a complex interaction between the availability and/or cost of the food and hunger state of the organism. However it can be defined and summarized in a demand function which relates the price and food intake (Figure 1-1). A demand function is a mathematical relationship that describes the effects of prices on the quantities of commodities purchased (Lea, 1978). The slope of the demand function is determined by the amount of the effort that an animal will emit to obtain a commodity as the price for that commodity increases. It is also called the elasticity of the demand, defined as the ratio of the proportional changes of dependent and independent variables (Lea and Roper, 1977). It should be noted that economists usually draw the curve as the y-axis because it is the dependent variable, but in the experimental designs in psychology, the price (reinforcement schedule) is more or less the independent variable so that is why we draw it in the x-axis and the demand quantity (the dependent variable) on the y-axis. The slope of the demand function, the elasticity of demand, changes in response to various factors. In eating behavior these factors could be things such as availability and substitutability of alternatives (Lea and Roper, 1977), the incentive value or amount of the food (Killeen, 1995), the deprivation level of the individual (Hursh *et al.*, 1988), presence of others and amount of attached cost (Johnson *et al.* 1993) etc. Many studies using these types of designs have shown that food intake does change under different

economic conditions of food availability (Sumpter *et al.*, 1999; Rovee-Collier *et al.*, 1982; Hursh, 1980; Hursh *et al.*, 1988).

Under fixed ratio (FR) schedules, each unit of food costs a fixed number of behavioral responses and operant behavior is used to emulate cost for food in laboratory settings. FR schedules are believed to be the most direct and are the most commonly used method to set the price of a commodity (Bauman *et al.*, 1996; Hursh, 1984). Thus in all of our studies presented here, we will use fixed ratio reinforcement schedules. The term fixed ratio will be combined with the term unit price, (the term that we presented earlier) as 'fixed unit price' or 'FUP'. By using these cost regimens, researchers aim to understand how animal's preference for food is shaped by the different economic environments and by extrapolation to the human condition, how an individual's food preferences may be affected in these economies where different types of food are usually available at all times. In this type of continuous access protocol, parameters of meal size and frequency may also be determined as food intake is organized in meals so increased food intake could be realized by increased meal size, meal frequency, or both. Thus, different experiments designed in this project will be discussed and analyzed in terms of demand functions and meal patterns.

Obesity/Positive Energy Balance on Food Demand

Obesity is one of the biggest threats to contemporary human health. At least 400 million adults globally are obese and ~700 million adults are estimated to become obese by 2015 (WHO, 2005). In addition the same report reveals that, based on the NHANES (National Health and Nutrition Examination Survey), obesity increase was highest in the last decade compared to the previous time periods. This clearly indicates that the effects of changes in the food environment in terms of its availability and cost because it

is such a short period of time for any mutations in the genetic pool population-wide. Thus in this dissertation we will examine the effects of the changes in the economic settings of the food environment by using the demand functions as indirect indication of the food motivated behavior. We hypothesize that demand for food for lean versus obese individuals will be different as it has been suggested that the reinforcing value of high-calorie foods are higher for obese individuals than for normal-weight individuals as overweight/obese individuals work harder for high-calorie snacks compared to normal-weight individuals (Giesen *et al.*, 2010; Temple *et al.*, 2009). Thus an animal model of these reports would be particularly necessary and beneficial for a more accurate understanding and reliable predictions for the reinforcing value of food in obese versus non obese individuals.

Melanocortin System and Obesity

Although we are interested in the effects of the environment on the rising obesity epidemic, we do not ignore the effects of genetics. Eventually we aim to combine both genetic and environmental approaches to come up with better therapeutic solutions. Although studies of animal models have identified several genes with measurable effects on body weight and composition, it has been found that polymorphisms in the melanocortin type 4 receptors (MC4R) of central nervous system are implicated in ~6% of early onset or severe adult obesity cases (Adan *et al.*, 2006; Lubrano-Berthelier 2003, Govaerts *et al.*, 2005, Vaisse *et al.*, 2000). As the most common known monogenic cause of human obesity, we chose MC4R mutant animals (MC4R^{-/-}) in this project as the genetic model of obesity.

The arcuate nucleus of hypothalamus (Arc) integrates and distributes to other parts of the brain the neural and hormonal signals from the periphery that reflect

metabolic status further into the brain. Within the Arc, neurons containing melanocortins (MCs), products of the proopiomelanocortin (POMC) prohormone, are activated by leptin, a long term satiety hormone secreted in response to increased adiposity in the body (Schwartz *et al.*, 1997). The biological effects of these MCs are mediated by five known melanocortin receptors (MC1-5R) all belong to the family of seven transmembrane G-protein coupled receptors (GPCRs) (Abdel-Malek *et al.*, 2001; Cone *et al.*, 1996).

MC1R is activated by melanocyte α -MSH and has a key role in determining skin and hair pigmentation (Abdel-Malek *et al.*, 2001). On the other hand unlike the other four MCRs, MC2R is an adrenocortical adrenocorticotrophic hormone receptor and has a role in secretion of steroid hormone along with some ambiguous functions affecting adipose tissue which also differs in humans and animals (Gantz and Fong, 2003). The last MC receptor, MC5R was found to have a role in sebaceous gland secretion (Hatta *et al.*, 2001). However, the effects on coordinated energy homeostasis are mediated by the central MC3R and MC4R, and these are expressed in several regions of the CNS.

MC4Rs have a major role in the regulation of feeding behavior and body weight and are expressed predominantly in paraventricular nucleus of hypothalamus (PVN), area postrema (AP) and nucleus of the tractus solitarius (NTS). It is a 332-amino acid protein encoded by a single exon gene localized on chromosome 18q22 and signals through the activation of adenylyl cyclase in response to its endogenous agonist ligand, α -melanocyte-stimulating hormone (α -MSH) which is a neuropeptide produced by proconvertase 1-dependent cleavage of POMC (Lubrano-Berthelie *et al.*, 2003). There is strong evidence that the action of both leptin and insulin relies upon α -MSH signaling

since administration of antagonists to MC3R/MC4R blocks each of their actions in the brain (Woods, 2005). Thus, studies demonstrated nonredundant roles for the MC3R and MC4R in energy homeostasis.

MC3R (on chromosome 20q13.2) is expressed in many areas of the CNS (and in several peripheral tissues, including the adipose tissue, heart, skeletal muscle, kidney, stomach, duodenum, pancreas and placenta (Gantz and Fong, 2003; Chajlani, 1996). However, the highest concentrations of these receptors are found in Arc and they are presumed to function as autoreceptors on POMC cell bodies at this site. The dorsal part of the ventromedial hypothalamus (VMH) that is presumed to receive projections from the Arc is another structure where the MC3R are abundantly located (Cowley *et al.*, 2001). In addition, high levels of MC3R mRNA are expressed in the ventral tegmental area which is a site involved in processing of the information on reward systems (Kishi and Elmquist, 2005). Interestingly, MC3Rs are not found to be present in the NTS or AP (Blevins and Baskin, 2010).

The activity of the MC system is also regulated by an endogenous antagonist agouti-related protein (AgRP), which acts at MC3R and MC4R. It is produced exclusively in the Arc in rodents, monkeys, and humans and a single dose of AgRP that is administered into the brain near the Arc increases food intake (Kishi and Elmquist 2005). An important side note is that although often described as a competitive antagonist (which binds to a receptor but does not activate it), AgRP acts in fact as an inverse agonist (which binds to receptor but has the opposite action than the agonist) on constitutively active MC3Rs and MC4Rs (Haskell-Luevano and Monck, 2001). In contrast to POMC neurons, AgRP neurons are activated during negative energy

balance initiating eating and inhibited by leptin. Common literature suggests that leptin stimulates MC signaling, resulting in increased energy expenditure (oxygen consumption and diet induced thermogenesis) and decreased energy intake whereas opposite effects by AgRP are inhibited by leptin. An inverse agonist and an agonist acting on the same receptor indicate how tightly these systems are regulated. A large body of evidence of the functional roles of these receptors comes from the genetic mutation studies in mice which have elucidated distinct and complementary roles for MC3R and MC4R in food intake and body weight (Branson *et al.*, 2003).

Diet-Induced Obesity

As we stated above whereas genetic factors play a key role in which individuals will develop obesity, the determinants of obesity seem to be a multifactorial phenomenon and it may be caused by environmental or genetic factors, and/or combinations of the two. The environmental factors such as a sedentary lifestyle and the overconsumption of a high fat content diet especially make critical contributions to the present high rates of excessive adiposity in our population (Chandler *et al.*, 2005). Thus, while not a direct cause, high fat content diet may promote obesity (Willett and Leibel 2002).

Bjorntorp (1993) termed visceral obesity 'the civilization syndrome' as the ingredients of positive energy balance, including low physical activity, stress induced hormonal changes, nicotine and alcohol consumption are frequent features of our modern, urbanized society. Thus even when pronouncing the importance of genetics, one should also argue the influences by environmental factors leading obesity. For this reason, in paradigms in which we used genetic mutations leading an obese phenotype, we also employed diet-induced obese (DIO) animal models in order to determine the

contributions of either the excessive adipose tissue accumulation in the body or the metabolic changes as determinant of the behavior of lean versus obese individuals in various economic food environments.

Melanocortin System and Cholecystokinin

It has been found that large meal sizes characterize many genetic animal models of overeating and obesity. This suggests that some aspect of satiation or satiety may be impaired. During meals, gut (stomach and intestine) signals such as distension of the stomach or cholecystokinin (CCK) trigger nerve impulses via glutamatergic vagal afferent fibers traveling to the hindbrain. Specifically CCK which is a gut-derived satiety factor is believed to restrain meal size by stimulating the hindbrain sites, NTS and/or by direct action in the area postrema (AP) (Appleyard *et al.*, 2005; Berthoud, 2002; Berthoud *et al.*, 2006; Fan *et al.*, 2004; Rinaman *et al.*, 1993) along with that of α -MSH release in the NTS from terminals of POMC neurons.

CCK is generated as food is the gastrointestinal tract and acts within the time of a single meal. Exogenous administration of CCK decreases meal size in a dose dependent manner without causing illness. Some of the CCK enters the blood as a hormone that stimulates the exocrine pancreas and gall bladder as discussed above; but some of the CCK also diffuses locally in a paracrine fashion within the intestinal wall to generate action potentials through the vagus nerve which transmits a message to the AP/NTS signifying the ingestion and upcoming absorption of the food.

There is now strong evidence that MC4Rs modulate release of glutamate from vagal afferents in the NTS by a presynaptic mechanism (Wan *et al.*, 2008); in most NTS cells, the number of spontaneous excitatory postsynaptic events was enhanced by

MC4R stimulation. One functional aspect of α -MSH action may be to augment CCK and/or other vagally-mediated satiety signals.

Negative Energy Balance and Food Demand

There are many central and peripheral signals involved in energy homeostasis and regulation of food intake, and understanding these mechanisms may lead to effective treatments in the control of obesity. Along with the readily available high-calorie/high-fat foods leading DIO discussed above, the lack of necessity for a more physically active lifestyle in today's industrial societies may contribute to the obesity epidemics that we suffer nationwide in U.S. and also globally is a common assumption that obesity may be related to decreased physical activity. In addition to a role for energy input (food intake) in body weight control, elective energy output (physical exercise) has been shown to reduce the risks for obesity and obesity-related diseases in animals and humans (Dishman *et al.*, 2006; Wolfe *et al.*, 1993).

Studies with animals models showed that exercise prevents or delays onset of obesity in young obesity-prone rats, this was hypothesized to be related to the effects of exercise on the development of the neurocircuitry controlling obesity (Hansen *et al.*, 2008; Rhodes *et al.*, 2005). It has also been shown to slow the weight gain in some genetic models of obesity (Irani *et al.*, 2005). However, although obesity has been related to high fat dietary content and lack of physical activity, the methods to treat human obesity still rely predominantly on dietary restriction, which has proven difficult to sustain. One purpose our multifactorial approach reflected in this project is to analyze the effects of unit price changes, physical activity (voluntary running), dietary obesity and the interaction of the three on demand functions, meal patterns and body weight.

Running Wheel Activity as Food foraging. Free-living animals must learn how to search for food in order to survive and often perform a series of behaviors including traveling and capture before consuming food. In addition to its survival value for the access of food, physical activity has been shown to have rewarding value in rodents. Thus, rats will press a press lever in order to access running wheels (Belke, 1997; Belke and Wagner, 2005; Iversen, 1993). In our studies here, running was used both as a voluntary activity and an obligatory cost to access to food. As a reminder previously we proposed the terms approach cost which is analogous to travel or effort and unit price that is the effort expended once the food item or patch is reached (Atalayer and Rowland, 2009). In the same study we showed that when concurrent approach cost and unit price were imposed, food demand again varied relatively little across the range of parameters studied. On the other hand meal number and meal size were profoundly affected by approach cost (Figure 1-2). The operant behaviors for approach cost and unit prices used were nose poke and lever press responses, respectively.

In that earlier study (Atalayer and Rowland, 2009) we compared the two most commonly used operants and demonstrated that when averaged across various unit price schedules (fixed, various, progressive)¹, nose poke operant response did result in a slightly higher food intake than the lever press, but both the mice in both groups changed intake comparably across the various types and increments of unit price schedules, suggesting that nose poke and lever press may be used interchangeably as cost devices for the study of food demand (Figure 1-3). These findings agree with

¹ Fixed unit price (FUP) in which each unit of the item costs a fixed amount (e.g., number of responses), variable unit price (VUP) in which each food item costs a mean amount but the actual cost of each item varies around that mean and progressive unit price (PUP) in which successive food items within an episode of feeding become progressively more costly.

previous studies in rodents comparing nose poke and lever press operants in which acquisition of the nose poke response often occurs more rapidly and emits slightly more responses (David *et al.*, 2001; Ettenberg *et al.*, 1981). Thus when the experimenter prefers to reduce the time of training in mouse studies, a nose poke operant might be more useful. As much as these are the two operants that have been dominantly employed in these types of studies, we recently hypothesized that when the effects of approach cost is analyzed in a laboratory setting, a running wheel activity would emulate a more natural situation compared to other types of commonly used operants and would comprise a relatively more ecologically valid operant, thus may result in either different or more accurate analyses of such parameters of neuroeconomic analysis of food intake in animal models.

Nevertheless establishing ecological validity is beyond the scope of this project. One should keep in mind that, one may not be sure that the results with the running wheel activity in comparison to any other operant behavior is more accurately emulating the cost for food that the animals face in the nature. For that, observational studies in the natural habitat of these species should be referenced. However one of the aims of this project is to find out whether the various operants that are used in this type of research may be resulted in substantially different meal patterns and food intake.

Summary

Obesity and its related metabolic disorders have become an intractably large financial burden to modern societies in terms of health care costs and loss of productivity. There has been considerable focus on predisposing genetic factors, including polymorphisms of MC4R, but it is likely that most obesity is more directly linked to the ubiquitous availability of energy dense foods. We will show that a model of

genetic obesity may be highly responsive to economic cost; this result suggests that changes to the food environment can be effective in curbing food intake and/or choice in both normal and genetically disadvantaged obese populations.

It is unrealistic to believe that a purely physiological manipulation such as gene or pharmacotherapy will be effective in the face of a “toxic” food environment. It is more realistic to ask how the obesity epidemic might be reversed by physiological manipulations in conjunction with radical changes of approach by the health delivery and food industries. The present study is thus presented as an early and small step in the preclinical analysis of this type of approach.

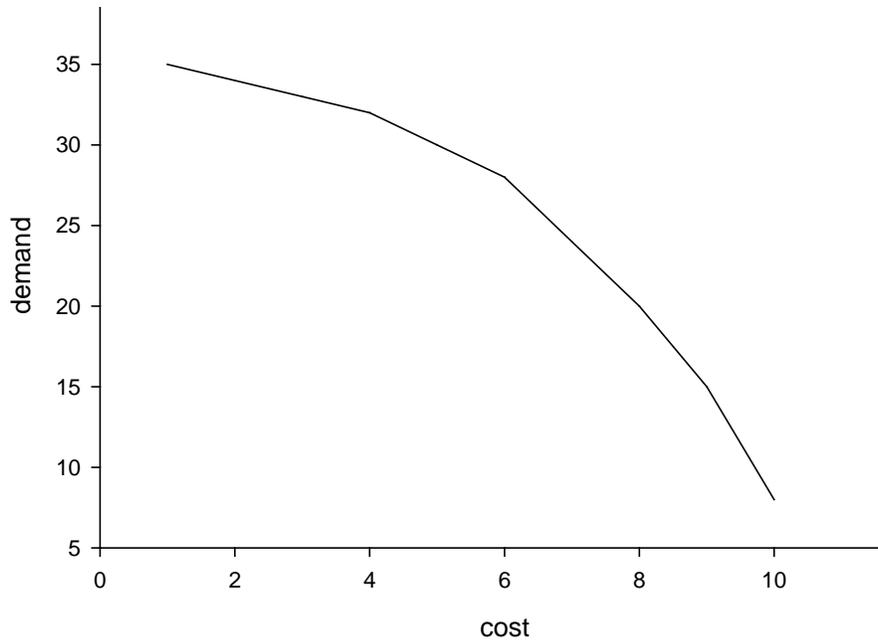


Figure 1-1. The demand function. The figure summarizes the relation between the cost and demand. The slope of the function is determined by the amount of the effort that a person or an animal will work to obtain a commodity as the price for that commodity increases (the numbers are arbitrary and the graph was purely conceptual).

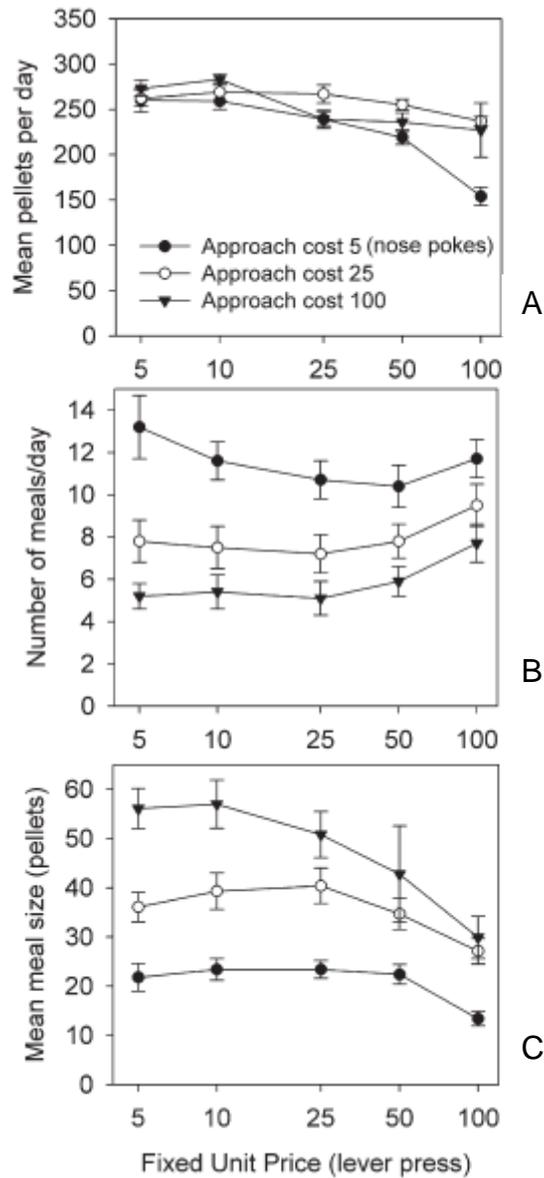


Figure 1-2. Concurrent approach costs and unit prices imposed. A) Mean (\pm SEM) food intake, B) meals per day, C) and meal size of 7mice in three approach cost and five fixed unit price conditions (log scale).

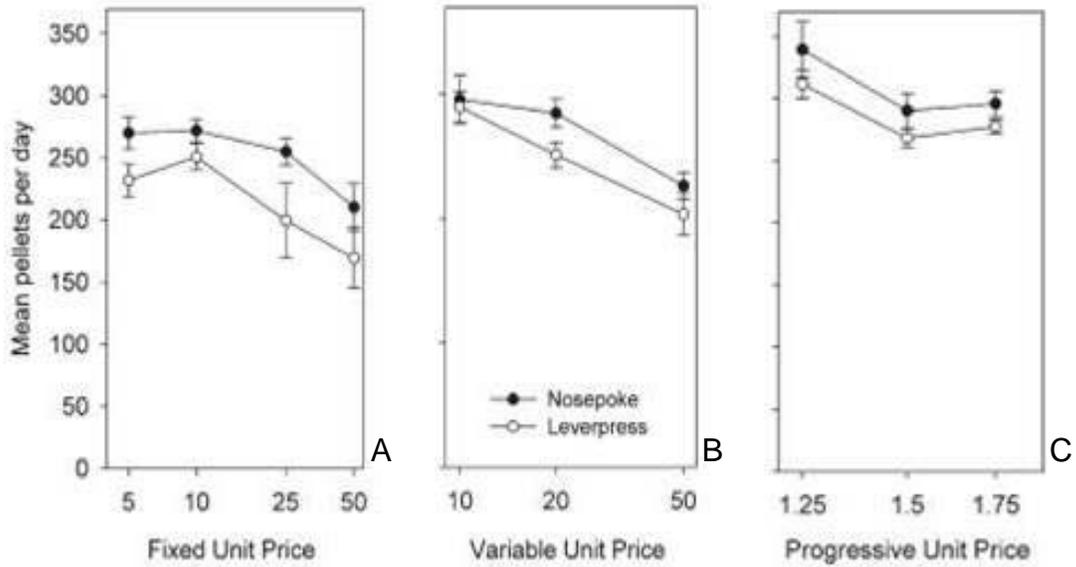


Figure 1-3. The comparison between nose poke and lever press operants. Mean (\pm SEM) number of pellets taken per day as a function of unit price under A) fixed, B) variable, and C) progressive ratio schedules. Data are for separate groups (Ns=8) required to emit either nose poke or lever press responses to obtain food, but for the same animals is left-to-right progression across costs and schedules. Note that the fixed price axis is scaled logarithmically.

Table 1-1. Appetitive costs, alternative terms, and definitions

Term used in this paper	Alternate term(s)	Functional description or examples
Approach cost (AC)	Procurement cost	Searching for a remote food source.
	Anticipatory activity	May have low probability of success.
	Travel cost	May include moving food to a hoard.
	Distal foraging	
Unit price (UP)	Consummatory cost	Effort in proximity to food such as
	Ratio requirement	hulling seeds or nuts; picking fruit.
	Proximal foraging	Relatively high probability of success.

CHAPTER 2 GENERAL METHODS

Animals

Mice (*Mus musculus*) have become an important species for contemporary basic research into physiology and behavior because of their potential for genetic manipulation. The identification of several genes involved in obesity has generated considerable interest in studies of food intake in mice, yet the number of published works that have examined meal parameters is still small and the results are often inconsistent (Atalayer and Rowland, 2009). This may be in part because mice present some procedural challenges. However, the cost efficiency and genetic variety of mice species constitute advantage over other animal models especially when larger sample sizes are considered.

In most of the experiments, we did not include female mice in our design to avoid the possibility that estrous cyclicity would interact with our analysis of meal patterns. In our previous studies using operant response methods in lean and genetically obese mice (Vaughan and Rowland, 2003; Vaughan *et al.*, 2005; Atalayer and Rowland, 2009), we studied the effect of changes in approach cost (we and others (Collier *et al.*, 1972) previously termed this procurement cost) at a constant but low unit price (previously, consummatory cost); those studies showed that meal parameters in mice are highly sensitive to approach, very similar to results reported in rats (Collier *et al.*, 1972). Animal use is approved by a campus-wide IACUC and is compliant with the recommendation of the Guide for the Care and Use of Laboratory Animals (1996).

Diets

For 1-2 weeks between receipt of the animals and the start of the experiments, animal were housed individually in polycarbonate home cages with Purina 5001 Chow pellets and tap water available ad libitum. Methods, in which discrete small food items are presented sequentially, as in standard operant behavior protocols, are particularly appealing because they minimize food spoiling behavior for which laboratory mice often show a proclivity. A few such studies have appeared in the recent literature (Chi and Powley, 2003; Donovan *et al.*, 2007; Fox and Byerly, 2004; Tabarin *et al.*, 2007; Vaughan *et al.*, 2005), but most of these used a low and constant unit price, and did not generate demand functions. Thus for the experiments, mice obtained 20 mg nutritionally-complete pellets (Purina Test Diet 5TUM, a grain-based tablet with 10.4% kcal from fat and 24.1% from protein) when they completed a price that was specified by the particular reinforcement schedule that was in effect. The diets that were used in specific experiments (e.g., diet-induced obesity, acute food intake protocol for CCK action) will be described at each section for each experiment.

Apparatus

Operant chambers. When not in experiments, all mice were individually housed in standard polycarbonate cages containing a 2-3 cm layer of bedding (SaniChips, Teklad, Madison, WI) placed on standard racks and were provided with material for nest building (Nestlet, AnCare, Bellmore, NY). In the experimental procedures for analyzing demand functions, twenty-four operant chambers (Med Associates: 13x13x12 cm with Plexiglas and metal walls and stainless steel grill floor plus solid nesting platform) enclosed in ventilated, noise attenuating cabinets with the same 12:12 light cycle as the vivarium (a 15 watt bulb in a standard light fixture run from a 24 hr timer) were used. All

chambers were equipped with one lever press and one nose poke operant device, located 2cm above the floor, situated on one wall on either side of a food aperture. Depending on the design, both or only one of these operants were active during a particular protocol as stated in the method sections of each experiment.

The nose poke unit includes a yellow light emitting diode stimulus light at the back of the access port, and a source and detector. According to the manual for the chambers, whenever the beam is broken with an approximate of 0.64cm length of animal nose entry, it is detected as a response by the system. Water was supplied from sipper tubes mounted on the wall opposite side to the food magazine and the two operant devices. The vivarium was temperature ($23\pm 2^{\circ}\text{C}$) and humidity (40-70%) controlled, with a 12:12 light cycle (lights on 0700). During the experiments, mice lived in test chambers for 23 h per day. The mice were weighed daily and kept in empty holding cages during a 1 h cleaning period without food, although water was available.

The Psychology department vivaria are part of the centralized University of Florida Animal Care program with full AAALAC accreditation. For various procedures, we modified the location of some of the operants and/or cues and/or house lights based on the design which will be described at each section for each experiment.

Running wheels. Eight chambers were additionally fitted with an external running wheel with free wheel resistance of 4–5 g and 55.8 cm outer circumference (Med Associates, St. Albans, VT: ENV-3042; 1km~1800 revolutions) to which the mice had free and voluntary access. Revolutions were recorded by a counter on the running wheel and also by the computer program.

Data analysis and software. A record of the total pellets obtained by mice and number of responses (nosepoking, leverpressing, running wheel activity) were acquired by the MED-PC IV computer software (MED Associates, St. Albans, VT). The computer recordings allowed an analysis of the number of meals and the amount eaten at each meal. Early studies on meal patterns in rodents have defined a meal as an eating episode initiated after at least 40 min of non-eating period (Kissileff, 1970; LeMagnen, 1992; Clifton, 2000). In mice, spontaneous food intake occurs in a number of eating bouts separated by periods of non-eating or meal-to-meal intervals (MMI) that typically are at least 30 min in length (Clifton, 2000). In our previous work, we compared two different MMI criteria (15 and 30 min) to define a meal and results showed that the meal distribution in mice was critically dependent on the MMI criterion, regardless of the operant employed (Atalayer and Rowland, 2009). Although determining which of the MMIs is a more accurate representation for `a meal` for mice, 15min MMI seemed to better agree with some of the earlier studies with mice (Petersen and McCarthy, 1981; Vaughan and Rowland, 2003; Fox and Byerly, 2004; Vaughan *et al.*, 2005). Thus we used this criterion in all of our analyses. Note that on average an animal will have stopped in the middle of the interval preceding a zero entry and will have started eating in the middle of the interval following a zero entry; thus, this 15 min meal criterion in fact defines an average minimum inter-meal interval of 30 min, but with a range from 15-45 min. After the numbers of meals per day were determined for each mouse, the mean meal size was derived by dividing the number of total pellets by the number of meals for each day.

The raw data from the computer recordings, showed how many responses were made and the number of pellets earned at each 15 min each day. After the numbers of meals for each mouse were counted by the experimenter for each day of each schedule from the raw data, the mean meal size was derived by dividing the number of total pellets by the number of meals for the particular day. In our previous study we found that mice can adjust the changes in cost schedules typically in ~2 days (Atalayer and Rowland, 2009). The mean for number of meals, total pellets earned and meal sizes were computed for each mouse and for each reinforcement schedule by averaging them over four days. Parameters were analyzed for significance with SPSS computer software by using repeated measures analysis of variance (ANOVA), one-way ANOVAs, t-tests and post hoc Bonferroni and/or LSD and/or Tukey when it is necessary. In all cases, $P < 0.05$ was considered significant.

CHAPTER 3 POSITIVE ENERGY BALANCE AND FOOD DEMAND

Introduction

Previous work from our laboratory demonstrated that MC4R^{-/-} mice were hyperphagic and took larger meals under specific low cost schedules (Vaughan *et al.*, 2006a). However, a systematic analysis of increasing costs and full demand functions were not determined in that study. In the first part of Chapter 3 full demand functions in wild type (WT), MC3R^{-/-}, MC4R^{-/-} and DKO mice will be examined. Studies suggest that hyperphagia in MC4R^{-/-} occurs to support that excessive body weight increase that is essentially caused by a metabolic deficiency in these mice (Marie *et al.*, 2000). Thus in the second part, the same type of analysis is conducted with diet-induced obese (DIO) mice to compare their results with those from genetically obese mice to determine if the demand functions are the sole product of the higher level of adipose tissue in obese animals or whether the metabolic consequences of MCR mutations do play a role. In the last section of Chapter 3, the effects of CCK will be determined along with the comparison of the induction of c-Fos activity as an indication of cellular activity by CCK in the brain of these various mice with deletion of MC3R and/or MC4R.

Melanocortin 3 and 4 Receptor Knock Out Mice

MC4R^{-/-} mice. Homozygous deletion (-/-) of the MC4R results in an obese phenotype including hyperphagia, indicating that this receptor has a role in limiting food intake and stimulation of energy expenditure. They also exhibit significant hyperinsulinaemia and alterations in metabolic rate, maintain normal levels of lean body mass and are longer than WT. (Chen *et al.*, 2000; Huszar *et al.*, 1997; Haskell *et al.*, 2009). Heterozygous MC4R^{+/-} mice present an intermediate average increase in

weight, females being more severely affected than males; however they display a broad variability in phenotype with an adult weight ranging from that of WT to that of homozygous MC4R^{-/-} mice (Vaisse *et al.*, 2000).

Studies have shown the contribution of MC4R and MC3R to the regulation of metabolism. Marie *et al.* (2000) reported increased body weight of the MC4R^{-/-} female mice at 12 wk of age, and of the MC4R^{-/-} male mice at 10 wk although they found no significant hyperphagia in these mice. At the end of 14 weeks, MC4R^{-/-} mice of both genders were heavier than sex-matched WT, and as a consequence they started to eat more. Their result indicates that MC4R^{-/-} gained weight because of the changes in their metabolism and that their hyperphagia must occur to maintain their higher weight level.

They also measured the oxygen consumption which is another indication of metabolic rate and reported that MC4R^{-/-} mice consume 20% less oxygen than WT (Marie *et al.*, 2000). Although these mice were leptin-resistant, young non-obese MC4R^{-/-} mice are partially sensitive to the effects of leptin which suggests that leptin resistance is at least partially a consequence of the weight gain in these animals. Thus, as they are somewhat sensitive to leptin as they get older and heavier they eventually become fully leptin resistant (Marie *et al.*, 2000). In addition, another measure for metabolic rate is diet-induced thermogenesis, the ability of leptin to induce uncoupling-1 protein (UCP1) mRNA in brown adipose tissue (BAT), a heat-producing tissue (Himms-Hagen, 1979). Marie *et al.* (2000) showed that partially leptin resistant young MC4R^{-/-} mice were unable to produce UCP1 mRNA in BAT in response to peripheral leptin administration. This suggests that the mutation has a metabolic effect because MC4R^{-/-} mice show insensitivity to leptin-mediated thermogenesis even before they become

completely leptin-resistant. Thus these animals not only have an obese phenotype, but also a metabolic deficiency that was resultant of the genotypic manipulation.

In the pilot study for this project with a small sample size and both genders, we found that the slopes of the demand functions (elasticity of demand as previously described) of MC4R^{-/-} mice were significantly different than in WT littermates. The heterozygous deletions in MC4R (MC4R^{+/-}) resulted in an intermediate level of variance of demand functions between that of homozygous deletion and intact receptor conditions in mice (unreported data). For this reason, to capture the main contrast between the genotypes and the effects of the receptor deletions in addition to a simplicity principle, we did not include the MC4R^{+/-} mice in this project. Our hypothesis, based on our preliminary data, was that the obese mice would be hyperphagic and that their demand function would be less elastic for increasing costs for food.

MC3R ^{-/-} mice. In contrast, homozygous deletion (-/-) of the MC3R does not result in increased body weight, but may be associated with higher adiposity although not being hyperphagic and also despite being slightly hypophagic based on some reports (Chen *et al.*, 2000). Fat pad extractions indicated that nutrients are preferentially stored as fat depots rather than lean mass, resulting in what is called a “feed efficiency” in MC3R^{-/-}. They are also reported to be hypoactive compared with WT controls in terms of the ambulatory activity and this may partly explain their obesity (Chen *et al.*, 2000; Cummings and Schwartz, 2000).

It has been shown that the growth of both female and male MC3R^{-/-} mice was normal until approximately 26 weeks of age, at which time they became slightly heavier than WT although they possess normal bone mineral content, but the average length of

femur bones was significantly shorter than that of age-matched WT. These suggest that growth is stunted in the absence of MC3Rs (Chen *et al.*, 2000). Also, the absence of MC3R does not result in large changes in metabolic rate as the body temperatures of both male and female MC3R^{-/-} mice were normal. The effects of reduced expression of aforementioned MC3R in the peripheral tissue, may contribute to the phenotypes observed. We expected a similar meal patterns and demand functions but more adiposity in MC3R^{-/-} mice compared with that of WT animals.

DKO (double-knockout). Based on the reports that have been published to this date on both MC4R^{-/-} and MC3R^{-/-} mice, one may speculate that DKO mice eat excessively, due to absent MC4R signaling, and store ingested calories more efficiently, due to the absence of both receptors (Cummings and Schwartz, 2000). Chen *et al.* (2000) included a description of mice with deletions of both type 3 and 4 receptors (double knockout, DKO) but to our knowledge further characterization has not been published. To examine whether the MC3R and MC4R systems may interact, we have now bred mice with deletions of both type 3 and 4 receptors. We hypothesized that DKO mice would show a higher level of hyperphagia and the demand functions would be significantly different than that of WT mice. However since this was one of the very few studies employing this newly bred genotype of mice, we were not sure if these mice would be significantly different from the MC4R^{-/-} mice in terms of the measures used in this project.

Diet-Induced Obese Mice

Because of the aforementioned effects of homozygous deletion of MC4Rs, we also decided to conduct the same experiment with the animals that have the same obese phenotype as MC4R^{-/-} but not the underlying genotypic expression of the

metabolic deficiency. The question was if the meal patterns and the slope of the demand functions were shaped by the accumulated high levels of fat in these obese animals and/or metabolic deficiency has a role in it with a possible interaction effect.

Effects of CCK

Protocols using peripheral CCK and injection of MC4R agonists into the NTS reduce food intake. This leads to the general assumption that one functional aspect of α -MSH action may be to augment CCK and/or other vagally-mediated satiety signals. In this case, absence of MC4R should diminish the satiety effect of CCK or other gut-related signals, and this has been reported by some investigators (Blevins *et al.*, 2009). Previous work in our lab has not supported this prediction (Vaughan *et al.*, 2006b). MC4R^{-/-} mice showed slightly greater inhibition of liquid food intake after CCK injection compared to wild type mice, or after oral preload of the same diet. We wanted to generalize this finding to solid diet and expected to find similar results to our previous works in our lab, although in the literature the general view was that MC4R^{-/-} mice were less sensitive to the effects of exogenous CCK (Adan *et al.*, 2004)

Subjects and Housing

MC3R^{-/-} mice on a mixed C57B6/129 background were generated by breeding heterozygous pairs from a colony originating at Merck Pharmaceutical Co. MC4R^{-/-} mice, also on a 129/B6 background, were generated by breeding heterozygous pairs from a colony originating at Millenium Pharmaceuticals Co. DKO mice were generated by crossing the MC3R^{-/-} and MC4R^{-/-} mice. All mice were genotyped from a tail snip taken at weaning (Huzsar *et al.*, 1997). Wild type controls were the littermates (+/+) derived from our in house breeding colony. No consistent differences in meal patterns or intakes were found between males and females, so these data were not analyzed for gender

differences. These colonies are maintained in the Cancer Genetics vivarium at the University of Florida. At 3-6 mo of age, mice were moved to the Psychology vivaria for the test procedures. Both facilities are managed by a centralized and accredited animal care program.

For the DIO protocol, sixteen male C57BL/6 (B6) mice were purchased at 3 mo of age (Jackson Laboratories; Bar Harbor, ME) and housed in Psychology Department vivarium at the University of Florida. B6 mice were used because this strain has been shown to be a particularly good model for human metabolic syndrome and obesity when fed with high-fat content diet, but remains lean and physically normal when fed a low-fat diet (Surwit *et al.*, 1988; Samuelsson *et al.*, 2008).

General Methods

Twenty-four behavior test chambers were used for testing. All chambers were equipped with a nose poke device with a small cue light above. The nose poke device was used in these procedures as the operant behavior for the effort cost imposed on food. The devices were located approximately 2 cm above the floor and adjacent to a food receptacle. These data files allowed an analysis of the number of meals and the amount eaten at each meal. Data were accumulated in 15 min time bins throughout each 23 h period leaving 1 h for cleaning and weighing the mice. Each cost schedule was applied for 4 days. The experiment was run in two replications to increase the sample size, with 17 and 23 mice respectively in each phase and with each genotype represented. The data from both studies were similar and were combined for analysis.

Procedures

Experiment 1: Food Demand Analysis in MCR Knockout Mice

A total of 40 naïve mice of MC4R^{-/-} (N=12; 6 females, 6 males), MC3R^{-/-} (N=8; 3 females, 5 males), DKO (N=11; 7 females, 4 males) and WT (N=9; 3 females, 6 males) littermates were used. Mice were 4-9 mo of age during these tests. The WT group included both MC3R^{+/+} and MC4R^{+/+}, but no difference was evident so their data were combined. No consistent differences in meal patterns or intakes were found between males and females, so these data were not analyzed for gender differences. The experiment was run in two replications to increase the sample size, with 17 and 23 mice respectively in each phase and with each genotype represented. The data from both studies were similar and were combined for analysis, as planned.

Prior to the main protocol, to habituate mice to the chambers and the novel pellets, a 4-day training period was applied in which pellets were delivered contingent upon FUP2 (each pellet delivery is contingent on the completion of two responses) with the same nose poke operant. Mice were deemed to have learned the contingency when they earned enough pellets over 23 h to maintain their body weight, typically within 2 days (Atalayer and Rowland, 2009). No food deprivation was imposed at any time during the experiment. During the main protocols, mice lived in test chambers for 23 h per day and obtained a 20 mg pellet when they completed n nose poke responses as specified by a programmed FUPn schedule of reinforcement. After training, mice were tested using an increasing series of unit costs (FUP 2, 5, 10, 25, 50) with 4 days at each unit price.

At the end of the study in the second batch of mice (N=23), and after about 1 week of free feeding to regain any lost weight, mice were anesthetized (Sleepaway) and the

subcutaneous, visceral and pericardial (thoracic) white adipose tissue depots were dissected and weighed.

Experiment 2: Food Demand analysis in Diet-Induced Obese Mice

A total of 16 naïve B6 male mice were randomly divided in two dietary groups of 8, high-fat (HF) and low-fat (LF). The diets were purchased from Research Diets (New Brunswick, NJ): HF #D12492, 34.9% fat and 60% energy; LF # D01060501, 4.3% fat and 10% energy. For the next 9 weeks, mice received the designated diet in their home cages. Throughout this period HF fed mice gradually gained more weight than LF fed mice and displayed significantly higher body weight and distinct DIO. After this phase, all mice were run in the operant test chambers where they worked for 20 mg pellets (low fat) pellets for the same training period and main protocol as in Experiment 1.

Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice

Three CCK studies were performed. In the first, WT, MC4R^{-/-} and DKO were included. In the second, MC4R^{-/-}, MC3R^{-/-} and corresponding WT from two different age ranges were tested. In the third, after at least one week of free chow intake, mice from the first study were used to study induction of c-Fos immunoreactivity.

In the first study, mice were 4-9 mo of age at the time of testing. Food intake was measured in 30 min test sessions using a “dessert” protocol in which the mice were adapted to receive a highly preferred food treat at the same time each day. The dietary treats were Fruit Crunchies (Bio-Serv; No. F05798; 52% carbohydrate, 20% protein, 6% fat by weight, with carbohydrate fraction ~75% mono- and disaccharides), 190-mg spherical pellets about same macronutrient composition as chow and available in grape, apple, and orange flavors (Rowland and Robertson, 2005). During training, the mice received 10 pellets of mixed flavors in a 10 ml glass beaker, hung inside the cage from

a metal stirrup. On the first day, the beaker was left in place for as long as needed for robust intake to occur. Thereafter, the time per day was tapered rapidly to 30 min. We found that most (~90%) WT mice readily consumed Crunchies (Rowland and Robertson, 2005) whereas ~25% of MC4R^{-/-} and DKO mice did not eat this novel food even after many days of presentation. Thus, sufficient mice were initially trained so that at least 6 reliable eaters were obtained for each genotype.

Test intakes were recorded as the number of Crunchies eaten; bedding was searched for half pellets, but these or smaller crumbs were minimal: most eating of this size of food item seems to be largely all-or-none. After about 1 week adaptation to the dessert regimen, as well as to handling, a baseline was established by recording intake for 3 consecutive days. On test days, mice were injected subcutaneously at about 1300 h with either the vehicle (saline, .02 ml/10 g body weight) or CCK (6 µg/kg). These doses were chosen on the basis of our previous work (Vaughan *et al.*, 2005). The procedure was repeated 5-7 days later, but with the alternate injection. Half the mice received saline first and half received CCK. There was no order effect, so data were combined for analysis. Intakes after vehicle or peptide were expressed as % of the baseline for each individual, and data were analyzed using one-way ANOVA and post-hoc Tukey tests ($P < 0.05$). CCK (as the sulfated octapeptide) was purchased from Sigma Chemical Co (St Louis, MO). Intake after drug was also analyzed as % of the intake by each individual after vehicle injection. No food deprivation was imposed in this protocol.

In the second study, MC3R^{-/-} and MC4R^{-/-} mice of two age ranges were used, young adults (3-4 mo) and middle-aged (7-8 mo). Littermate MC3R^{+/+} and MC4R^{+/+}

were tested as controls as far as possible, but no middle-aged MC4R+/+ were available. To address in part weight differences between groups, two doses of CCK (3 and 6 µg/kg) were tested. Further, to be consistent with studies from other laboratories (Fan *et al.*, 2004; Blevins *et al.*, 2008), we used an overnight (18 h) food deprivation protocol with a standard chow pellet presented as the test food. As before, injections were made 5 min before food was presented and intake was measured after 30 min. Three tests were conducted at 1 week intervals, with each mouse receiving vehicle, 3 or 6 µg/kg CCK, in random order. Data will be presented as absolute intakes and expressed as % of the individual intakes on the vehicle test day. These data were subjected to ANOVA and post-hoc Tukey tests, with a significance criterion of $P < 0.05$.

Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice

Using the mice that had been in the first study but now 1-2 mo older, we examined induction of c-Fos immunoreactivity by CCK. This was performed in several batches, with all genotypes represented in each batch; no differences were found as a result of batch, so data were combined, as planned. On each test day, chow pellets were removed from the home cages 1-2 h beforehand to prevent recent spontaneous meals.

Mice were injected with CCK (6 µg/kg) or saline vehicle and 1 hr later were anesthetized (Sleepaway, 1 ml/kg; Fort Dodge), and perfused transcardially with heparinized saline followed by paraformaldehyde. Brains were removed, stored in paraformaldehyde overnight, and then sliced by vibratome into coronal 75 µm sections at the levels of the paraventricular nucleus (PVN) of the hypothalamus and the area postrema (AP) and adjacent nucleus of the tractus solitarius (NTS). Sections were then incubated with primary (c-Fos 4 polyclonal; Santa Cruz) and secondary (biotinylated goat anti-rabbit IgG, Zymed) antibodies, and the reaction product visualized using ABC

(Vector Labs), as we have described in detail elsewhere (Li and Rowland, 1993). Sections were mounted on slides and c-Fos positive cells in regions of interest were examined under a microscope and counted manually by two observers; to ensure a blind procedure, animal numbers were obscured during this phase. The counts from the two observers showed <5% variation and were averaged.

Data Analyses

A repeated measures analysis of variance (ANOVA; SPSS) was used with genotype as a between-subject variable and the increasing schedules as a within-subject variable. Follow-up Tukey tests were employed to assess specific differences.

For acute feeding tests and the fat pad analysis, one-way ANOVA (SPSS) and post hoc Tukey tests (where appropriate) were used to examine the genotype effects on the % suppression of food intake or the dissected body fat content. For the c-Fos studies, the numbers of Fos-stained cells in each region of treated mice were compared across genotypes using one-way ANOVAs and Tukey post-hoc contrasts.

Results

Experiment 1: Food Demand Analysis in MCR Knockout Mice

The demand for food as a function of unit price is shown in Figure 3-1. There were differences in intake as a function of unit price ($P < 0.001$) with intake declining as unit price increased. There was a significant main effect of genotype ($P = 0.006$) and a genotype x unit price interaction ($P_s < 0.001$). As is evident from panel A of Figure 3-1, at the lower costs (FUP2, FUP5, FUP10) the MC4R^{-/-} and the DKO mice ate more food per day than either MC3R^{-/-} or WT mice ($P_s < 0.001$). However, as unit cost increased further, the intakes of the MC4R^{-/-} and DKO declined more sharply than those of MC3R^{-/-} or WT, so that at the highest price (FUP50) the former groups consumed the

least ($P < 0.05$). Panel B of Figure 3-1 expresses the intake of each group as % of their intake at the lowest cost (FUP2); intakes at the highest cost (FUP50) had fallen by 40-50% in MC3R^{-/-} and WT, while intakes of the MC4R^{-/-} and DKO had fallen by ~70%.

The meal parameters are shown in Figure 3-2. The number of meals (panel A) taken per day did not differ as a function of genotype but declined as unit prices increased ($P_s < 0.001$ for all 4 genotypes). Mean meal size (panel B) was comparable in MC3R^{-/-} and WT and did not decline significantly across the unit cost series in these genotypes. In contrast, the mean meal size of the MC4R^{-/-} and DKO dropped rapidly as unit cost increased ($P_s < 0.001$). Thus, the decline in total food intake as unit prices increased (Figure 3-1) was the result of fewer and smaller meals in MC4R^{-/-} and DKO, whereas in WT and MC3^{-/-} it was a result of fewer meals only (Figure 3-2).

Body weights and fat pads. The body weights of these animals during the operant protocol are shown in Figure 3-3. Panel A shows the individual weights at the beginning and end of the cost series, and panel B shows the mean group cumulative changes in weight at each cost. DKO animals were uniformly obese at the start of the study, the MC4R^{-/-} were variably obese, and MC3R^{-/-} and WT had comparably low weights. All animals lost some weight during the FUP series, but the greatest absolute loss was in the MC4R^{-/-} and DKO animals (Figure 3-3B). One-way ANOVAs on the cumulative changes were significant ($P_s < 0.001$) at FUP50. Interestingly, the greatest individual weight losses were not consistently associated with the highest initial weights (Figure 3-3A).

These differences in body weight were reflected in dissected body fat (Table 3-1 and Figure 3-4). The mean fat from the combined subcutaneous and visceral depots

rose from a mean of 3% body weight in WT to 11.6% in MC4R^{-/-} and 21.7% in DKO. The thoracic cavity also contained visible fat in MC4R^{-/-} and DKO: while not a large mass compared with the other pads, thoracic fat mass (<0.02 g in WT and MC3R^{-/-}) was 0.06±0.01 g (mean±SEM) in MC4R^{-/-} and 0.13±0.02 g in DKO.

Experiment 2: Food Demand analysis in Diet-Induced Obese Mice

Prior to the diet regimen, mice designated for HF and LF groups had similar weights (24.2±0.4 and 24.2±0.3 g respectively, mean±SEM). At the end of 9 weeks, the HF and LF groups weighed 45.6±0.7 g and 35.3±1.5 g ($P<0.001$).

The demand for food as a function of unit cost is shown in Figure 3-5A. There were differences in intake as a function of unit price ($P<0.001$) with intake declining as unit price increased. The only significant difference between the DIO (previously HF) and LF mice in terms of total pellets earned per day was during FUP50 ($P<0.05$) as DIO mice received less pellets than LF mice. The meal parameters are also shown in Figure 3-5. The number of meals (Figure 3-5B) taken per day did not differ as a function of phenotype but declined as the unit prices increased ($P_s<0.001$ for both phenotypes). Mean meal size (Figure 3-5C) did not change significantly across the unit cost series in HF mice. In contrast, the mean meal size of the LF mice increased as unit cost increased ($P<0.05$).

Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice

In the first study, using the dessert protocol, mean intake of Crunchies in the baseline period did not differ as a function of genotype. The mean (±SEM) numbers of Crunchies eaten were: WT 8.5±0.4, MC4R^{-/-} 7.1±0.7, DKO 7.2±0.6. After vehicle injection, mean intakes of the WT and MC4R^{-/-} groups were within 5% of their baseline, but the intake of the DKO group was reduced significantly both from their baseline and

from the absolute intake of the other two groups ($P_s < 0.05$) to 4.4 ± 5 and 5.4 ± 8 Crunchies (64% of their baseline). Intakes following peptide injections were thus analyzed both as % baseline and % vehicle (Table 3-2). The anorectic effect of CCK was at least as great in the MC4R^{-/-} and DKO mice as in WT. CCK reduced food intake from vehicle and baseline in each group ($P_s < 0.05$). While the suppression of intake in the MC4R^{-/-} and DKO groups was slightly greater than for the WT, only the DKO was significant ($P < 0.05$) relative to WT and only when expressed as % baseline.

The deprivation-chow protocol was used in the second study, and the results are shown in Table 3-3 and Figure 3-6. Table 3-3 shows the absolute intakes, which differed significantly across groups ($P < 0.001$). Post-hoc tests showed that the intake of young (3-4 mo) and middle-aged (10-11 mo) MC4R^{-/-} differed significantly ($P_s < 0.02$) from each other, but neither differed from the corresponding MC4R^{+/+} (for which only young mice were available). Two-way ANOVA showed that intakes were lower in MC3R^{-/-} than in MC3R^{+/+} groups, and were lower in young than middle-aged cohorts. The higher dose of 6 $\mu\text{g}/\text{kg}$ suppressed intake relative to the vehicle condition in all groups; the lower dose of 3 $\mu\text{g}/\text{kg}$ suppressed in all groups except for young MC3R^{-/-} and MC3R^{+/+}. Mean intakes as % vehicle are shown in Figure 3-5A. At the lower dose, the young MC3R^{-/-} and MC3R^{+/+} differed from all other groups (one-way ANOVA; $P_s < 0.01$) while at the higher dose there was no group difference in % suppression. Since the MC4R^{-/-} groups weighed about 2x more than the MC4R^{+/+} control (Table 3-3), for a given dose they received about twice the absolute amount of CCK. Thus, approximately the same absolute amounts of CCK were received by the MC4R^{+/+} after

6 µg/kg and MC4R^{-/-} after 3 µg/kg, respectively; the % intakes were comparable in these conditions (Figure 3-6B).

Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice

The effect of CCK-8 on Fos-ir in PVN is shown in Table 3-4. Fos was induced strongly in WT, MC4R^{-/-} and DKO mice, but less so in MC3R^{-/-} ($P < 0.05$), with this latter group not significantly different from saline-treated controls. This dose of CCK induced very strong Fos in the AP and NTS of all 4 genotypes, and accurate counting was not possible.

Discussion

Experiment 1: Food Demand Analysis in MCR Knockout Mice

MC4R^{-/-} and DKO mice were obese and hyperphagic relative to WT, but only under the specific economic conditions of low cost for food. The elastic nature of their demand functions for food was not replicated in DIO B6 mice. The hyperphagia of MC4R^{-/-} and DKO mice, when present, was due mainly to large meal size. This suggests that the disruption of the MC4R gene, and independently of obesity, is sufficient to disrupt normal physiological controls of and/or environmental effects on food intake and meal size.

These findings in MC4R^{-/-} mice are consistent with but expand considerably on previous reports from our laboratory. Thus, using a FUP3 schedule, we found that MC4R^{-/-} ate ~33% more food per day than WT, and with larger meal size (Vaughan *et al.*, 2006b). Hyperphagia and increased meal size was also found using progressive ratio schedules of pellet cost; under progressive schedules, MC4R^{-/-} paid a higher average cost per pellet than WT (7.7 vs 5.6 lever presses under one condition, see Vaughan *et al.*, 2006b, Table 2). However, under a procurement-consummatory dual

cost foraging protocol (Vaughan *et al.*, 2005), intake of MC4R^{-/-} was identical to that of WT and MC3R^{-/-} across a range of procurement costs but low (FUP 5,10) per pellet costs. Together with the present data, these findings suggest that MC4R^{-/-} are hyperphagic when proximate or unit costs per pellet are less than ~10 responses. If unit costs increase further, including when part of the cost is distal or 'approach', then the mice are no longer hyperphagic and may eat less than WT. As a result they lose weight (see Vaughan *et al.*, 2005, Figure 3) but that weight loss does not appear to influence their food-directed operant behavior. In this regard, the first meal is smaller in MC4R^{-/-} after food deprivation (Blevins *et al.*, 2008) and from Table 3-3 it can be derived that young MC4R^{+/+} ate 26.2 g/kg body weight/30 min compared with 11.8 g/kg in MC4R^{-/-}. It follows that MC4R^{-/-} may be relatively insensitive to afferent information about adiposity, consistent with the observation by Marsh *et al.* (1999) that MC4R^{-/-} mice did not show anorexia to either peripheral or central injection of leptin.

This is to our knowledge the first detailed report of intake and feeding patterns in DKO, but our data agree with Chen *et al.* (2000) that they are significantly more obese than MC4R^{-/-}, a difference that is due almost exclusively to more adipose tissue (Table 3-1: note that the fat pads dissected do not account for all body fat). At 26 weeks of age, Chen *et al.* (2000) reported that female and male DKOs were 27 and 13% heavier, respectively, relative to MC4R^{-/-} counterparts. The sex difference in weight in MC4R^{-/-} was no longer evident in DKO. Our body weight data closely confirm those observations (Figure 3-3). We also noted considerable pericardial fat in the DKO mice.

Experiment 2: Food Demand analysis in Diet-Induced Obese Mice

DIO and control mice in Experiment 2 showed generally similar, although flatter demand functions than the WT in Experiment 1. Interestingly, despite their obese state

at the start of the FUP series, the DIO mice showed the same total intake and parameters as the non-obese (low-fat, LF) group. Note that for both groups, this involved a change to a common grain-based, low carbohydrate diet, and so it suggests that the diet and the economy determined intake more than the weight of the animal. Further, the DIO mice lost weight as FUP increased but there was no tendency to eat less than LF except at the highest cost. Clearly, the demand curves in DIO mice are inelastic or flat compared with the steep functions in MC4R^{-/-} and DKO.

Experiment 3: Behavioral Effects of Exogenous CCK-8 in MCR Knockout Mice

Electrophysiological, biochemical, and behavioral studies suggest that activation of MC4Rs in the NTS/AP and/or dorsal motor nucleus of the vagus potentiates the effect of vagally-mediated satiation signals such as CCK (Appleyard *et al.*, 2005; Fan *et al.*, 2004; Wan *et al.*, 2008; Blevins and Baskin, 2010). This is consistent with our demonstration of a large meal phenotype in MC4R^{-/-} and DKO at low food costs. It follows that the satiating effect of CCK-8 should be reduced in MC4R^{-/-} and DKO, but probably not in MC3R^{-/-}, relative to WT. That result has been reported in MC4R^{-/-} in two publications (Fan *et al.*, 2004; Blevins *et al.*, 2008) both using a food deprivation and chow protocol similar to our own. In contrast, using a 12 hr fast and a liquid diet, we found that the intake in MC4R^{-/-} was suppressed by various doses of CCK-8 at least as effectively as in WT (Vaughan *et al.*, 2006a).

In the study by Fan *et al.* (2004), mice were 9 wk of age and so obesity presumably had not fully developed. The dose they used (3 nmol/kg, close to our lower dose) reduced 30 min food intake in MC4R^{-/-} by ~40% relative to saline, less than in WT (~70%) and not significant. Their feeding test lasted 3 hr, and at later times the suppressant effect of CCK had completely disappeared in MC4R^{-/-} but not in the WT.

Thus, at the short interval we used (30 min). Fan's MC4R^{-/-} were in fact partially responsive to CCK. Blevins *et al.* (2008) reported a dose-response analysis, with mice receiving 0.75, 2.5 or 7.5 nmol CCK-8/kg lean body mass, and a 30 min food intake test. They found a suppression of food intake in WT at all 3 doses, but in MC4R^{-/-} only the highest dose produced a significant reduction relative to vehicle injection. However, at the highest dose, the % suppressions were not markedly different in WT and MC4R^{-/-} (43 and 36%, respectively). Our doses were not based on lean body mass, but Blevins (personal communication, June 2009) graciously provided data that the lean body mass of his WT and MC4R^{-/-} mice averaged 78% and 63% of body weight, respectively. Thus, the highest dose that his mice received transform to 5.85 and 4.72 nmol/kg (~5.5 and 4.5 µg/kg) in WT and MC4R^{-/-} respectively, a dose that is intermediate between our two doses. In our study, there is no evidence in either young or middle-aged MC4R^{-/-} for reduced sensitivity to CCK (Figure 3-6). This was also evident in our non-deprived (Crunchies) protocol (Table 3-2), which showed that DKO were also responsive to CCK-8. None of the published or present protocols indicate that MC4R^{-/-} are completely unresponsive to exogenous CCK, but in some protocols there may be a reduced responsiveness. Consistent with this, we were unable to find differences in Fos expression in AP/NTS of either MC4R^{-/-} or DKO compared with WT. Finally, in a study in which we stimulated endogenous release of CCK and other meal related factors, we found that oral preloads suppressed subsequent intake equivalently in WT and MC4R^{-/-} (Vaughan *et al.*, 2006a).

The relative sparseness of MC3R in the hindbrain, as well as the above results in DKO, would lead us to expect that MC3R^{-/-} mice would show normal satiation

responses to CCK-8. This was in fact reported by Fan *et al.* (2004) using mice 5-10 mo of age. However, our results revealed an unexpected insensitivity to CCK-8 (Figure 3-6). In the young cohorts, both the MC3R^{+/+} and ^{-/-} genotypes were unresponsive to the lower dose of CCK-8 and ^{-/-} showed a smaller effect than ^{+/+} at the higher dose. In middle-aged cohorts, the low dose was comparably effective in MC3R^{+/+} and ^{-/-}, but again at the higher dose the ^{-/-} were less responsive. In a preliminary study, using even younger mice (~2 mo) and a higher dose of CCK-8 (9 µg/kg), we found complete insensitivity in MC3R^{-/-}.

Experiment 4: C-Fos Inducing Effects of CCK-8 in MCR Knockout Mice

The Fos-ir data give no indication for a reduced CCK activation in AP/NTS in any genotype relative to WT, but they do suggest a reduced activation in PVN of MC3R^{-/-}. If peripheral CCK-8 is activating the PVN exclusively via the NTS/AP as a relay, this result would suggest that the relay has reduced effect in MC3R^{-/-} either because the synaptic transmission is reduced or the target neurons are tonically inhibited, for example because inhibitory POMC arcuate afferents have increased firing rate due to loss of functional MC3R autoreceptors. If this tonic inhibitory signal were carried through MC4Rs, then DKO mice would be insensitive to any increase in arcuate firing due to the MC3R^{-/-} deletion. In any event, the reduced sensitivity of MC3R^{-/-} to both CCK anorexia and c-Fos expression in PVN, at least warrant the speculation of a functional connection. This hypothesis requires further investigation, as well as the corollary that overexpression of MC3R might increase sensitivity to CCK.

Chapter Discussion

In sum, our data show more evidence for insensitivity to CCK-8 in MC3R^{-/-} than in MC4R^{-/-}, and that this may be age-dependent. Thus, putative insensitivity to CCK is not

a viable explanation for the large meal phenotype in MC4R^{-/-} because MC3R^{-/-} should show comparably increased meal size, but they do not. In addition, the elastic demand functions of the genetically obese mice indicated that although MC4-R signals hunger, the phenotypic expression of its deficiency was affected by the economic structure of the food environment. This was an unexpected finding because in human studies it has been shown that the incentive value of food in obese humans were reported to be higher and that these individuals were more willing to pay higher prices for food (Temple *et al.*, 2009; Saelens and Epstein, 1996). This was in agreement with our results here only when the price was low. As the prices increased, obese mice decreased their efforts to get the food. In an early study by Hamilton and Brobeck (1964), it has been shown that although to a lesser extent than rats, ventromedial hypothalamus-lesioned monkeys decreased their efforts as the fixed unit price increases comparably more than the sham-lesioned monkeys. Elastic demand in MC4R^{-/-} and DKO was also shown to be more reflective of their meal sizes and this was not due to obesity per se as diet-induced obese mice had significantly flatter demand functions compared to the genetically obese mice.

The literature on MC3R^{-/-} mice is rather inconsistent. Chen *et al.* (2000) had reported modestly lower free food intake in MC3R^{-/-} relative to WT and were suggested to be highly susceptible to diet-induced obesity when exposed to a high-fat diet. Female MC3R^{-/-} mice were reported to consume normal amounts of food, yet gaining more body weight than either WT or MC3R^{-/+}. This has not been generalized to male mice in their study. One study suggests that MC3R^{-/-} mice are unusually susceptible to diet induced obesity when fed with high fat food (Cummings and Schwartz, 2000). This was

somewhat counterintuitive to the literature on MC3R^{-/-} as it has been reported by many that these mice are hypoactive and strictly maintain their weight similar to a WT mouse. Thus, we replicated this idea by feeding MC3R^{-/-} with high-fat content food to see if their weight increase will be any different than that of WT mice. Our data, from a small number of animals (unreported), was not consistent with this view however; there was some tendency for higher fat accumulation in MC3R^{-/-} in subcutaneous depots.

We expected male MC3R^{-/-} mice to accumulate more adipose tissue when fed with HF diet than WT mice. We used MC3R^{-/-} (N=4) and WT (N=4) mice for this protocol. Same HF diet in Experiment 2 was used here. For the next 11 weeks, mice received the designated diet in their home cages. Prior to the diet regimen, both genotypes had similar weights (WT; 24.5±1.0 and MC3R^{-/-}; 25.8±1.6 g, mean±SEM). At the end of 11 weeks, there was not a significant difference between their weights (WT; 34.6±2.3 and MC3R^{-/-}; 34.3±2.6 g, mean±SEM). There was also no significant difference in the fat pad weight in MC3R^{-/-} mice compared to the WT.

The large meal size phenotype in MC4R^{-/-} and DKO, appeared not to be related to compromised CCK action as we found no evidence for a diminished action of CCK in MC4R^{-/-} and DKO when compared to WT mice. This was shown in various doses and also with manipulating various methodological factors in order to generalize the findings and to match it with previous reports. The data from c-Fos studies examining the effects of CCK supported the behavioral finding as the number of c-Fos induced cells were comparable in DKO, MC4R^{-/-} as in WT, thus, insensitivity to CCK is concluded to not to be a viable explanation for the large meal phenotype in MC4R^{-/-} and DKOs. In MC3R^{-/-} mice however, c-Fos induced cells were markedly less in PVN which was an

unexpected finding as MC3R have not been reported to be located in this structure. In a recent study (unreported data) we found enough evidence to believe that the normal activation in PVN is presumably mediated by MC3Rs, and whereas the normal activation in AP is through MC4Rs. In this MTII study we tested the acute anorectic effect of centrally-administered MTII (a MC3 and MC4R agonist), and subsequently examined the induction of Fos-immunoreactivity in select brain regions. As expected, MTII had no anorectic effect in DKO mice, but was anorectic in MC3R^{-/-}, MC4R^{-/-}, and WT. MTII decreasing food intake in both MC3R^{-/-} and MC4R^{-/-}, suggests that stimulation of either receptor is sufficient enough to produce anorexia. Fos-ir was induced strongly by MTII in PVN and AP in WT mice. In MC3R^{-/-} the induction in PVN was minimal and the effect in AP was as strong as in WT, whereas the reverse pattern was seen in MC4R^{-/-}. C-Fos was induced strongly in AP in all genotypes, but was reduced in PVN of MC3R^{-/-} relative to other genotypes. These results from this MTII c-Fos study suggest a double dissociation of region of action. Fos-ir was absent in the PVN of MC3R^{-/-}, but was normal in MC4R^{-/-}, suggesting that MC3Rs alone underlie the induction of Fos in the PVN. In contrast, in the AP, only the MC4R^{-/-} mice had reduced Fos-ir relative to WT, suggesting that MC4Rs alone underlie the induction of Fos induction in the AP.

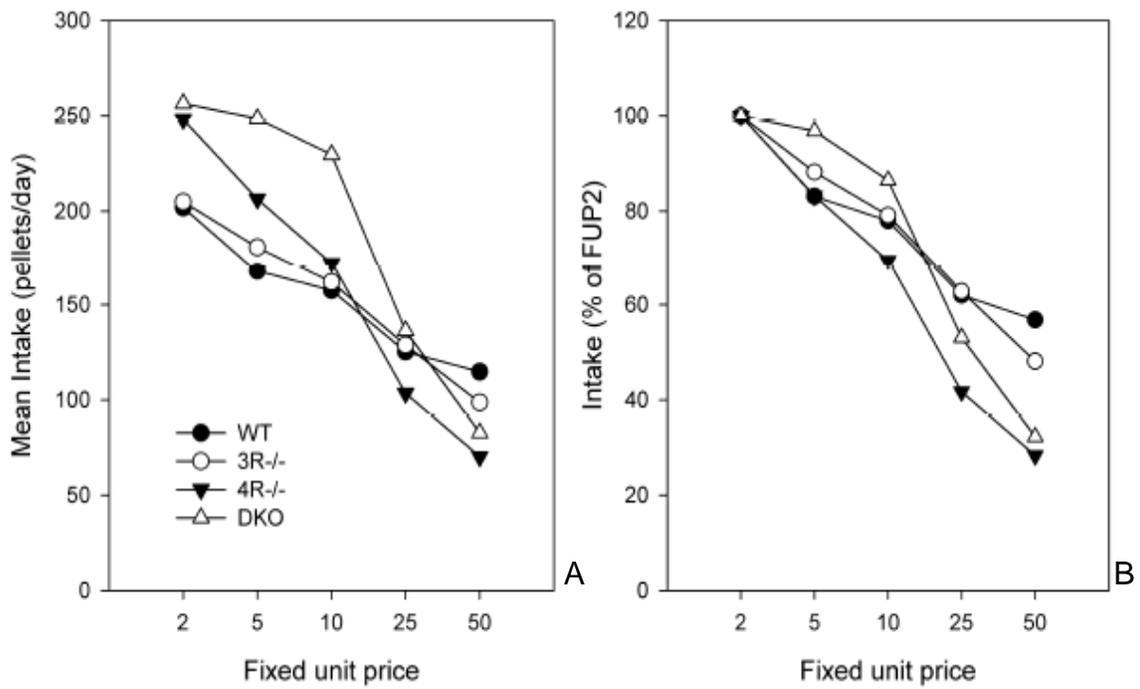


Figure 3-1. Daily (23 h) food intake, A) shown as number of 20-mg pellets eaten, in WT, MC3R-/-, MC4R-/- and DKO mice exposed to increasing fixed unit prices (nose pokes) per pellet. Each datum is the average of 4 days at each price step and 8-12 mice per genotype. B) The panel shows the intake in absolute terms; the right panel shows intake expressed relative to intake at the lowest cost.

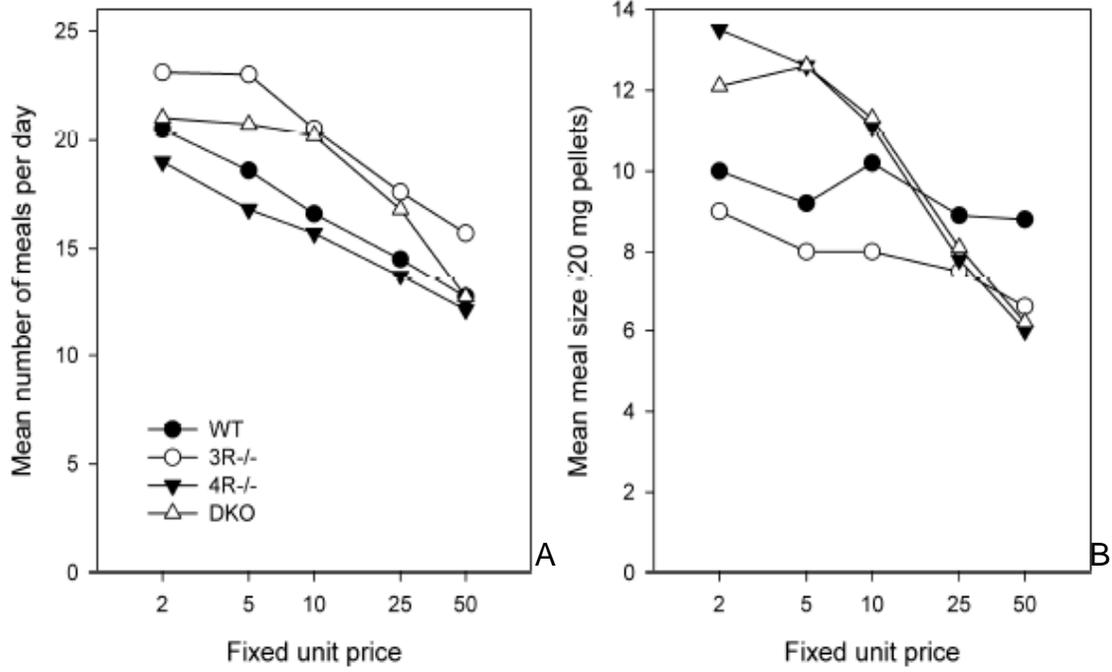


Figure 3-2. Intake data from Figure 3-1 analyzed for A) number of meals and B) mean meal size.

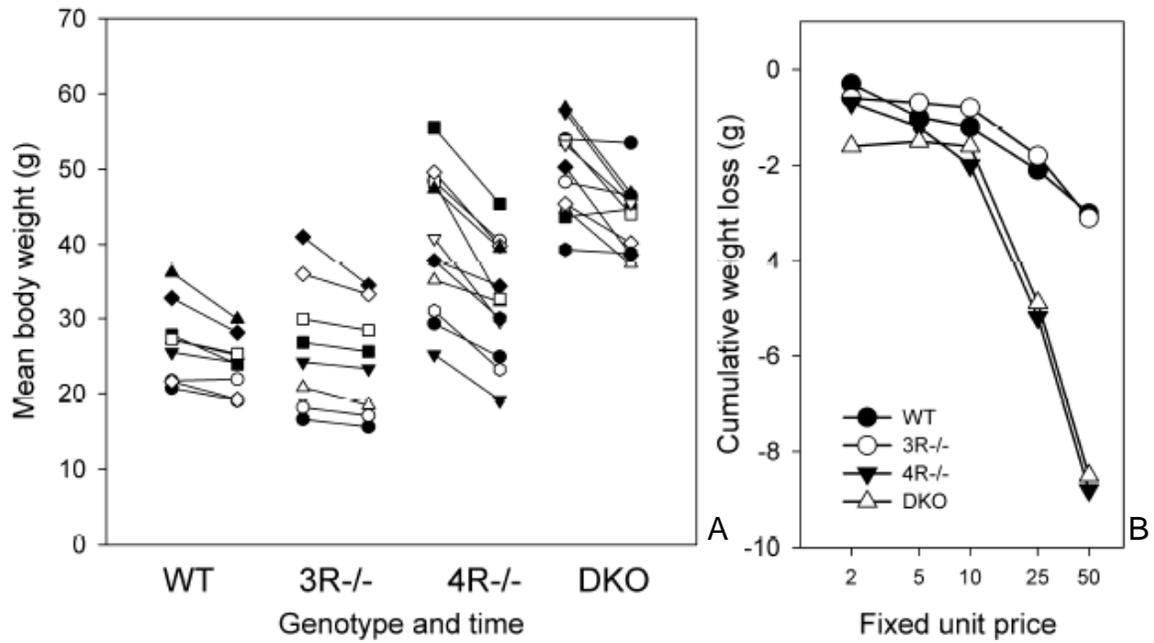


Figure 3-3. Weight changes of mice during the demand series. A) Weights of individual mice (different symbols) of each genotype at the beginning of the study (first 2 days of FUP2) and at the end of the study (last 2 days of FUP50). The lines thus join the start and end weights for each mouse. B) Weight loss as group means, the cumulative body weight changes from initial by the end of each demand schedule.

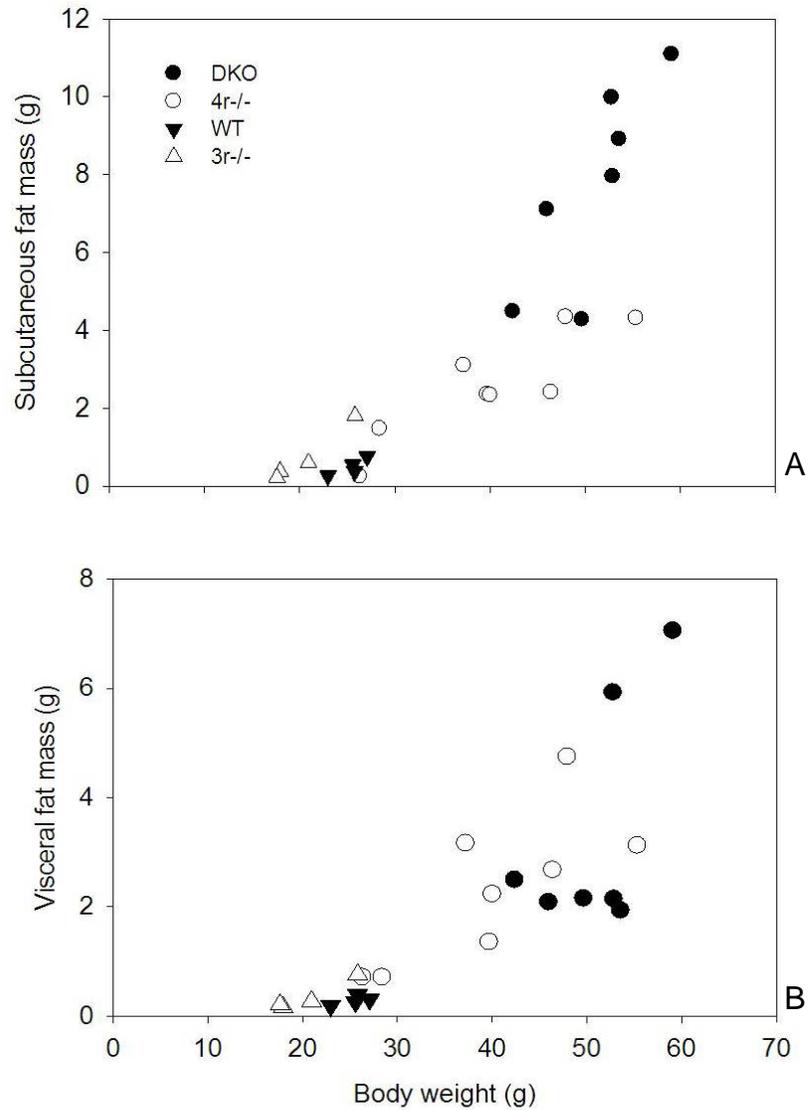


Figure 3-4. Scatterplots showing fat pad amounts. A) Subcutaneous and B) visceral fat pad weights in individual WT, MC3R^{-/-}, MC4R^{-/-} and DKO mice as a function of body weight. Fat pad weight was strongly correlated with body weight, but note that in DKO the subcutaneous fat is more consistently elevated than visceral fat.

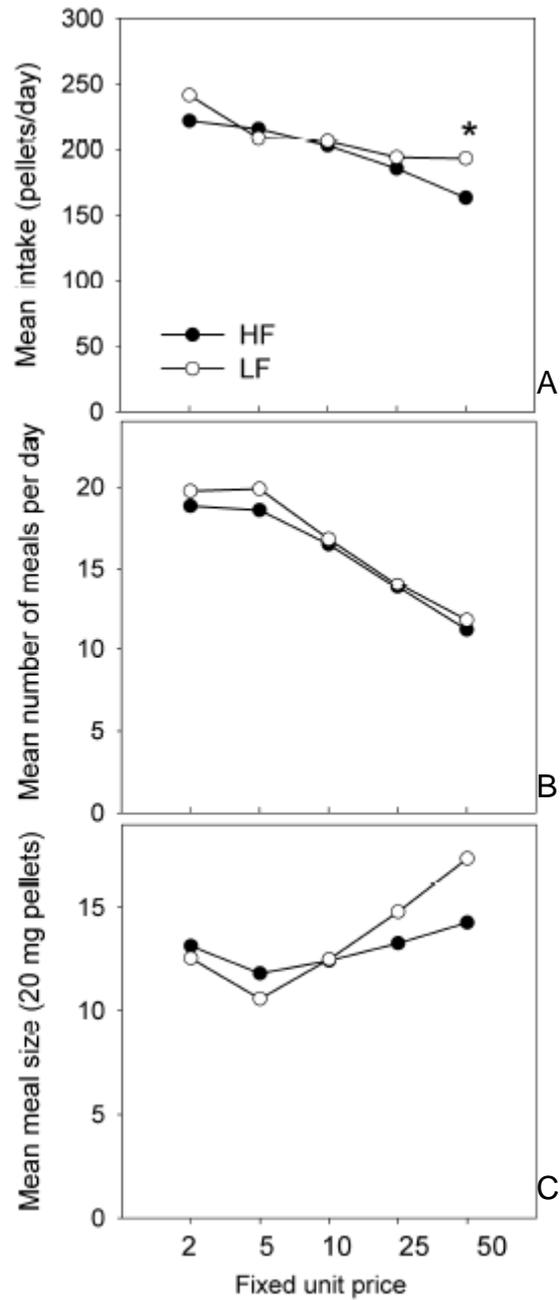


Figure 3-5. A) Daily (23 h) food intake, as number of 20-mg pellets eaten, in DIO (HF) and LF mice exposed to increasing fixed unit prices (nose pokes) per pellet. Each datum is the average of 4 days at each price step. Intake data were further analyzed for B) number of meals and C) mean meal size.

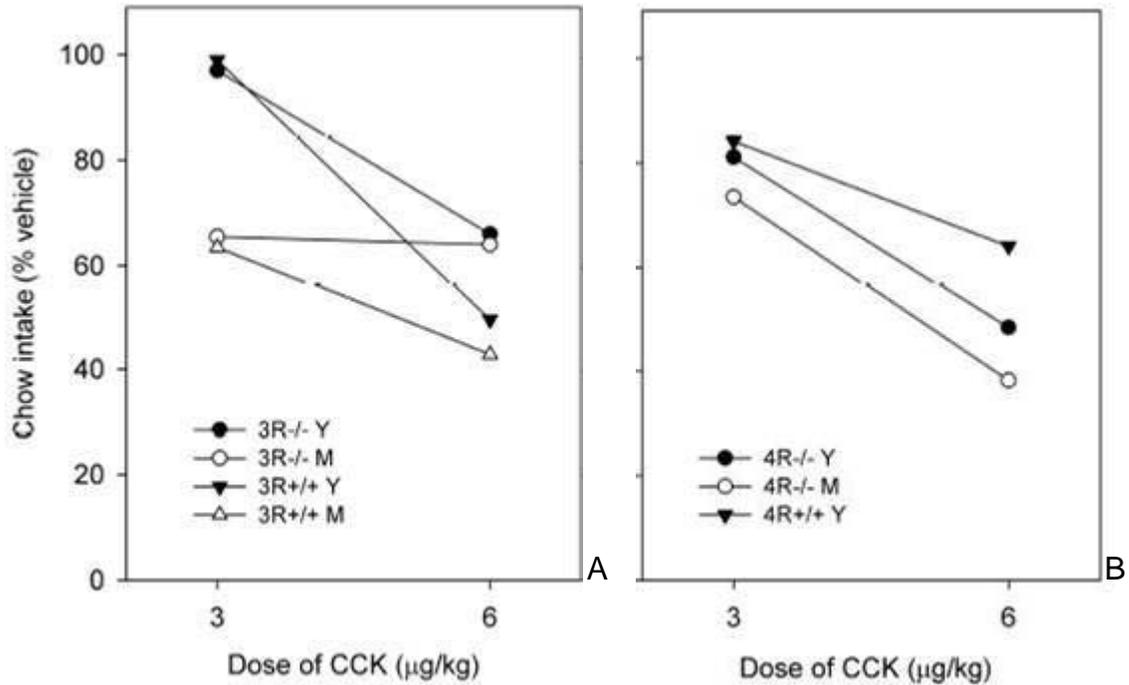


Figure 3-6. Effect of CCK-8 on intakes of chow in 30 min tests after 18 h food deprivation. Intakes are expressed as mean % intake after injection of vehicle; error bars are not shown for clarity, but average about 10% of the means. A) Data from young adult (Y: 3-4 mo) and middle-aged (M: 7-8 mo) MC3R^{-/-} and MC3R^{+/+} mice. B) Data from Y and M (10-11 mo) MC4R^{-/-} and Y MC4R^{+/+} mice. The numbers of mice per group were 4-10 as shown in Table 3-3.

Table 3-1. Fat content of mice from Experiment 1

Genotype (N)	Body wt (g)	Dissected fat (g)	Fat (% body wt)
WT (4)	25.3±.9 ^a	0.8±0.1 ^a	3.0±.4 ^a
MC3R ^{-/-} (4)	20.6±1.9 ^a	1.1±.5 ^a	4.9±1.7 ^a
MC4R ^{-/-} (8)	40.4±9.8 ^b	4.9±.9 ^a	11.5±1.7 ^b
DKO (7)	50.4±2.2 ^c	11.1±1.7 ^b	21.5±2.4 ^c

Shown are mean±SEM (N in parentheses). Statistical difference ($P<0.05$) between groups is indicated by different superscripts.

Table 3-2. Effect of CCK-8 (6 $\mu\text{g}/\text{kg}$) on intake of crunchies by undeprived mice

	Test Intake (% baseline)	Test Intake (% vehicle)
WT (5)	49 \pm 5	46 \pm 5
MC4R ^{-/-} (5)	28 \pm 9	25 \pm 8
DKO (6)	29 \pm 4*	47 \pm 7

Shown are mean \pm SEM (N in parentheses) * $P < 0.05$, vs corresponding WT datum.

Table 3-3. Effect of CCK-8 on chow intake by deprived mice

Genotype	Age (mo)	N	Body weight (g)	Intake after vehicle (g)	Intake after CCK (3 µg/kg)	Intake after CCK (6 µg/kg)
MC3R ^{-/-}	3-4	5	21.9±1.2	0.53±.04	0.53±.07	0.38±0.13
MC3R ^{-/-}	7-8	5	26.0±0.9	0.72±.09	0.48±0.1	0.47±.06
MC3R ^{+/+}	3-4	4	18.9±1.2	0.69±.06	0.68±.07	0.35±0.11
MC3R ^{+/+}	7-8	10	23.6±0.8	0.81±.04	0.52±.05	0.35±.06
MC4R ^{-/-}	3-4	7	42.5±1.2	0.50±.05	0.40±.05	0.24±.03
MC4R ^{-/-}	10-11	5	57.1±4.6	0.76±.03	0.57±.08	0.28±.03
MC4R ^{+/+}	3-4	7	25.6±0.6	0.67±.04	0.56±.05	0.41±.02

Shown are mean±SEM. Food intake in grams. Body weights are mean of 3 weeks, pre-deprivation.

Table 3-4. Fos-positive cells in paraventricular hypothalamus following CCK-8 (6 μ g/kg)

Group (N)	Fos-positive cells
WT (6)	235 \pm 50*
MC3R-/- (6)	122 \pm 26
MC4R-/- (4)	269 \pm 18*
DKO (6)	218 \pm 38*

Shown are mean \pm SEM (N in parentheses). * P <0.05 differs from saline-treated controls (36 \pm 5, N=4).

CHAPTER 4 NEGATIVE ENERGY BALANCE AND FOOD DEMAND

Introduction

Although there is a great deal of evidence in the literature showing the effects of exercise as a treatment for obesity and for maintaining a healthier life style (Colbert *et al.*, 2006; Melanson *et al.*, 2009), the economic structure of the food environment that humans and animals live in has not been taken into account. Some studies suggest that obesity is to a great extent an economic issue (Drewnowski, 2004). In addition in our studies above we have shown that environment and genetics both play roles on the eating behavior and obesity, thus when examining the health benefits of exercise on obesity and fat accumulation one should also include the economic setting of the food environment.

In the first experiment (Experiment 5) we used an animal model of voluntary running wheel activity and compared the demand functions and body weights of running and non-running (sedentary) mice. The second experiment (Experiment 6) included a foraging paradigm in which we employed two types of food costs that were defined in Chapter 1. Thus the food was contingent upon running wheel activity that was used as the approach cost (AC) and a concurrent fixed unit price (FUP) with a lever press response. We compared the effects of running wheel activity as opposed to nose poke response as the AC requirement. We also included a control group under a low cost FUP5 with a nose poke operant. The aim of this experiment was to investigate whether mice with high workloads of wheel running activity would exhibit different demand functions, as they may pay higher costs for food where the running is the effort

compared to nose poke as a common but less effortful type of operant behavior (Atalayer and Rowland, 2009; David et al., 2001).

Subjects and Housing

A total of 45 naïve male albino (CD1; Harlan, Indianapolis IN) were used in the protocols. CD1 mice (2-3 mo) were chosen because they are of mixed origin and have food intake near the middle of a range of strains of mice (Bachmanov *et al.*, 2002). Between the receipt and the experiments, mice were kept under the conditions explained in general methods in Chapter 2. During the experiments, mice lived in test chambers for 23 h per day. The mice were weighed daily and kept in empty holding cages during a 1 h cleaning period without food, although water was available.

General Methods

Behavioral test chambers were used for testing. In addition to the nose poke operants, 8 chambers were additionally fitted with an external running wheel in the test chambers as described in the general methods section of Chapter 2. Mice had free access to the wheels. Data were accumulated in 15 min time bins throughout each 23 h period.

Procedure

Experiment 5: Voluntary Running Wheel Activity and Food Demand

A total of 15 CD1 mice were divided randomly in two groups, one sedentary (N=8) and the other with access to running wheels (runners, N=7). Prior to the main study, in order to habituate mice to the operant chambers and to the novel pellets a 1 h training period was applied with free food available in the food receptacle at no cost and, in the runner group, access to the wheel was blocked by a removable wall panel. Then, for the next 1-2 days and with wheel access still blocked, a FUP2 was in effect in which

one food pellet was delivered contingent upon two nose poke responses. Mice were deemed to have learned the contingency when they earned enough pellets over 23 h to maintain their body weight; this typically occurred within 2-4 days. No food deprivation was imposed at any time during the experimental period.

After this training with the response device, access to the wheels was allowed for the runner group, and all mice were exposed an incrementing series of food cost schedules each of which was in effect for 4 consecutive days. The FUP were 2, 5, 10, 25 and 50 nose poke responses per pellet.

At the end of the food demand study, the mice were euthanized (Sleepaway) and the subcutaneous, visceral and pericardial (thoracic) white adipose tissue depots were dissected and weighed.

Experiment 6: Running Wheel Activity as Food Foraging

A total of 30 mice were used in this protocol which was completed in two batches. In the first part, 24 mice were randomly divided in three groups (Ns=7-8). Two experimental groups (runGR and pokeGR) were tested in a concurrent schedule of AC and FUP in operant chambers. The third group was a control group representing an ad libitum food intake condition in test chambers with only a low cost (FUP5) imposed upon throughout the protocol with a nose poke operant. Prior to the main study, in order to habituate mice to the operant chambers and to the novel pellets a 1 h training period was applied with free food available in the food receptacle at no cost. Then, for the next 1-2 days a fixed unit price of five responses (FUP5) was in effect in which a food pellet was delivered contingent upon five responses on the designated device.

For the runGR, running wheel activity was designated as the approach cost (AC) response and lever press was chosen as the fixed unit price (FUP) response. For the

pokeGR, a nose poke operant was used as the AC response and lever press was also chosen as FUP response. For the third group which was the ad libitum food intake simulation control (adlibGR) a nose poke operant was used as the FUP response. For this group no AC was applied. A nose poke operant with a low cost (FUP5) was chosen to create a minimal effort requirement for the animals. For the present purposes, this group will be representing a 'free food' condition in an operant chamber environment. The nose poke operant was chosen for reasons that we reported previously (Atalayer and Rowland, 2009). Following separate initial trainings at FUP5 with the designated operants for each group, as the main protocol runGR was tested in a series of approach cost (AC 25, 50, 100, 500, 1000) with a constant FUP5, each of which was in effect for 4 consecutive days. The approach costs for the pokeGR were AC5, 10, 25, 50, 100 with the same constant FUP5.

During the protocol, after completion of the designated AC on the running wheel (for runGR) or nose poke device (for pokeGR), a cue light was illuminated above the lever operant, indicating its availability for food responding at the prevailing FUP5. In addition, a house light was illuminated concurrently at the same time and for the duration with the cue light. Subsequently, whenever 15 min elapsed without a response on the lever, a meal was declared finished (as 15 min is our meal-to-meal interval criterion) then the lever was inactivated and the cue and the house light extinguished. Only a cue light that was lit at all times above the nose poke operant was used for the adlibGR, and the lever press operant was inactive at all times for this group. Total pellets and responses were recorded every 5 min throughout 23 h sessions.

At the end of the above protocol, a second batch of running mice (N=7) was added

to the protocol in order to increase the sample size for runGR. Data from both batches for two runGR groups were combined in later analysis in terms of mean number of pellets, meal size, meal numbers and total daily revolutions.

Data Analysis

Spilled pellets found beneath the cage floor were subtracted from the total earned; in every case spillage was small (typically <5% of the number earned). After the numbers of meals per day were determined for each mouse, the mean meal size was derived by dividing the number of total pellets by the number of meals for each day. Data were treated using a repeated measures analysis of variance (ANOVA; SPSS) with the activity level (sedentary versus runner) as a between-subject variable and the cost schedules as a within-subject variable. For Experiment 6, operant type (nose poke and running wheel activity) was used as the independent variable. Follow up Bonferroni and/or LSD tests were employed to assess specific differences.

Results

Experiment 5: Voluntary Running Wheel Activity and Food Demand

Running revolutions, meal numbers, meal sizes and the number of pellets consumed did not differ significantly across the 4 days that a given schedule was in force (Figure 4-1). Thus, to reduce the data for presentation, revolutions and meal parameters were averaged across the 4 days to give a single mean value for each mouse at each price (FUP), and group means (\pm SEM) computed from these individual means.

The numbers of pellets taken are shown as the demand functions in Figure 4-2A and Table 4-1 shows the caloric energy intake in both groups. Two-way ANOVA revealed that intake was significantly different between the runner and sedentary groups

($P < 0.001$), and the interaction between unit price and running condition was also significant ($P < 0.001$). The comparison between food intake of sedentary and runner mice showed significant differences at FUP10, 25 and 50 (all $P < 0.05$) and a difference in the reverse direction at FUP2 ($P < 0.05$). The number of pellets consumed per day by the sedentary mice declined monotonically as unit price increased while the intake of runner mice was relatively constant. Thus, the following pairwise comparisons showed that at every increase in the unit price, the intake of sedentary mice not running mice, decreased significantly (FUP2 compared with FUP5, 10, 25, 50; all $P_s = 0.001$, for all the other possible FUP comparisons; all $P_s < 0.05$).

The corresponding meal size and frequency data are shown in Figure 4-2B and 4-2C. Runners took consistently fewer meals than sedentary mice (Figure 4-2B). Pairwise comparisons indicated that this was significant at each unit price condition ($P < 0.05$ at each FUP and $P < 0.001$ at FUP5) except FUP50. Thus, two-way ANOVA for meal frequency revealed a between-subject effect of exercise and also a main effect of unit price increases for both groups as number of meals decreased significantly as the unit price increased ($P < 0.001$).

Meal size analysis showed runners ate consistently larger meals than sedentary mice (Figure 4-2C). Pairwise comparisons between the meal sizes of runner and sedentary mice showed that runners ate larger meals at each unit price condition ($P < 0.05$ at each FUP and $P < 0.001$ at FUP10). Thus, two-way ANOVA for meal size revealed a between-subject effect of exercise ($P < 0.001$) and but no main effect of unit price increase as both groups consumed relatively constant meal sizes throughout the protocol.

All mice ran substantial amounts, usually in excess of 5 km/day, and voluntary running wheel activity did not change as a function of unit price (Table 4-2). Nose poke responses on the other hand changed as a function of the unit price increases ($P < 0.001$) for both sedentary and runner mice (Figure 4-3). However, this was more pronounced in the running group because they consumed more pellets, as indicated above.

Body weights and fat pads. The average body weight of runner and sedentary groups was the same at the beginning of the experiment. As soon as wheels were available, runners lost weight and conserved a significantly lower mean body weight throughout the whole experiment (Figure 4-4A). All body weights, except at the beginning of the experiment, were significantly different between the groups ($P_s < 0.05$). The difference in body weight at the end of the experiment was reflected in dissected body fat (Figure 4-4B), and especially in subcutaneous pad (individual fat pad data not shown). Runner mice had significantly less body fat compared to sedentary mice ($P < 0.05$).

Experiment 6: Running Wheel Activity as Food Foraging

Total daily revolutions, lever press responses, meal numbers, meal sizes and the number of pellets consumed did not differ significantly across the 4 days that a given schedule was in force (Figure 4-5). Thus, to reduce the data for presentation, revolutions and meal parameters were averaged across the 4 days to give a single mean value for each mouse at each foraging cost condition (ACnFUP5) and group means (\pm SEM) computed from these individual means.

Table 4-2 shows the running wheel activity. All mice ran substantial amounts, approximately 5 km/day except the lowest foraging cost condition (AC25FUP5). Panel A

of Figure 4-6 shows the mean number of running revolutions (the approach cost response for runGR) and panel B of Figure 4-6 shows the number of nose pokes (the approach cost response for pokeGR) per day. One-way ANOVA analyses showed that the total number of approach cost (AC) responses for runGR (running wheel revolutions) and pokeGR (nose pokes) increased as the AC ratio increased ($P < 0.001$ and $P < 0.001$ respectively). For runGR mice, follow-up LSD indicated that at their lowest AC condition (AC25FUP5), mice ran significantly less than at any other cost conditions, and they ran comparable amounts for all the other AC conditions (Figure 4-6A). However, for the pokeGR, follow-up LSD showed that the nose poke responses were comparable at all the cost conditions except at their highest AC condition (AC100FUP5). At this highest cost, nose poke responses increased significantly compared to any other cost condition (Figure 4-6B). In addition, when compared as number of responses emitted throughout the protocol, one-way ANOVA showed that AC ratio emitted by the running wheel operant by runGR was significantly higher than the nose poke operant by the pokeGR ($P < 0.001$) (Figure 4-7). Note that the experimenter imposed AC ratio for runGR was higher than the AC ratio for the pokeGR.

One-way ANOVA showed that lever press responses on the other hand did not differ between the two groups (runGR and pokeGR) however did change as a function of the increases in the approach cost for both runGR (Figure and 4-8A) and pokeGR mice (Figure 4-8B) ($P_s < 0.05$). Follow-up LSD indicated that runGR mice increased their lever press responses significantly at the two highest cost conditions (AC500FUP5 and AC1000FUP5) ($P_s < 0.05$). PokeGR increased their lever press responses at the highest cost condition (AC100FUP5) ($P = 0.003$). Note that although the approach costs

increased for runGR (running wheel responses) and pokeGR (nose poke responses), the fixed unit price (lever press responses) was kept constant at FUP5.

The numbers of pellets taken are shown as the demand functions in Figure 4-9. One-way ANOVA revealed that intake was significantly different between the three groups ($P < 0.001$). Follow-up LSD showed that runGR mice ate more than adlibGR and pokeGR mice and also adlibGR ate more than pokeGR mice ($P_s < 0.001$). The number of pellets consumed per day by the pokeGR declined towards the highest AC condition (Figure 4-9A) while the intake of runGR was relatively constant (Figure 4-9B).

The corresponding meal frequency and size data are shown in Figure 4-10 and 4-11. One-way ANOVA showed a significant main effect for groups for meal frequency analysis ($P < 0.001$). Follow-up post hoc LSD revealed that adlibGR had significantly more meals compared to runGR ($P < 0.05$) and pokeGR ($P < 0.001$). RunGR also took consistently more number of meals than the pokeGR mice ($P < 0.001$). One-way ANOVA also indicated that there was also an effect of the AC increase on the meal frequency for only runGR ($P < 0.001$). Panel A of Figure 4-10A shows that runGR took similar number of meals throughout the protocol except at the highest cost condition (AC1000FUP5). A follow-up LSD showed that at this cost, number of meals decreased significantly ($P < 0.001$). Panel B of Figure 4-10 shows that number of meals for pokeGR did not change as a function of AC ratio increase. Note that in Figure 4-10B, at the highest cost condition for pokeGR (AC100FUP5) there was a high within-group variability.

The corresponding meal size data are shown in Figure 4-11A and 4-11B. One-way ANOVA showed a significant main effect for groups for meal frequency analysis

($P < 0.05$). Follow-up post hoc LSD revealed that adlibGR had significantly smaller meal sizes compared to runGR ($P = 0.006$) and pokeGR ($P < 0.05$). RunGR and pokeGR had comparable meal sizes throughout the protocol. One-way ANOVA also showed that there was also an effect of the approach cost increase on the meal sizes ($P < 0.001$). Panel A of Figure 4-11 shows that runGR had similar mean meal sizes per day throughout the protocol except at the two highest cost conditions (AC500FUP5 and AC1000FUP5). Follow-up post hoc LSD indicated that at these cost conditions, runGR mice increased their mean meal sizes significantly ($P < 0.001$). Panel B of Figure 4-11 shows that pokeGR had a highly significant decrease in their mean meal sizes at their highest cost condition (AC100FUP5) ($P < 0.001$). Note that for each parameter, data were shown in separate figures for the two groups as different x-axis values were needed because the cost schedule ratios are different for the runGR and pokeGR.

Body weights. Table 4-3 shows the caloric energy intake in all three groups. One-way ANOVA showed that the average weight of all three groups did not differ significantly at the beginning of the experiment. However post hoc LSD showed that runGR was slightly heavier than the adlibGR initially ($P = 0.38$). One-way ANOVA revealed that weight was significantly different between the groups at the end of the experiment, ($P < 0.001$). A follow up LSD showed that runGR and adlibGR mice had comparable weights whereas pokeGR weighed significantly less than both these groups (Figure 4-12).

Experiment 5 and 6: Voluntary Running versus Running as Food Foraging Effort

Table 4-2 (m) and Figure 4-13 (km) show voluntary and foraging running wheel activity from both experiments. Although voluntary running mice seemed to run somewhat more, it was not significantly different from foraging mice. One-way ANOVA

showed that voluntary runner mice ate significantly fewer number of pellets than the foraging mice throughout the protocol ($P=0.001$). However, mean meal sizes and daily number of meals did not differ between the two groups.

Discussion

Experiment 5: Voluntary Running Wheel Activity and Food Demand

Present work reports meal patterns of mice under negative energy balance when given different "economic" environments regarding food availability. More specifically the mice were exposed to increasing degrees of difficulty in obtaining food. The degree of difficulty was in the form of the number of operant behaviors, nose-pokes that the mice had to perform in order to receive food pellets. Supporting of our previous works in our lab, the harder the animal had to work for food the less they ate (Atalayer and Rowland, 2009, 2010a, 2010b).

Running mice lost weight and in this negative energy balance, runner mice consumed more pellets per day and ate bigger but fewer meals compared to the sedentary mice. They seemed to rapidly adjust their meal patterns and food intake in order to incorporate voluntary exercise into their daily routine in the operant chambers. Although they ate more, runner mice maintained a lower body weight throughout the protocol indicating that their increase in food intake was not enough to compensate for the extra energy expenditure. They also had significantly less body fat than sedentary mice, especially in subcutaneous depots. Our results agree with studies in the literature reporting that exercising mice consume significantly more energy than non-exercising mice, yet they experience weight loss and a decrease in body fat (Vaanholt et al., 2007)

In our experiments no food restriction was applied at any time and food was always contingent upon some level of cost (n number of nose pokes) which generated,

as in previous studies (Atalayer & Rowland 2009; Bauman, 1991; Collier, 1985; Hursh, 1980) a characteristic demand function. However, the shape of these functions which define elasticity of demand function differed between running and sedentary groups. Demand functions in runner mice were less elastic than those of sedentary mice. One may argue that because of their negative energy balance, the food commodity was more crucial for the runner mice than the sedentary mice. Thus, their demand for the food commodity was insensitive to the effects of the increases in the cost. In other words, the incentive value of the reward (food) was higher for the running mice when they were in a negative energy balance state. Incentive value is one of the factors for determining the willingness of the individual for spending effort in order to get a particular reward/reinforcement (Berridge, 1996). It has been shown that the elasticity of demand functions can be affected by manipulations of such factors (Hursh, 1984; Killeen, 1995)

Although running mice expended more effort in order to maintain a higher daily food intake compared to the sedentary mice, their energy intake still was not enough to fully compensate the energy loss resulted from voluntary running. As a consequence they lost weight. However, they did not decrease the level of running regardless of food intake and the associated costs. This would be consistent with the speculation that appetitive behavior may increase running as a food foraging activity (Vaanholt *et al.*, 2007). Although the running wheel activity was voluntary in this protocol, the animals may be increasing their running wheel activity as a foraging type of behavior each time the unit price increases.

Many studies demonstrate that exercising lean mice (and rats) usually do not lose

weight with wheel running since they compensate for their extra energy expenditure by increasing their intake (Krawczewski *et al.*, 2009). However, our runner mice lost weight, presumably because of a failure to increase their overall intake when the fixed unit price for food increased after a certain level. This indicates that these mice under the experimental conditions of increasing food prices in addition to a voluntary running wheel activity may be performing differently than most other lean rodents given ad libitum access to food and a running wheel. Thus another control group would have been helpful to emulate an ad libitum condition for food availability. With this control group, it would be possible to see the effects of voluntary running alone on the body weight change. We are currently collecting this data with both same weight and lighter mice. These mice are exposed to the lowest cost FUP2 that is kept constant throughout the protocol to simulate an 'ad libitum' food intake condition in the operant chambers. They are also allowed to run voluntarily as in the original protocol. This will help for the interpretation on whether the weight change in the runner and sedentary groups at the end of the experiment is the result of voluntary running activity or increase in the unit price alone or the interaction between the two. Pilot results from mice which weighed less than the voluntary running mice in the original protocol indicated that unit price increase is in fact playing a role in the weight change in addition to the running wheel activity. These mice in this pilot study which were under a very low cost (FUP2) were able to maintain their body weight throughout the protocol and there was not any decrease in their weight at the end of the experiment. However, this may be initial weight-dependent as the average initial weights of the mice in the original protocol were markedly heavier than these mice in this pilot study. Thus results from the same weight

group will be important to resolve this issue and we are currently collecting this data.

Experiment 6: Running Wheel Activity as Food Foraging

In this protocol, two different operants as the AC response were compared. Both AC responses had a concurrent lever press response requirement as the FUP in order for the animal to receive the food. One group was required to run in order to activate a lever which, when pressed 5 times (FUP5), delivers food. The other group was required to nose poke to activate the lever (FUP5). Results clearly indicated that with running mice were able to emit higher costs for food foraging compared to the nose poke behavior. With the running wheel operant in order to activate a lever to receive food, mice seemed to acquire inelastic demand functions and were able to keep up with working at the higher ratios. Mice that were required to nose poke for activating the lever (pokeGR), as in our previous study (Atalayer and Rowland, 2009), had highly elastic demand functions; as the approach cost increased the demand decreased to a level where animals were not able to maintain their body weights and were in a negative energy balance. Presumably, had we gone to higher AC for pokeGR especially, a further decline in demand would have occurred as it does in rats (Hursh, 1984), but we were limited in our ability to do this because of restrictions on maximal weight loss (~15%) due to welfare considerations. In our previous works (Atalayer and Rowland, 2009) we have shown that mice tested with a nose poke operant acquired slightly more pellets compared with lever press as fixed unit price responses under various reinforcement schedules in the same strain of mice (CD1). These findings agree with studies in rats comparing nose poke and lever press operants in which acquisition of the nose poke response often occurs more rapidly, but response rates in trained rats are usually comparable (David *et al.*, 2001; Ettenberg *et al.*, 1981). We previously showed

that nose poke can acquire more responses than a lever press operant (Atalayer and Rowland, 2009). The present study shows that running wheel activity acquires even more number of responses than a nose poke operant so mice can bear higher cost ratio requirements compared to both a lever press and a nose poke operant. This is in agreement with the idea that running wheel activity is a more relevant activity for food foraging (Vaanholt *et al.*, 2007). In other words, one may argue that mice did seem to be more motivated to “work for food” when the foraging activity is somewhat relevant to what they would be doing in their natural habitat. Another explanation would be that the running wheel activity may be rewarding on its own (Belke, 1997; Belke and Wagner, 2005; Iversen, 1993). Thus, mice may be more motivated to work not only for food but also for the running activity itself.

Chapter Discussion

Voluntary exercise was shown to be a good stimulant of food intake in mice, even when costs are associated with food procurement. This increased intake occurs mostly through the increased meal size. When food was contingent on running wheel activity, mice seemed to be able to emit higher ratios for cost for food compared to a nose poke response as shown in Experiment 6. The comparison of the running wheel activity between the Experiment 5 and 6 indicated that these mice ran similar distances, although voluntary mice seemed to run more this was not statistically significant.

In contrast to the general agreement that exercise causes a negative energy balance and hence increases food intake in mice, the picture in rats is less clear. For example, Levin & Dunn-Meynell (2003) found that runners did not increase their chow intake or adjust their expenditure in order to compensate for their negative energy balance. They therefore lost weight but defended this lowered level of weight gain

against food restriction. Exercise may reset energy homeostasis so that a lowered level of weight gain is defended (Levin & Dunn-Meynell, 2003; Krawczewski *et al.*, 2009). The commonality between rats and mice is that the adaptation in food intake does not fully offset the increased energy expenditure. Thus, in Experiment 5 animals were motivated to nose poke more in order to eat more pellets to compensate for the energy expenditure on the running wheel (voluntary) and generate inelastic demand functions compared to the sedentary animals. This was not the case for the sedentary animals. Their demand functions were elastic which means that as the fixed unit price increased their food intake decreased. However, they were still heavier at the end of the protocol. In addition, when food is contingent upon the running wheel activity as the approach cost response, animals also showed inelastic demand functions compared to when the nose poke was the approach cost response.

For the animal, absolute approach cost may be important not only in terms of what type of a response it is but also in terms of the elapsed time from the start of responding till when the food is delivered. The amount of time spent in the two different responses compared was not measured in this study, but an average of 600 nose pokes per day spent to initiate 6 meals (Atalayer and Rowland, 2009) would very likely account for less than 2 min of total approach cost response time per meal. More detailed temporal analysis will be needed to assess further the contribution of elapsed time on meal parameters. Consistent with this analysis, time itself has been shown to be an effective cost parameter in rats (Mathis, Johnson & Collier, 1995; Collier, Johnson & Mathis, 2002). Lastly, these data give further support to the idea that foraging or appetitive cost should not be considered a unitary phenomenon (Rowland and Mathes, 2008), but

instead consists of at least two phases that affect behavior quite differently. We have introduced the terms approach cost and unit price to make this distinction clear insofar as it relates to behavioral economics, but presumably there are as yet unknown underlying neural structures that support such a distinction.

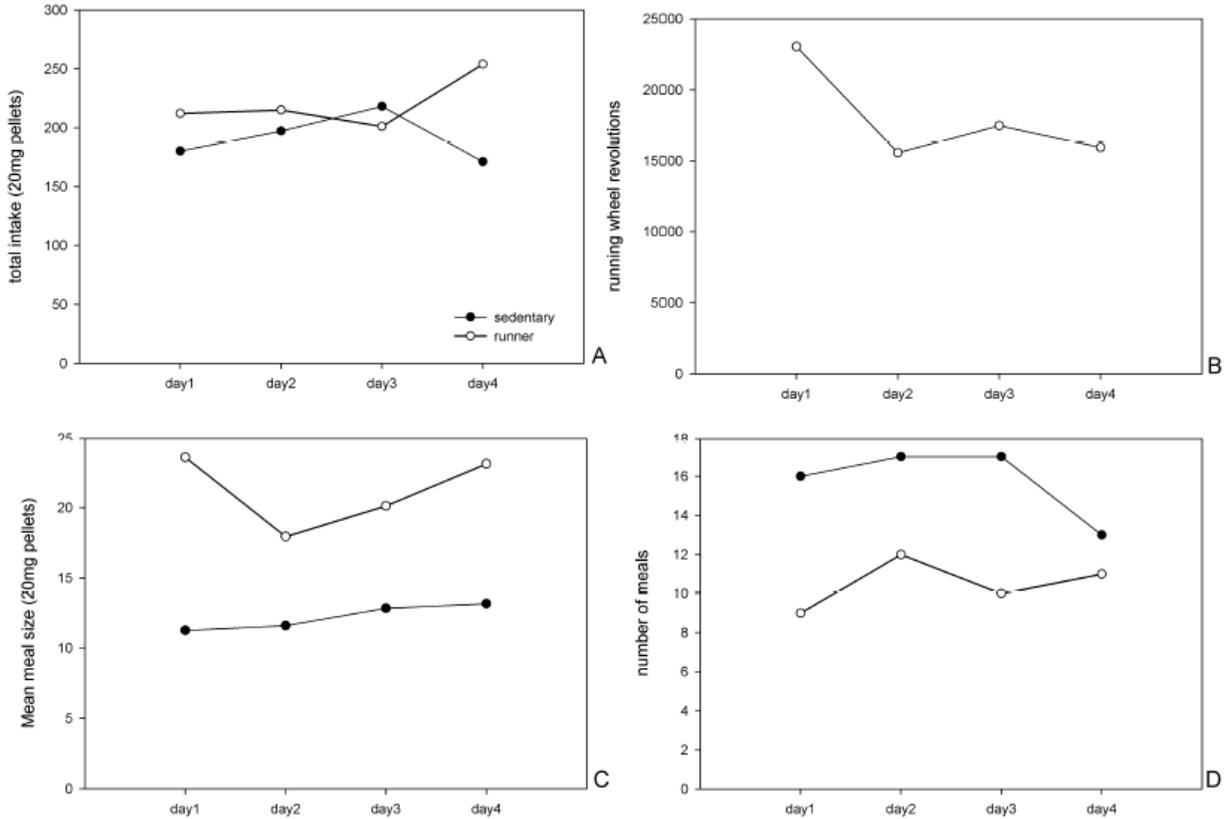


Figure 4-1. Individual data from a typical mouse to illustrate the day-to-day variability. A) total number of pellets consumed daily, B) running revolution (runner mouse only), C) meal size, D) meal frequency. Each data point at each figure is a daily value of each parameter of a prototype mouse from each of running and sedentary groups at a FUP25 cost schedule. Data points did not differ significantly across the 4 days that a given schedule was in force for each parameter; hence data were averaged across the 4 days for the statistical analyses.

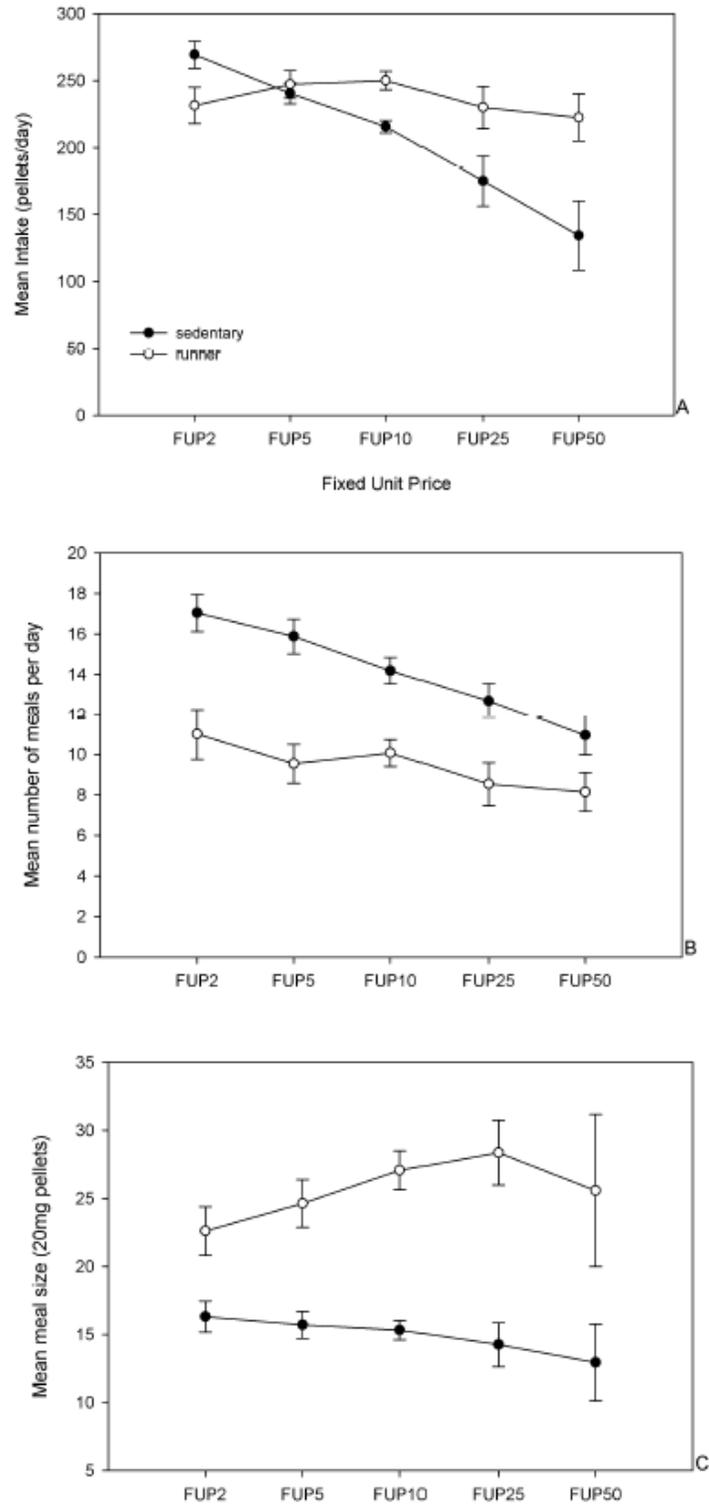


Figure 4-2. Mean (\pm SEM) A) daily food intake, B) number of meals taken, and C) meal size of sedentary and runner mice from Experiment 1 plotted as a function of each FUP for 20 mg food pellets. Each data point is an average of 4 days and 8 mice. See text for statistical interpretations.

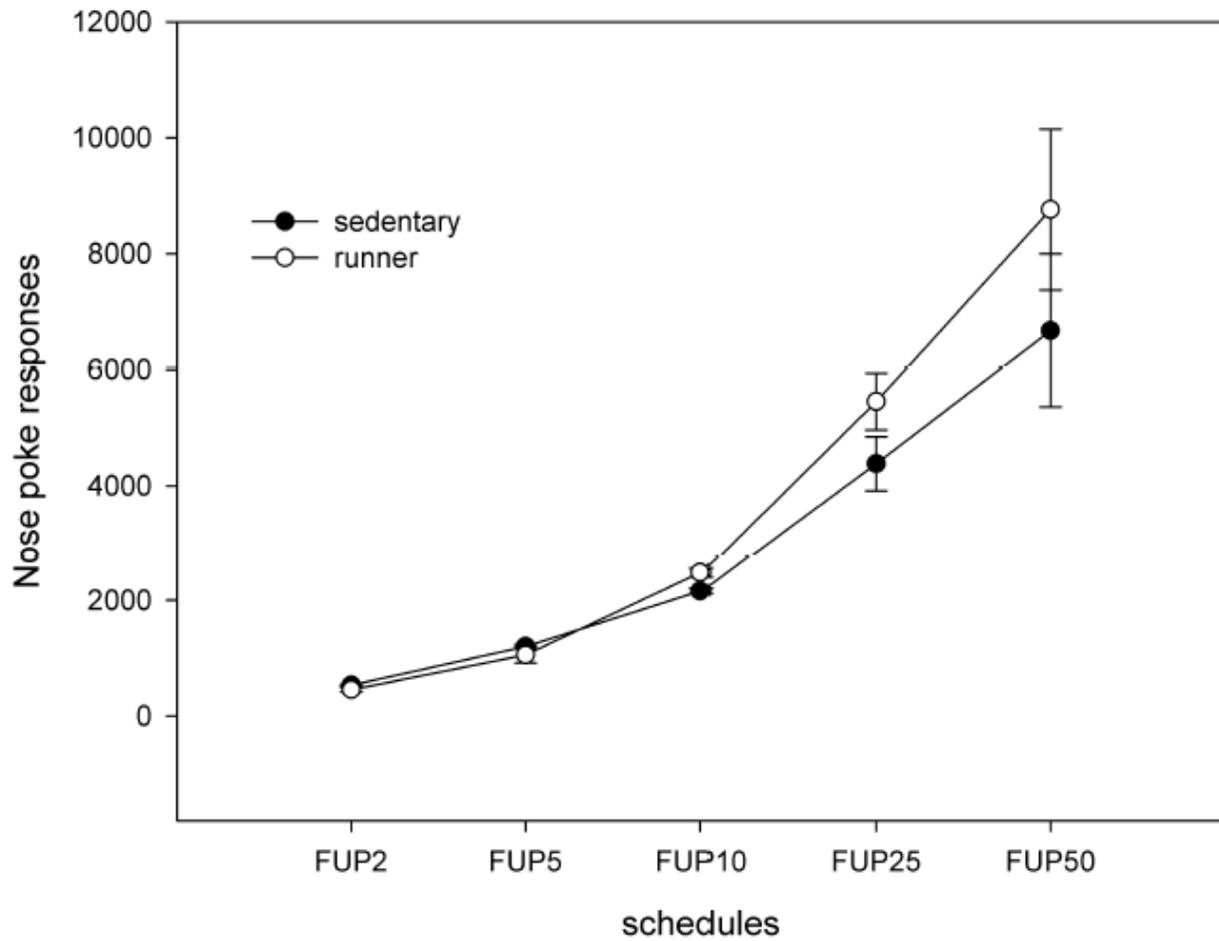


Figure 4-3. Nose poke responses. Mean (\pm SEM) nose poke responses emitted. Separate lines show the running and sedentary mice. Both groups were required to emit nose poke responses to obtain food.

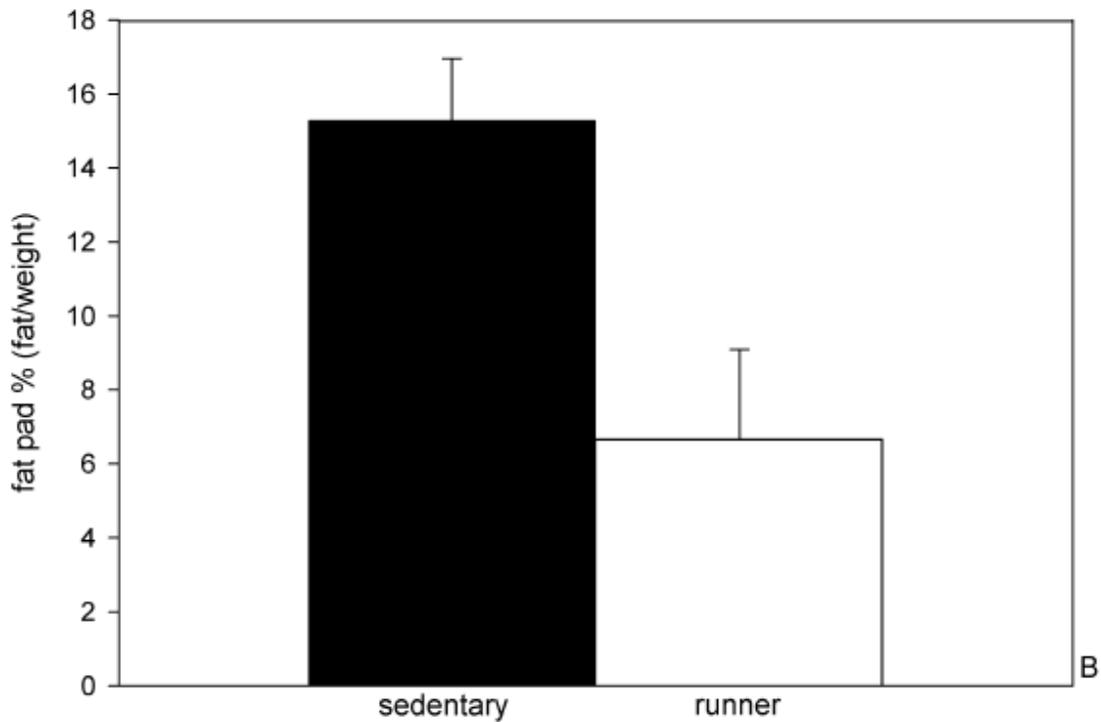
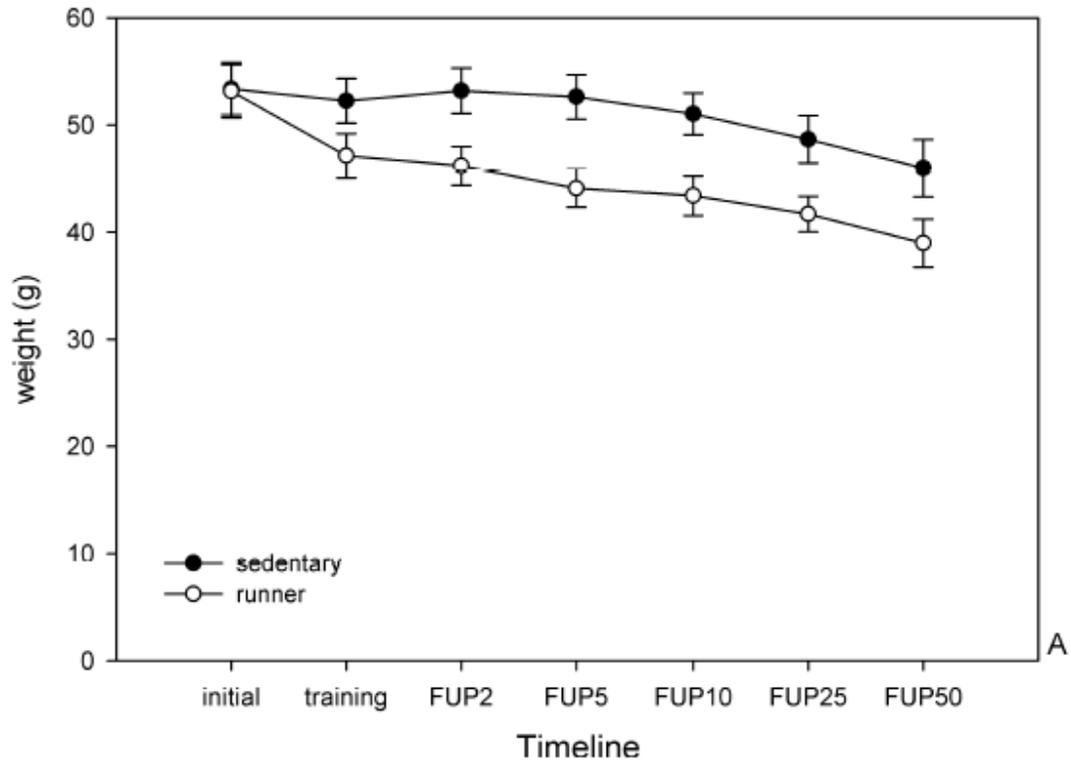


Figure 4-4. A) The mean (\pm SEM) body weight of sedentary and wheel running mice in Experiment 5 at various phases of the experiment. B) Dissected fat pads (subcutaneous + visceral + thoracic) mass, expressed relative to body weight, of sedentary and runner mice at the end of Experiment 5 ($P < 0.01$).

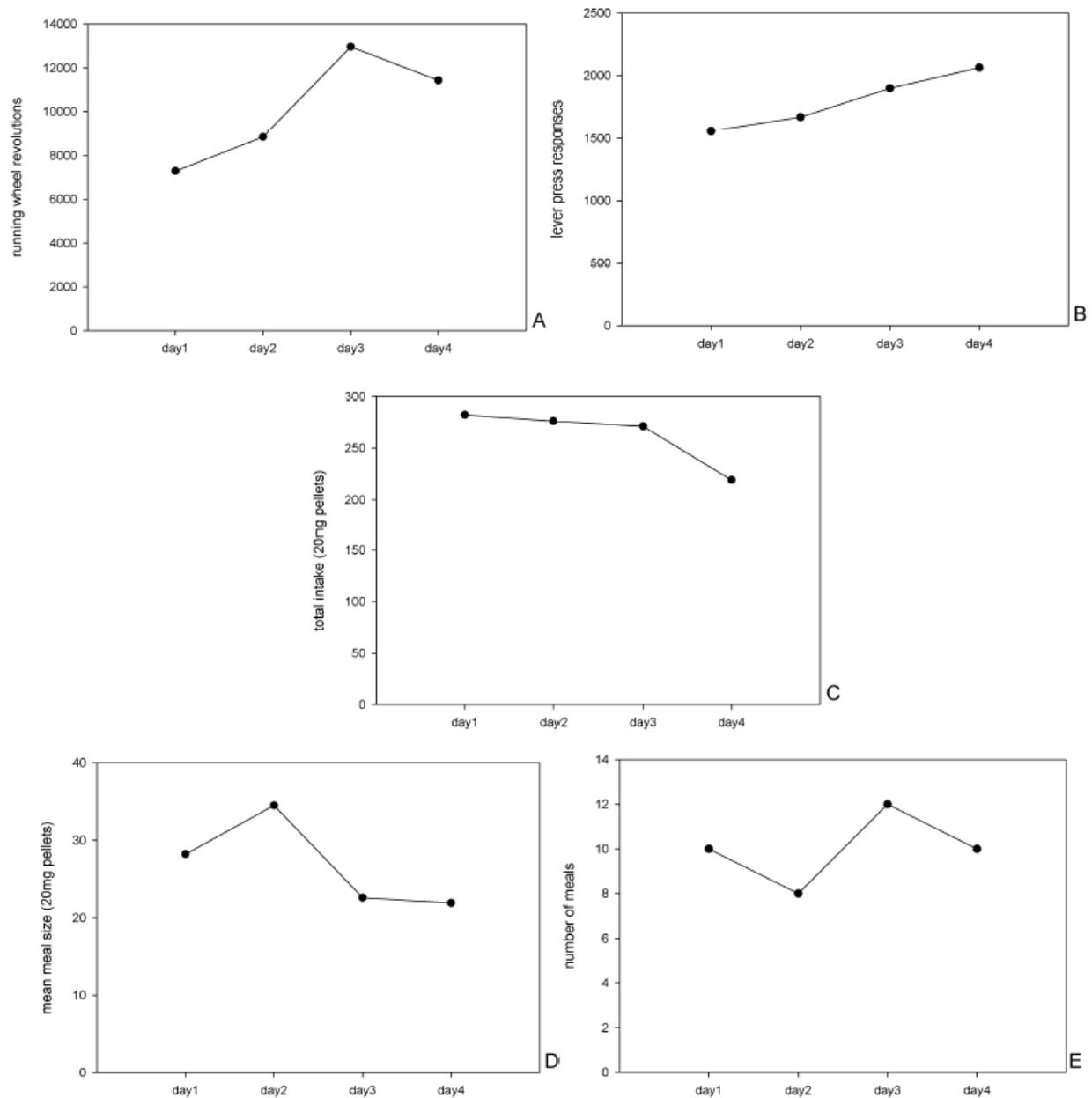


Figure 4-5. Individual data from a typical mouse to illustrate the day-to-day variability. A) total daily revolutions, B) lever press responses, C) total number of pellets consumed daily, D) mean meal sizes, E) meal frequency. Each data point is a daily value of each parameter of a prototype mouse from running group at AC100FUP5 cost schedule. Data points did not differ significantly across the 4 days that a given schedule was in force for each parameter; hence data were averaged across the 4 days for the statistical analyses.

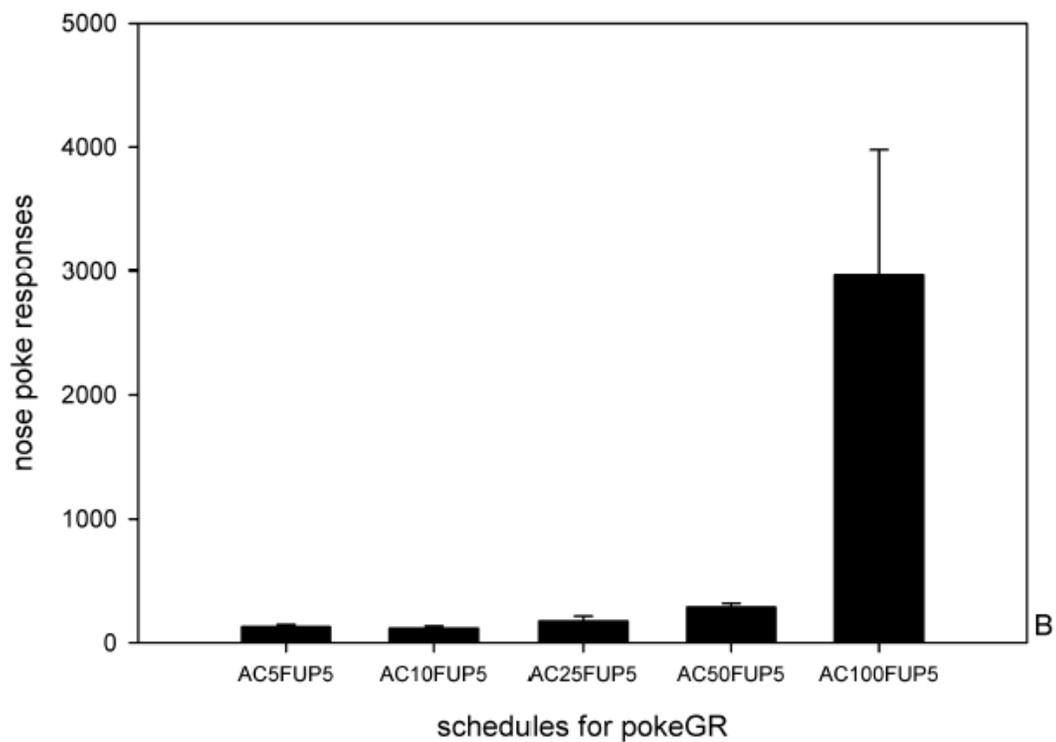
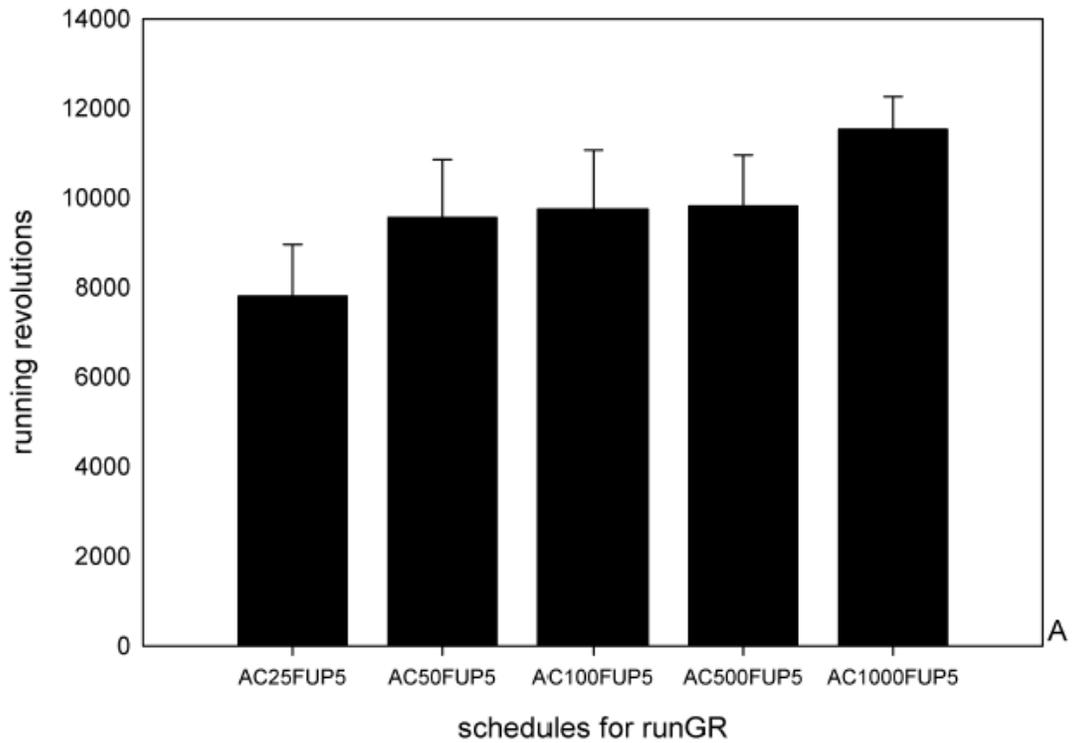


Figure 4-6. Bar plots for mean number of responses (\pm SEM). This figure shows A) number of running revolutions, approach cost response for runGR, and B) nose pokes, approach cost response for pokeGR.

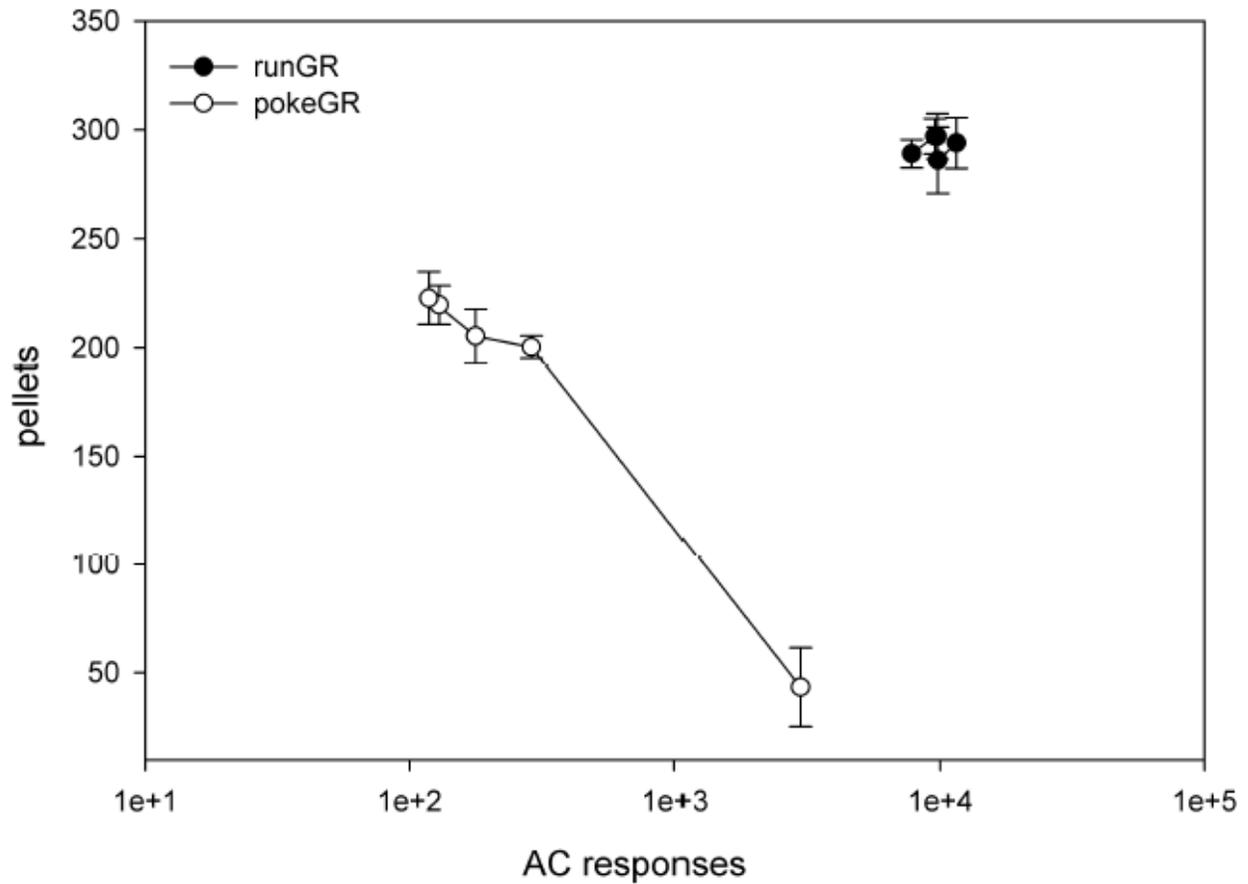


Figure 4-7. The number of responses emitted for both groups plotted against the pellets consumed on the y-axis in a log-log scale. When compared as number of responses emitted throughout the protocol, one-way ANOVA showed that AC ration emitted by the running wheel operant by runGR was significantly higher than the nose poke operant by the pokeGR ($P < 0.001$).

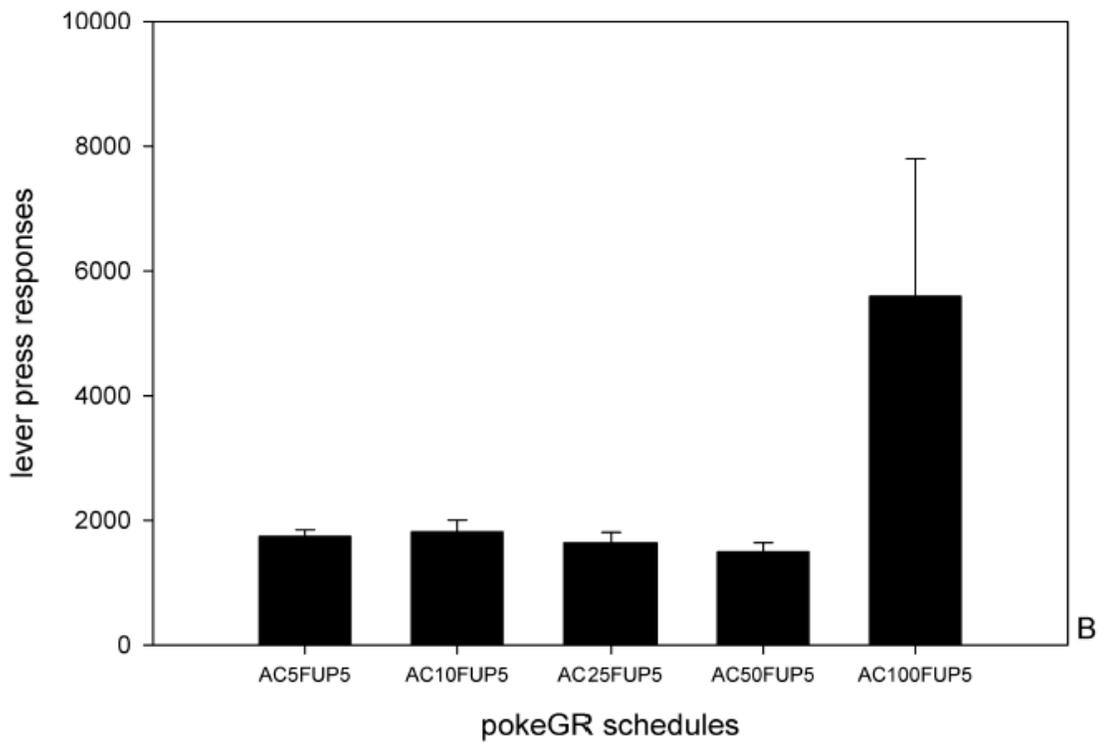
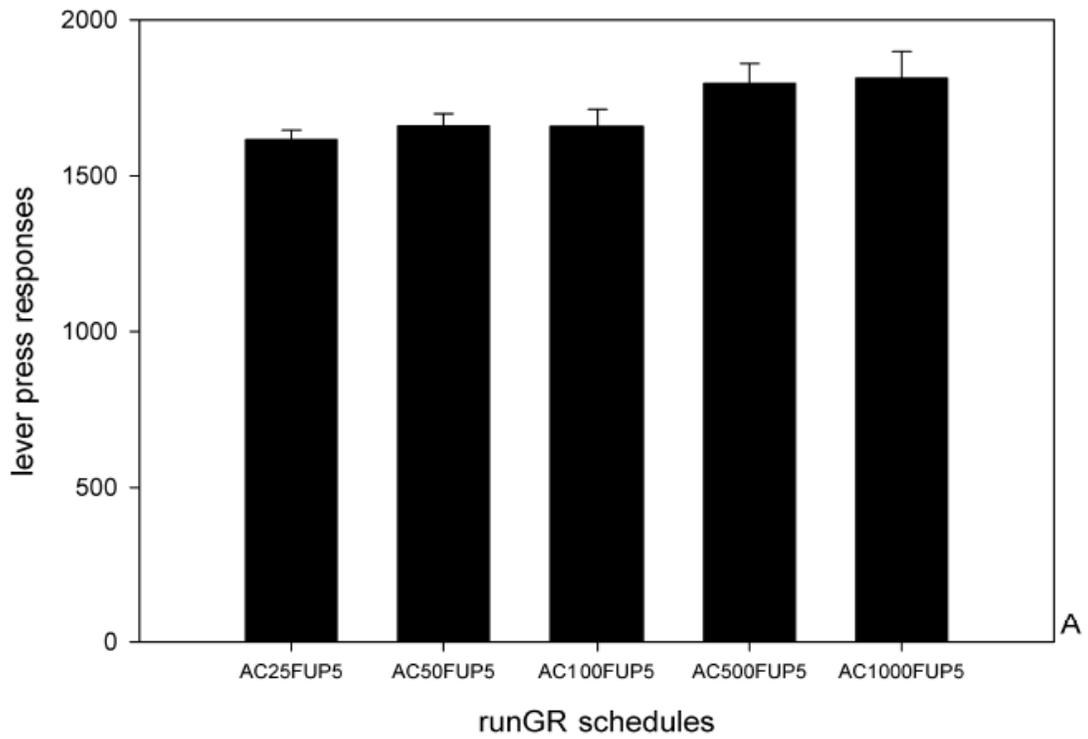


Figure 4-8. Lever press responses (FUP responses) for A) runGR and B) pokeGR.

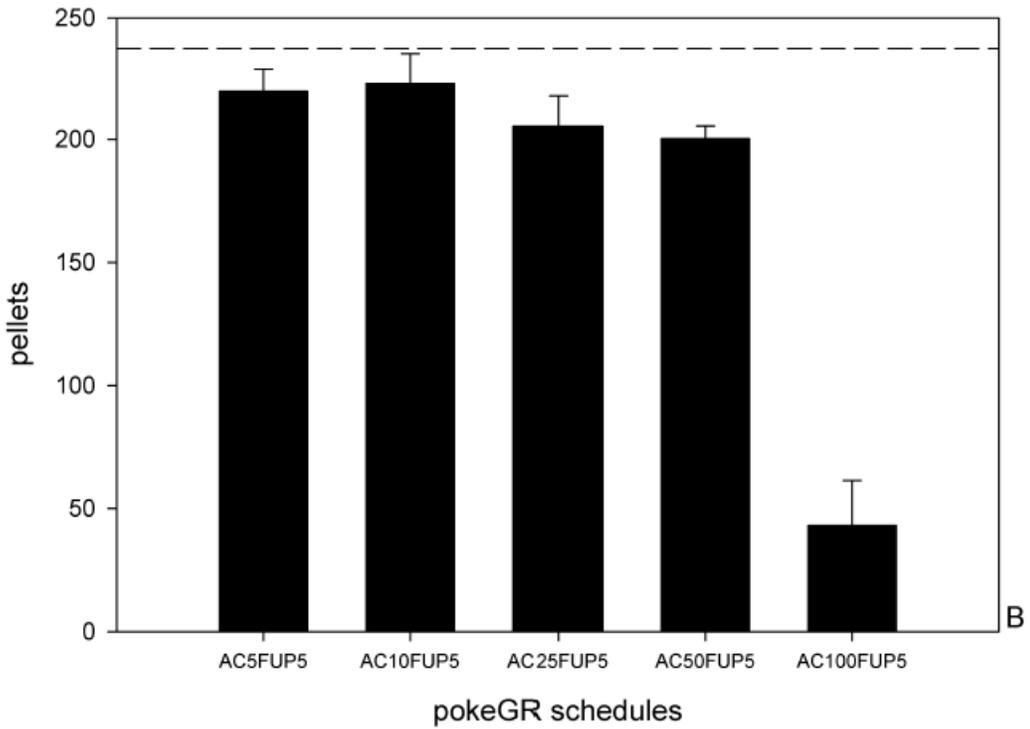
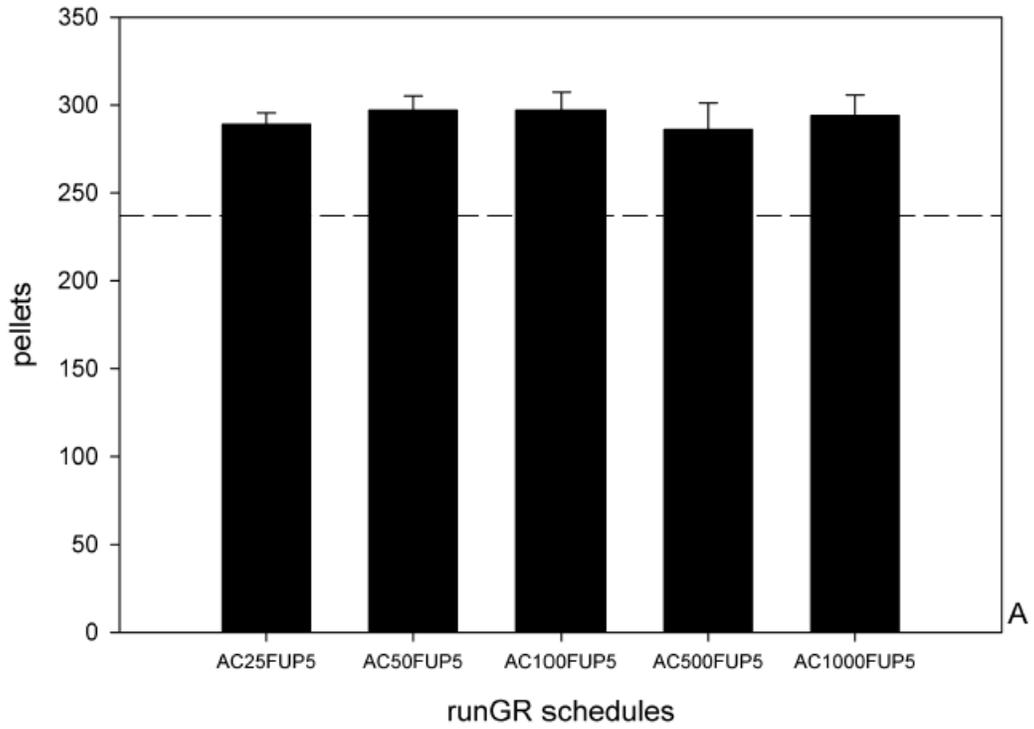


Figure 4-9. Mean (\pm SEM) number of pellets taken as the demand functions for A) runGR and B) pokeGR. The dashed line indicates the average for daily total number of pellets consumed by the adlibGR mice.

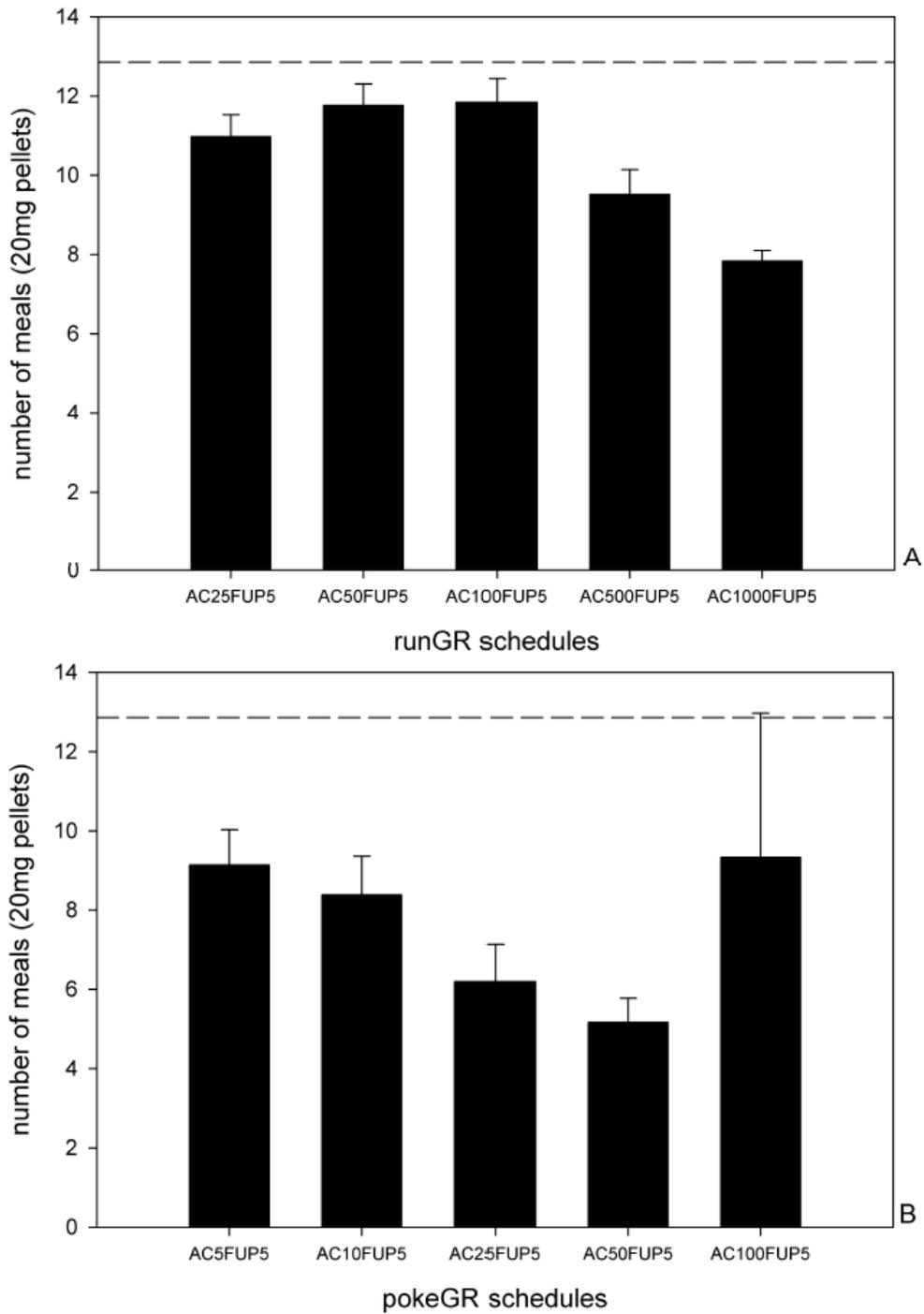


Figure 4-10. Mean number of meals (meal frequency) taken per day (\pm SEM) for A) runGR and B) pokeGR. The dashed line indicates average daily number of meals consumed by the adlibGR mice. Each data point is averaged at every 4 days to match the timeline of the approach cost increase schedule of the two experimental groups.

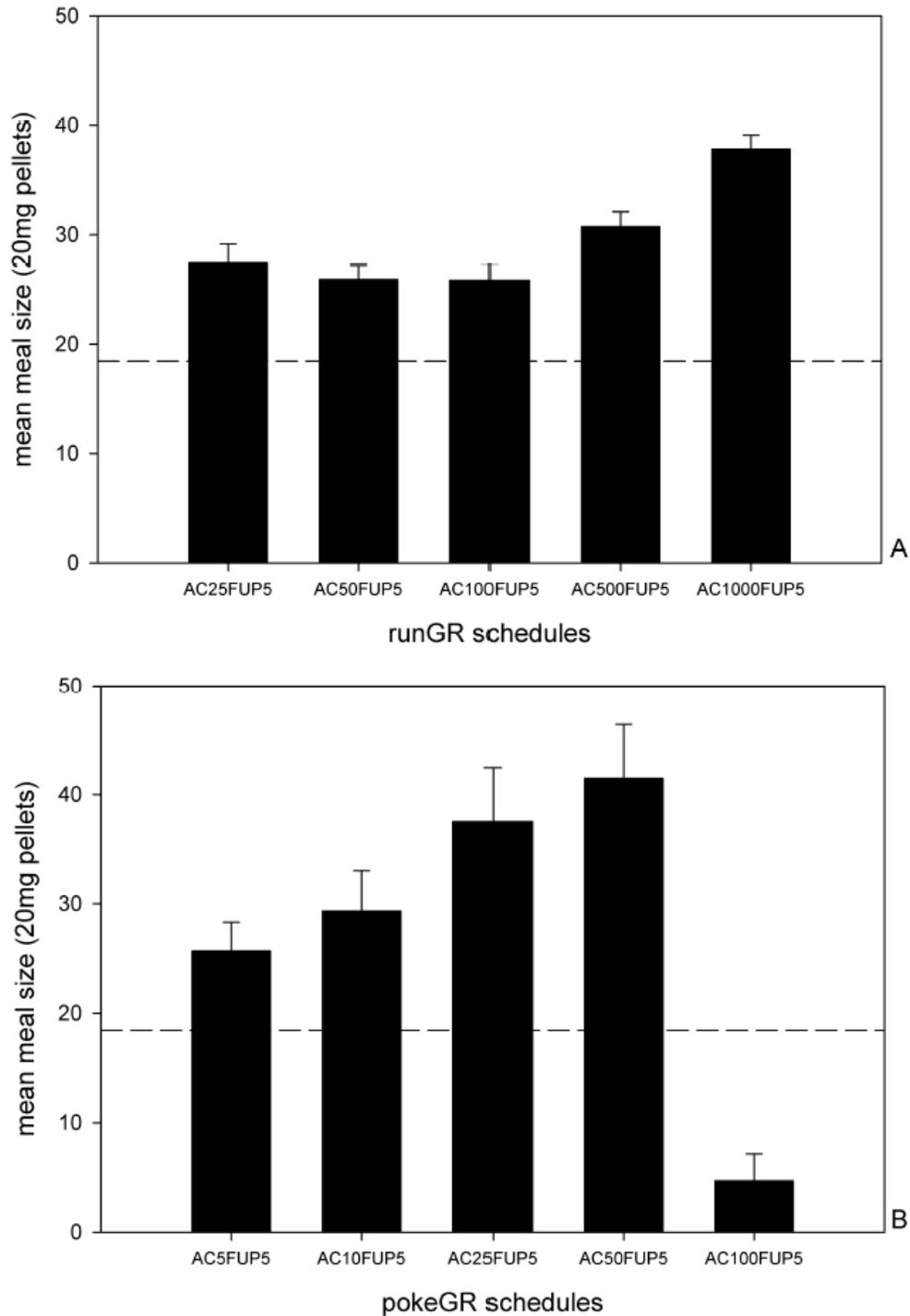


Figure 4-11. Mean daily meal sizes (\pm SEM) for A) runGR and B) pokeGR. The superimposed data points are total average daily meals sizes consumed by the adlibGR mice. Each data point is averaged at every 4 days to match the timeline of the approach cost increase schedule of the two experimental groups.

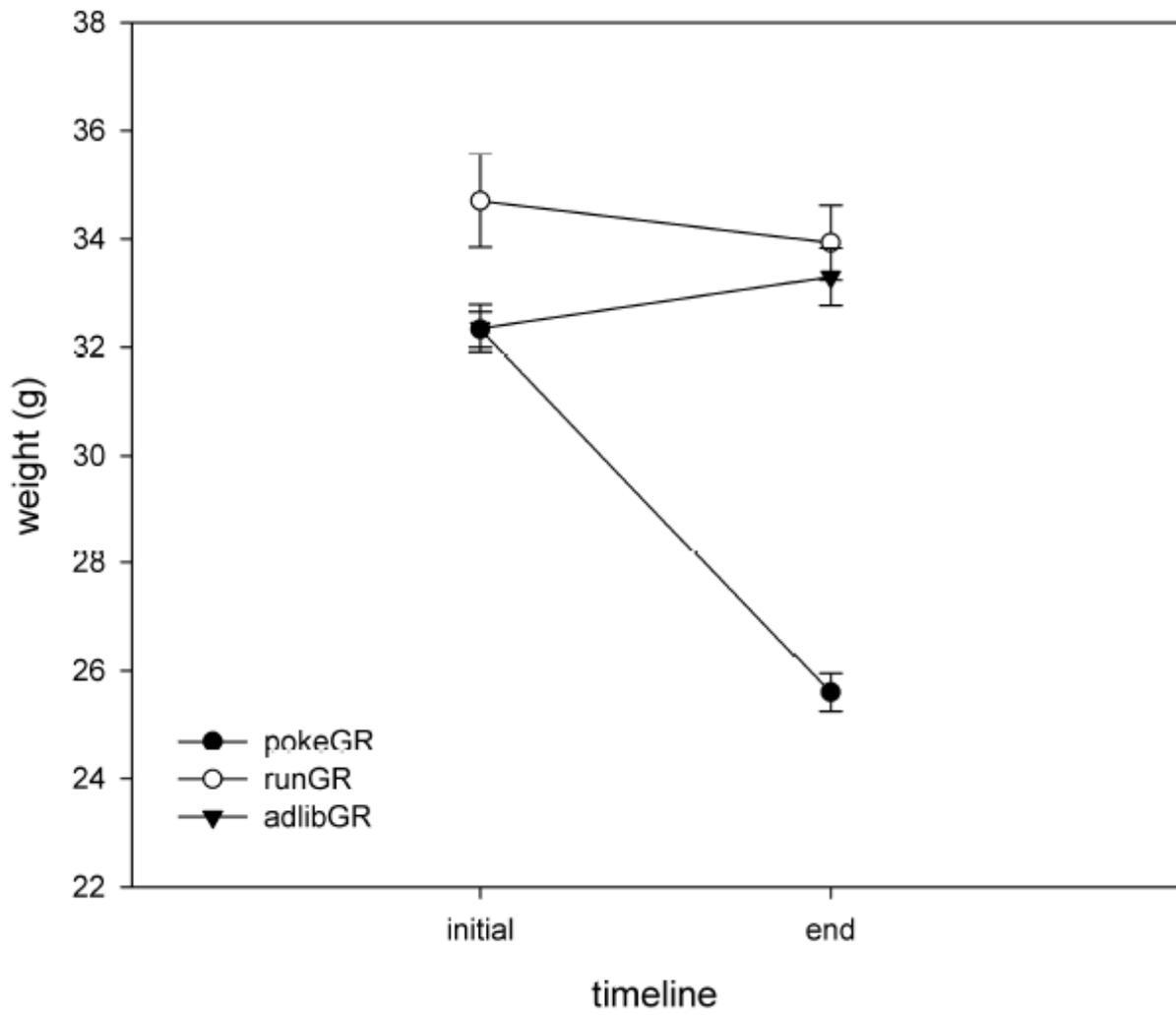


Figure 4-12. Body weight change.

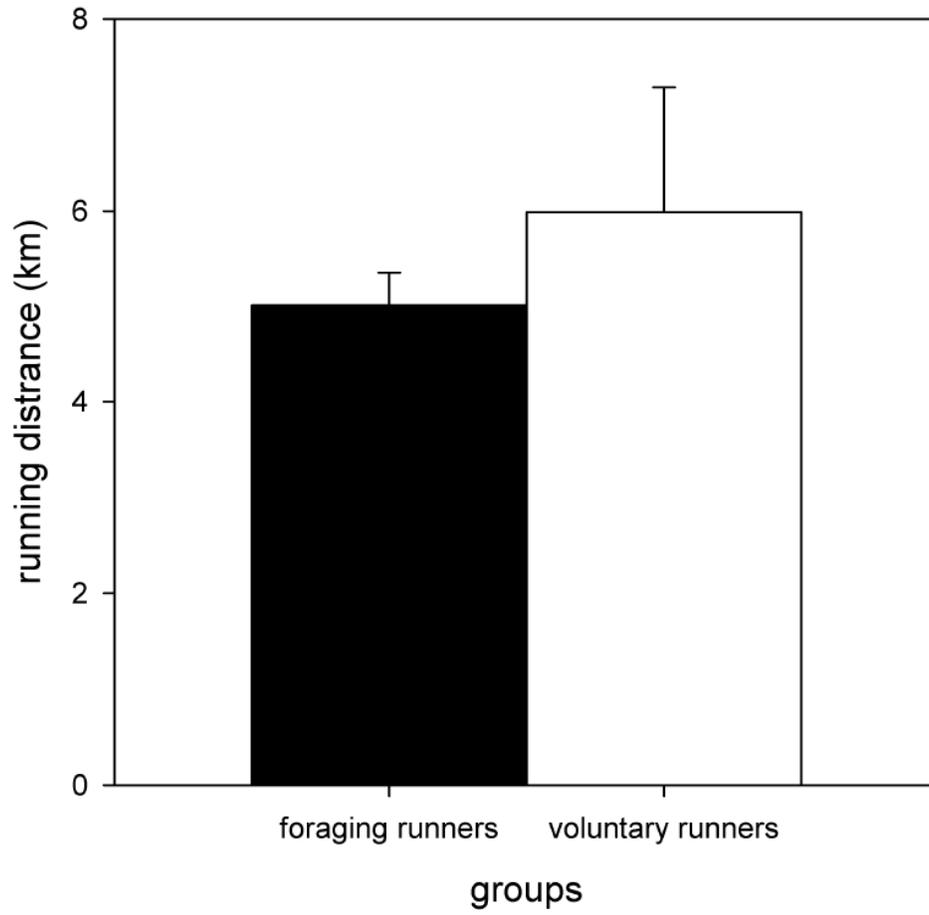


Figure 4-13. Voluntary and foraging running wheel activity from both experiments.

Table 4-1. Total caloric energy intake at each FUP schedule in Experiment 5

	Sedentary mice	Runner mice
FUP2	80.79±3.07	69.41±4.08
FUP5	72.14±2.31	74.12±3.06
FUP10	64.67±1.40	74.99±2.05
FUP25	52.54±5.60	69.02±4.73
FUP50	40.32±7.74	66.71±5.29

Note: Shown are both mean data from 4-day regimen of each schedule applied and also group mean±SEM (Kcal).

Table 4-2. Running distance (m/day) of mice in Experiments 5 and 6 as a function of the fixed unit price (FUP) for food

	Experiment 5: Voluntary running		Experiment 6: Foraging running
FUP2	3910±1163	AC25	4359±643
FUP5	8015±2111	AC50	5337±721
FUP10	6419±1575	AC100	5443±733
FUP25	5764±862	AC500	5478±637
FUP50	6255±1028	AC1000	6287±442

Note: Shown are group mean±SEM. There were no significant differences between FUPs (Experiment 5) and ACs (Experiment 6) within a study.

Table 4-3. Total caloric energy intake at each schedule in Experiment 6

	pokeGR		runGR		adlibGR
AC5FUP5	65.67±2.67	AC25FUP5	82.58±2.16		
AC10FUP5	66.59±3.61	AC50FUP5	83.04±1.68		
AC25FUP5	61.39±3.69	AC100FUP5	81.37±1.36	FUP5	70.9±2.3
AC50FUP5	59.89±1.55	AC500FUP5	75.16±3.20		
AC100FUP5	12.93±5.44	AC1000FUP5	79.98±0.71		

Note: Shown are group means ±SEM (Kcal).

CHAPTER 5 GENERAL DISCUSSION

Some of these findings are new, some support and some contradict previous work. One of the main findings of this study was that although MC4Rs signal hunger, the phenotypic expression of its deficiency can be affected by the economic structure of the food environment. The high elasticity in demand functions for food that have been shown in MC4R^{-/-} and DKO compared to WT and MC3R^{-/-} mice was caused by the changes in meal size rather than number of meals consumed per day. The diet-induced obesity protocol showed that this effect was not due to obesity per se but presumably more dominantly due to the genetic deletion of the MCR receptors. However even though the effect of obesity was found to be minimal compared to the effects of genetic deletion of MCRs and resultant metabolic deficiencies on the demand function differences between lean and obese mice, there was still a significant effect of the obesity per se when the cost for food was too high. At this highest FUP condition, diet-induced obese mice decreased their food intake significantly compared to the lean mice even though their demand for food was very similar under costs lower than FUP50. Thus, although it was more pronounced in genetically obese mice, it is still true to argue that the elasticity of the demand function of diet-induced obese mice is different than the lean mice. The positive energy balance state of the obese mice resulted in highly elastic demand functions as opposed to the negative energy balance state of the running mice. In the latter case, mice had inelastic demand functions that are resistant to the changes in the cost unlike the elastic demand functions that are very sensitive to the changes in the price. Thus, negative energy balance resulted in inelastic food demand. One

argument would be that energy balance is an important factor determining how much animals are motivated to work for food.

It would be particularly interesting to know what would be the demand functions when animals are in both conditions of negative and positive energy balance. We are currently investigating whether the effects of exercise on food demand vary between DIO and lean mice, and including both positive and negative energy balance in the same design. The initial data indicate that running did not accelerate, and may have slowed, weight loss of DIO mice upon return to a low fat diet. In this pilot study mice were divided randomly in four groups, as sedentary high-fat (SEDHF), sedentary low-fat (SEDLF), runner high-fat (RUNHF) and runner low-fat (RUNLF). HF diet groups were made fat previously by HF diet and their weight was comparable to our genetic obesity models (Atalayer *et al.*, 2010b). These mice were then replaced in the behavioral chambers for the analysis of the demand functions with or without the running wheels based on their assigned groups above.

These mice were different strain, C57BL/6 (B6) than the mice we used in Chapter 4 (CD1) experiments in the present work. B6 mice ran slightly, but not significantly more than the CD1 mice and, as shown in this study, daily voluntary running wheel activity did not change as unit prices increased. In addition, the running activity of initially diet-induced obese (HF) and LF mice did not differ statistically at any time during the study. During the 4 consecutive FUP phases with low fat 20mg 5TUM pellets that was also used in this present work, sedentary mice from the LF group maintained their body weight while those that had developed obesity from the HF diet prior to the operant protocol, lost weight almost to their original level. The runner mice from the LF group

lost a small amount of weight at the highest FUP only. Surprisingly, runner mice from the HF group lost less weight than their sedentary counterparts so that the end of each FUP they weighed more than their sedentary HF counterparts ($P<0.05$). Although not significant at the end of the experiment, RUNHF mice weighed more than SEDHF, while the opposite relationship was true in the corresponding LF groups. Thus, exercise and the attendant changes in food intake seemed to slow weight loss upon switching from a high to low fat diet, and independent of the economic conditions.

Overall, runner mice ate more pellets per day than sedentary mice ($P<0.05$) and pairwise comparisons were significant at each schedule except FUP5. A two-way ANOVA analysis showed a main effect for unit price and a running x unit price interaction ($P<0.001$ at each FUP). Meal pattern analysis showed that runner mice took bigger meals at each cost schedule, however this was significantly different from the sedentary group only at FUP25 and FUP50 ($P<0.05$). For meal sizes, two-way ANOVA analysis showed a main effect for unit price and a main effect for running ($P<0.05$ for both). For the corresponding number of meals, although runner mice took consistently fewer meals at each schedule, this did not result in statistical significance. In a two-way ANOVA, a main effect for unit price was found ($P<0.001$).

Although it was not statistically different, at the end of the experiment RUNLF mice weighed less than SEDLF mice. This was the same trend that was reported in Experiment 5 in Chapter 4 as runner mice had less fat pad amount compared to the sedentary mice. However the opposite trend seemed to be the case for the HF mice: RUNHF mice weighed somewhat but not significantly more than SEDHF mice. It should be noted that the weight loss in the HF groups is due primarily to the diet shift to low fat

5TUM chow pellets, but compounded by the unit price requirements. In addition, similar to the meal patterns of the runner mice in Experiment 5 in Chapter 4, both runner groups in in this pilot study (RUNLF and RUNHF) consumed bigger and fewer meals than their sedentary counterparts (SEDLF and SEDHF), resulting in greater total daily intake. Our results agree with studies in the literature reporting that exercising mice consume significantly more energy than non-exercising mice, yet they experience weight loss and a decrease in body fat. Bell, Spencer & Sherriff (1995) reported that despite increased food intake and statistically similar body weights, exercising high fat fed mice had significantly less body fat than sedentary high fat fed controls, a result that differs somewhat from the results of our pilot study in which the RUNHF group did not lose more weight than SEDHF mice.

There are several methodological differences that may account for the differences between our results and those of Bell, Spencer & Sherriff (1995). First, they used 6-wk-old female Swiss Albino mice as in our diet-induced obesity protocol we used male 9-mo-old C57BL/6 (B6) mice. Older B6 mice in our study were more prone to obesity, shown by the dramatic increase in body weight when fed with high fat diet (± 17 g) compared with the ~ 4 g weight gain in reported by Bell, Spencer & Sheriff (1995) It is quite possible that age and/or gender and/or strain might have played a role on the differential results in both studies (Pitts, 1984). Irani *et al.* (2005) reported that the primary effect of exercise on body weight of the old obese MC4RKO mice was not as dramatic as that observed for these same mice when allowed to exercise at a young age before becoming obese. In human studies a significant inverse cross-sectional relationship has been shown between activity energy expenditure and percent body fat

in males, whereas such relationship was not found to be apparent in females (Westerterp & Goran, 1997).

In this pilot work, sedentary diet-induced obese mice (SEDHF) ate the lowest amount of food of any group (especially at the start of the FUP phases) and steadily lost weight, approaching their pre-HF diet weights. As the phenotypic expression of a condition, diet-induced obesity was also shown to play a role on the effect of exercise on food intake and body weight changes. However, although, the sedentary lean mice (SEDLF) weighed more than the runner lean mice (RUNLF) at the end of the protocol, the runner HF fed mice (RUNHF) lost most of their weight end the end of the experiment at a similar level as the sedentary HF fed mice (SEDHF). This was particularly interesting because neither runner nor sedentary diet-induced obese mice defended their higher weights throughout the protocol and RUNHF mice weighed somewhat more than the SEDHF mice, albeit not significantly. Thus, exercise was not effective at promoting weight loss in already obese mice. This slightly differential effect of exercise on RUNLF and RUNHF compared to their sedentary counterparts was not caused by the differential amounts of energy expenditure on the running wheels as we found no statistical difference between the voluntary running wheel activity of RUNLF and RUNHF. Thus, although we found a preventative effect of exercise on body fat accumulation, exercise actually slowed weight loss in mice with a precondition of obesity.

In this dissertation, the attempt to combine the physiological conditions with the environmental economical settings is an important because of its high relevance to today's obesity epidemic. Although eating is a physiological process, environmental

factors cannot be and must not be ignored when determining how much an individual eats. The series of studies applied here shows that the economical setting is important on the eating behavior in a way that may reverse the effects of the factors that are conventionally known to affect eating in certain ways. In the experiment where we manipulated the economical structure of the food environment of the genetically obese and metabolically deficient mice in Chapter 3, although conventionally we were expecting that the obese mice would be hyperphagic (Lubrano-Berthelmer, Cavazos, Dubern, 2003) or more willing to work for food (Hamilton and Brobeck, 1964) it was clear that obese mice started to work less and eat less as a consequence when the economical setting was changed. It is not a novel idea that the food choices are economic concepts however this work is an original and a unique in that it reveals that not only the food intake is an economical concept but also the phenotypical expressions of genetic tendencies can be altered by these environmental changes and this work does more than just theorizing this idea. This type of research in real life settings as translational studies with human subjects would be very important and are currently being designed in various settings by some investigators (Nasser *et al.*, 2008) which may give insights for the human condition of obesity.

As a conclusion, although the elasticity of their demand functions were different there was something similar between the mice in positive energy balance and mice in negative energy balance: obese mice in positive energy balance were eating more than the lean mice; however note that runner mice that are in negative energy balance were also eating more than the sedentary lean mice. One may explain the hyperphagia in both these opposite energy balance conditions with the 'wanting versus liking' theory

(Berridge, 1996). Thus while the runner mice that are in a negative energy balance were hyperphagic compared to the sedentary mice because of the appetitive/incentive motives that are considered as 'wanting'. The obese mice that are in the positive energy balance on the other hand were hyperphagic because the pleasure/palatability motives that is considered as 'liking'. Of course both of the motivational processes may still be effective to a certain extent at the same time in both of energy balance conditions but it is plausible to assume that one motive may be more dominant over the other in a particular energy balance condition as stated above. This understanding may give us an idea on although both groups eat more than their control groups; the motivational processes underneath their hyperphagia may be different. The important difference between the mice in positive energy balance and the mice in negative energy balance is that although hyperphagic, genetically obese mice became almost hypophagic and ate less when the price for food was too costly (FUP50), however the mice that are running, maintained their daily food intake at the FUP50 (Experiment 5) and even higher to AC1000FUP5 (Experiment 6). This is particularly promising and hopeful in a way that although the appetitive motives of 'wanting' are very hard to override with environmental manipulations, the pleasure/palatable related motives of 'liking' can be overcome by such alterations. This is partly also the explanation of today's rising obesity epidemics in our obesigenic food environment. This work indicates that if an obesigenic food environment can lead metabolic deficiencies and obesity, a food environment that is promoting the healthy eating may also be very important and not useless as treatment for obesity.

LIST OF REFERENCES

- Abdel-Malek ZA, Scott MC, Furumura M, Lamoreux ML, Ollmann M, Barsh GS and Hearing VJ (2001) The melanocortin 1 receptor is the principal mediator of the effects of agouti signaling protein on mammalian melanocytes. *Journal of Cell Science* 114, 1019-1024.
- Adan RAH, Tiesjema B, Hillebrand JJG, la Fleur SE, Kas MJH, de Krom M (2006) The MC4 receptor and control of appetite. *Br J Pharmacol* 149:815–827.
- Appleyard SM, Bailey TW, Doyle MW, Jin YH, Smart JL, Low MJ, Andresen MC (2005) Proopiomelanocortin neurons in nucleus tractus solitarius are activated by visceral afferents: regulation by cholecystokinin and opioids. *J Neurosci* 25:3578-3585.
- Atalayer D, Rowland NE (2009) Meal patterns of mice under systematically varying approach and unit costs for food in a closed economy. *Physiol Behav* 98:85-93.
- Atalayer D and Rowland NE (2010a) Comparison of C57BL/6 and DBA/2 mice in food motivation and satiety. *Physiol and Behav* 99:679-683.
- Atalayer D, Robertson KL, Haskell-Luevano C, Anderson A, Rowland NE (2010b) Food demand and meal size in mice with single or combined disruption of melanocortin type 3 and 4 receptor. *Am J of Physiol-Regul, Comp and Integr Physiol* (in press).
- Bachmanov AA, Reed DR, Beauchamp GK, Tordoff MG (2002) Food intake, water intake and drinking spout side preference of 28 mouse strains. *Behav Genet* 32:435-443.
- Bauman R (1991) An experimental analysis on the cost of the food in a closed economy *J Exp Anal Behav*, 56:33-50.
- Bauman RA, Raslear TG, Hursh SR, Shurtleff D, Simmons L (1996) Substitution and caloric regulation in a closed economy. *J Exp Anal Behav* 65(2): 401-422.
- Belke TW (1997) Running and responding reinforced by the opportunity to run: effect of reinforcer duration. *J Exp Anal Behav*, 67:337-351.
- Belke TW, Wagner JP (2005) The reinforcing property and the rewarding aftereffect of wheel running in rats: a combination of two paradigms. *Behavioural Processes* 68:165–172.

- Bell RR, Spencer MJ, Sherriff JL (1995) Diet-induced obesity in mice can be treated without energy restriction using exercise and/or a low fat diet. *J Nutrition* 125:2356-63.
- Berridge KC (1996) Food reward: Brain substrates of wanting and liking. *Neuroscience and Biobehavioral Reviews*, 20(1): 1-25.
- Berthoud HR, Sutton GM, Townsend L, Patterson LM, Zheng H (2006) Brainstem mechanisms integrating gut-derived satiety signals and descending forebrain information in the control of meal size. *Physiol Behav* 89:517-524.
- Berthoud HR (2002) Multiple neural systems controlling food intake and body weight. *Neurosci Behav Rev* 26:393-428.
- Bjorntorp P (1993) Visceral obesity: a "civilization syndrome". *Obes Res* 1(3):206-22.
- Blevins JE and Baskin DG (2010) Hypothalamic-brainstem circuits controlling eating. *Frontiers in Eating and Weight Regulation. Forum Nutr. Basel, Karger* 63:133–140.
- Blevins JE, Morton GJ, Williams DL, Caldwell DW, Bastian LS, Wisse BE, Schwartz MW, Baskin DG (2008) Forebrain melanocortin signaling enhances the hindbrain satiety response to CCK-8. *Am J Physiol Regul Integr Comp Physiol* 296:476-484.
- Blevins JE, Morton GJ, Williams DL, Caldwell DW, Bastian LS, Wisse BE, Schwartz MW, Baskin DG (2009) Forebrain melanocortin signaling enhances the hindbrain satiety response to CCK-8. *Am J Physiol Regul Integr Comp Physiol* 296: 476–484.
- Branson R, Potoczna N, Kral JG, Lentjes KU, Hoehe MR, Horber FF (2003) Binge eating as a major phenotype of melanocortin 4 receptor gene mutations. *N Engl J Med* 348:1096–103.
- Chajlani V (1996) Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem Mol Biol Int* 38(1):73-80.
- Chandler PC, Viana JB, Oswald KD, Wauford PK, Boggiano MM (2005) Feeding response to melanocortin agonist predicts preference for and obesity from a high-fat diet. *Physiol Behav* 85:221-230.

- Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan X, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L, Metzger JM, Strack AM, Camacho RE, Mellin TN, Nunes CN, Min W, Fisher J, Gopal-Truter S, MacIntyre DE, Chen HY and Van der Ploeg LHT (2000) Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nature Genetics* 26:97-102.
- Chi MM, Powley TL (2003) C-Kit mutant mouse behavioral phenotype: altered meal patterns and CCK sensitivity but normal daily food intake and body weight. *Am J Physiol Regul Integ Comp Physiol* 285:1170-1183.
- Clifton PG (2000) Meal patterning in rodents: psychopharmacological and neuroanatomical studies. *Neurosci Biobehav Rev* 24:213-222
- Colbert LH, Mai V, Tooze JA, Perkins SN, Berrigan D and Hursting SD (2006) Negative energy balance induced by voluntary wheel running inhibits polyp development in APCMin mice. *Carcinogenesis* 27:2103–2107.
- Collier GH, Hirsch E, Hamlin P (1972) The ecological determinants of reinforcement in the rat. *Physiol Behav* 9:705-716.
- Collier GH (1985) Satiety: an ecological perspective. *Brain Res Bull* 14:693–700.
- Collier GH, Johnson DF, Hill WL, Kaufman LW (1986) The economics of the law of effect. *J Exp Anal Behav* 46:113–36.
- Collier GH, Johnson DF, Mathis C (2002) The currency of procurement cost. *J Exp Anal Behav* 78:31–61.
- Cone Rd, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA (1996) The melanocortin receptors: agonists, antaonists, and the hormonal control of pigmentation. *Recent Prog Horn Res* 51:287-317.
- Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* 411(6836):480-484.
- Cummings DE and Schwartz MW (2000) Melanocortins and body weight:a tale of two receptors. *nature genetics* 26:8-9.
- David J, Polis I, McDonald J, Gold LH (2001) Intravenous self-administration of heroin/cocaine combinations (speedball) using nose-poke or lever-press operant responding in mice. *Behav Pharmacol* 12:25–34.

- Dishman R K, Berthoud HR, Booth FW, Cotman CW, Edgerton VR, Fleshner MR, Gandeia SC, Gomez-Pinilla F, Greenwood BN, Hillman CH, Kramer AF, Levin BA, Moran TH, Russo-Neustadt AA, Salamone JD, Van Hoomissen JD, Wade CE, York DA, Zigmond MJ (2006) Neurobiology of exercise. *Obesity* 14:345–356.
- Donovan MJ, Paulino G, Raybould HE (2007) CCK1 receptor is essential for normal meal patterning in mice fed high fat diet. *Physiol Behav* 92: 969-974.
- Drewnowski A (2004) Obesity and the food environment Dietary energy density and diet costs. *Am J Preventive Medicine* 27:154-162.
- Ettenberg A, Koob GF, Bloom FE. (1981) Response artifact in the measurement of neuroleptic-induced anhedonia. *Science* 213:357–9.
- Fan W, Ellacott KLJ, Halatchev IG, Takahashi K, Yu P, Cone RD (2004) Cholecystokinin-mediated suppression of feeding involves the brainstem melanocortin system. *Nature Neurosci* 7:335-336.
- Fox EA, Byerly MS (2004) A mechanism underlying mature-onset obesity: evidence from the hyperphagic phenotype of brain-derived neurotrophic factor mutants. *Am J Physiol Regul Integ Comp Physiol* 286:994-1004.
- Gantz I and Fong TM (2003) The melanocortin system. *Am J Physiol Endocrinol Metab* 284: 468–474.
- Giesen JCAH, Havermans RC., Douven A, Tekelenburg M and Jansen A (2010) Will Work for Snack Food: The Association of BMI and Snack Reinforcement. *Obesity* 18(5):966-70.
- Govaerts C, Srinivasan S, Shapiro A, Zhang S, Picard F, Clement K, Lubrano-Berthelier C, Vaisse C (2005) Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function. *Peptides* 26:1909–1919.
- Guide for the Care and Use of Laboratory Animals. National Academy Press. (1996) Washington, D.C.
- Hamilton CL and Brobeck JR (1964) Hypothalamic hyperphagia in the monkey. *J Comp Physiol Psychol* 57(2):271-278.
- Hansen G, Madsen AN, Joergensen J, Vrang N, Fosgerau K, Larsen PJ (2008) Exercise modifies body weight set-point in diet-induced obesity-prone rats. *Appetite* 51:350–412.

- Haskell-Luevano C, Monck EK (2001) Agouti-related protein functions as an inverse agonist at a constitutively active brain melanocortin-4 receptor. *Regul Peptides* 99: 1–7.
- Haskell-Luevano C, Schaub JW, Andreasen A, Haskell KR, Moore MC, Koerper LM, Rouzard F, Baker HV, Millard WJ, Walter G, Litherland SA, Xiang Z (2009) Voluntary exercise prevents the obese and diabetic metabolic syndrome of the melanocortin-4 receptor knockout mouse. *FASEB J* 23:642-655.
- Hatta N, Dixon C, Ray AJ, Phillips SR, Cunliffe WJ, Dale M, Todd C, Meggit S, Birch-Machin MA, and Rees JL (2001) Expression, candidate gene, and population studies of the melanocortin 5 receptor. *The Journal Of Investigative Dermatology* 116(4):564-570.
- Himms-Hagen L (1979) Obesity may be due to malfunctioning of brown fat. *121:1361-4*.
- Hursh SR Economic concept for the analysis of behavior. (1980) *J Exp Anal Behav* 34:219-238.
- Hursh SR (1984) Behavioral economics. *J Exp Anal Behav*, 42(3): 435–452.
- Hursh SR, Raslear TG, Shurtleff D, Bauman RA, Simmons L (1988) A cost-benefit analysis of demand for food. *J Exp Anal Behav* 50:414-440.
- Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Smith FJ, Boston BA, Fang Q, Berkemeir LR, Gu W, Cone RD, Campfield LA, Lee F (1997) Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 88:131-141.
- Irani BG, Xiang Z, Moore MC, Mandel RJ, Haskell-Luevano C (2005) Voluntary exercise delays monogenetic obesity and overcomes reproductive dysfunction of the melanocortin-4 receptor knockout mouse. *Biochem Biophys Res Commun* 326:638-644.
- Iversen IH (1993) Techniques for establishing schedules with wheel running as reinforcement in rats. *J Exp Anal Behav* 60:219-238.
- Johnson DF, Triplehorn J, Collier G (1993) The effect of patch depletion on meal patterns in rats. *Anim Behav* 46:55-62.
- Killeen PR (1995) Economics, ecologists, and mechanics: The dynamics of responding under conditions of varying motivation. *J Exp Anal Behav* 64:405-431.
- Kishi T and Elmquist JK (2005) Body weight is regulated by the brain: a link between feeding and emotion. *Molecular Psychiatry* 10, 132–146.

- Kissileff HR (1970) Free feeding in normal and 'recovered lateral' rats monitored by a pellet-detecting eatometer. *Physiol Behav* 5:163-174.
- Krawczewski KA, Benoit SC, Tschoep M, Obici S (2009) Voluntary exercise and CNS control of energy balance. *Appetite* 52:815–868.
- Lea SEG and Roper TJ (1977) Demand for food on fixed-ratio schedules as a function of the quality of concurrently available reinforcement. *J Exp Analysis Behavior* 27:271-80.
- Lea SEG (1978) The psychology and economics of demand. *Psychological Bulletin* 85(3):441-446.
- LeMagnen J. *Neurobiology of feeding and nutrition*. (1992) Academic Press, San Diego.
- Levin BE, Dunn-Meynell AA (2003) Chronic exercise lowers the defended body weight gain and adiposity in diet-induced obese rats. *Am J Physiol Regul Integr Comp Physiol* 286:771–778.
- Li BH, Rowland NE (1993) Dexfenfluramine induces fos-like immunoreactivity in discrete brain regions in rats. *Brain Research Bulletin* 31:43-48.
- Lubrano-Berthelier C, Cavazos M, Dubern B (2003) Molecular genetics of human obesity-associated MC4R mutations. *Ann N.Y. Acad Sci* 994:49-57.
- Marie LS, Miura GI, Marsh DJ, Yagaloff K, and Palmiter RD (2000) A metabolic defect promotes obesity in mice lacking melanocortin-4 receptors *PNAs* 97(22):12339–12344.
- Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KAM, Fisher SL, Burn P, Palmiter RD (1999) Response of melanocortin-4-receptor-deficient mice to anorectic and orexigenic peptides. *Nature Genetics* 21:119-122.
- Mathis CE, Johnson DF, Collier GH. (1995) Procurement time as a determinant of meal frequency and meal duration. *J Exp Anal Behav* 63:295–311.
- Melanson EL, Gozansky WS, Barry DW, MacLean PS, Grunwald GK, and Hill JO (2009) When energy balance is maintained, exercise does not induce negative fat balance in lean sedentary, obese sedentary, or lean endurance-trained individuals. *J Appl Physiol* 107: 1847-1856.
- Morato S, Johnson DF, Collier G (1995) Feeding patterns of rats when food-access cost is alternately low and high. *Physiol and Behav* 57(2): 21-26.

- Nasser JA, Evans SM, Geliebter A., Pi-Sunyer FX, Foltin RW (2008) Use of an operant task to estimate food reinforcement in adult humans with and without BED. *Obesity* 16(18):1816-20.
- Petersen S and McCarthy JC (1981) Correlated changes in feeding behavior on selection for large and small body size in mice. *Behavior Genetics* 11(1):57-64
- Pitts GC (1984) Body composition in the rat: interactions of exercise, age, sex, and diet. *Am J Physiol Regul Integr Comp Physiol* 246:495-501.
- Rhodes JS, Gammie SC, Garland T Jr (2005) Neurobiology of mice selected for high voluntary wheel-running activity. *Integr Comp Biol* 45:438–455.
- Rinaman L, Verbalis JG, Stricker EM, Hoffman GE (1993) Distribution and neurochemical phenotypes of caudal medullary neurons activated to express cFos following peripheral administration of cholecystikinin. *J Comp Neurol* 338:475-90.
- Rovee-Collier CK, Clapp BA, Collier GH (1982) The economics of food choice in chicks. *Physiol Behav* 28(6):1097-1102.
- Rowland NE, Mathes CM (2008) Appetitive and consummatory aspects of food intake in rodents. Chapter in *Appetite and food intake: behavioral and physiologic considerations*. Edited by R.B.S.Harris & R.D.Mattes; Taylor & Francis, 43-59.
- Rowland NE, Robertson KL (2005) Effect of two types of environmental enrichment for singly housed mice on food intake and weight gain. *Lab Animal* 34:29-32.
- Saelens BE and Epstein LH (1996) Reinforcing Value of Food in Obese and Non-obese Women. *Appetite* 27, 41–50.
- Samuelsson AM, Matthews PA, Argenton M, Christie MR, McConnell JM, Jansen EHJM, Piersma AH, Ozanne SE, Twinn DF, Remacle C, Rowlerson A, Poston L, Taylor PD (2008) Diet-Induced Obesity in Female Mice Leads to Offspring Hyperphagia, Adiposity, Hypertension, and Insulin Resistance: A Novel Murine Model of Developmental Programming Hypertension 51:383-392.
- Schwartz, Michael W. Seeley, Randy J. Woods, Stephen C. Weigle, David S. Campfield, L. Arthur Burn, Paul Baskin, Denis G. (1997) Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes* 46(12):2119-5.

- Sumpter CE, William T, Foster TM (1999) The effect of differing response types and price manipulations on demand measures. *J Exp Anal Behav* 71:329-354.
- Surwit RS, Kuhn CM, Cochrane C, McCubbin JA, Feingloss MN (1988) Diet-induced type II diabetes in C57BL/6J mice. *Diabetes* 37:1163–1167.
- Tabarin A, Diz-Chaves Y, Consoli D, Monsaingeon M, Bale TL, Culler MD, Datta R, Drago F, Vale WW, Koob GF, Zorrilla EP, Contarino A (2007) Role of the corticotrophin-releasing factor receptor type 2 in the control of food intake in mice: a meal pattern analysis. *Eur J Neurosci* 26: 2303-2314.
- Temple JL, Bulkley AM, Badawy RL, Krause N, McCann S, and Epstein LH (1999) Differential effects of daily snack food intake on the reinforcing value of food in obese and nonobese women. *Am J Clin Nutr* 90:304–13.
- The world health report (2005)
<http://www.who.int/mediacentre/factsheets/fs311/en/index.html>. World Health Organization.
- Vaanholt LM, De Jong B, Garland TJ, Daan S and Visser GH (2007) Behavioural and physiological responses to increased foraging effort in male mice. *The Journal of Experimental Biology* 210, 2013-2024.
- Vaisse C, Clement K, Durand E, Hercberg S, Guy-Grand B, Froguel B (2000) Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest* 106:253–262.
- Vaughan CH, Rowland NE (2003) Meal patterns of lean and leptin-deficient obese mice in a simulated foraging environment. *Physiology Behav* 79(2):275-9.
- Vaughan CH, Haskell-Luevano C, Andreasen A, Rowland NE (2006a) Effects of oral preload, CCK or bombesin administration on short term food intake of melanocortin 4-receptor knockout (MC4RKO) mice. *Peptides* 27:3226-3233.
- Vaughan CH, Moore MC, Haskell-Luevano C, Rowland NE (2005) Meal patterns and foraging in melanocortin receptor knockout mice. *Physiology and Behavior* 84:129-133.
- Vaughan CH, Moore MC, Haskell-Luevano C, Rowland NE (2006b) Food motivated behavior of melanocortin-4 receptor knockout mice under a progressive ratio schedule. *Peptides* 27:2829-2835.

- Wan S, Browning KN, Coleman FH, Sutton G, Zheng H, Butler A, Berthoud HR, Travagli RA (2008) Presynaptic melanocortin-4 receptors on vagal afferent fibers modulate the excitability of rat nucleus tractus solitarius neurons. *J Neurosci* 28:4957-4966.
- Westerterp KR, Goran MI (1997) Relationship between physical activity related energy expenditure and body composition: a gender difference. *IntJ obesity* 21:184-188.
- Willett WC, Leibel RL (2002) Dietary fat is not a major determinant of body fat. *The American Journal of Medicine* 113(9):47-59.
- Wolfe AM, Gortmaker SL, Cheung L, Gray HM, Herzog DB, Colditz GA (1993) Activity, inactivity, and obesity: Racial, ethnic, and age differences among schoolgirls. *Am J Public Health*, 83:1625-1627.
- Woods SC (2005) Signals that influence food intake and body weight. *Physiol Behav* 86:709 – 716.

BIOGRAPHICAL SKETCH

Deniz Atalayer graduated from Bogazici University (Istanbul-Turkiye) in 2004 with a Bachelor of Science degree in psychology. In fall 2005, Deniz was admitted to the behavioral neuroscience program in psychology department at the University of Florida, and started to work with Dr. Neil Rowland. Her research interests include eating behavior and obesity research with genetic, neurological, physiological, and neuroeconomic approaches. She defended her master's thesis that was examining the effects of the use of different operants in the meal pattern analysis in mice in fall 2007, and continued for her Ph.D. in the same program which she will receive in August 2010. From there, Deniz will be pursuing her postdoctoral training at St. Luke's Hospital Obesity Research Center at the Columbia University.