Molecular and Physiological Adaptations to Weight Perturbation in Mice

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Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

under the Executive Committee

of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2011

ABSTRACT

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From a medical perspective, obesity may be defined as a degree of relative adiposity sufficient to derange metabolic physiology in a manner that negatively impacts the health of the individual. While population-based cut points based on body mass index (BMI) are frequently used as a means of identifying such individuals, this is an imprecise approach since the critical levels of adiposity in this regard differ substantially among individuals.

Our common genetic predisposition to increased adiposity, coupled with an environment conducive to positive energy balance results in an increasing prevalence of human obesity. Weight loss, even when initially successful, is very difficult to maintain due, in part, to a feedback system involving metabolic, behavioral, neuroendocrine and autonomic responses that are initiated to maintain somatic energy stores (fat) at a level considered 'ideal' by the central nervous system (CNS). Circulating leptin is an important afferent signal to the CNS relating peripheral energy stores with modulations in key leptin sensing area sensitivity possibly implicated in the functional and molecular basis of defense of body weight. These physiological responses, which include increased metabolic efficiency at lower body weight, may be engaged in individuals at different levels of body fat depending on their genetic makeup, as well as on gestational and post-natal environmental factors that have determined the so-called "set-point".

In the work presented in this dissertation the following aspects of the physiology of the defense of body weight were explored: 1) whether levels (thresholds) of defended adiposity can be raised or lowered by environmental manipulation; 2) the physiological and molecular changes

that mediate increased metabolic efficiency following weight loss, 3) leptin's role in setting the threshold; 4) the effects of ambient temperature on metabolic phenotypes of weight perturbed to assess whether torpor contributes to metabolic adaptation; and 5) whether changes in gut microbiota accompany changes in diet composition and/or body weight.

To assess whether the threshold for defended body weight could be increased or decreased by environmental manipulations (i.e. high fat diet & weight restriction), we identified bioenergetic, behavioral, and CNS structural responses of C57BL/6J in long term diet induced obese (DIO) male mice to weight reduction. We found that maintenance of a body weight 20% below that imposed by a high fat diet results in metabolic adaptation - energy expenditure below that expected for body mass and composition - and structural changes of synapses onto arcuate pro-opiomelanocortin (POMC) cell bodies. These changes are qualitatively and quantitatively similar to those observed in weight-reduced animals that were never obese, suggesting that the previously obese animals are now "defending" a higher body weight. Maintenance of a lower body weight for more than 3 months was not accompanied by remission of the increased metabolic efficiency. Thus, the consequence of long term elevation of body weight suggests an increase in defended body fat that does not abate with time.

Mice can enter torpor – a state of decreased metabolic rate and concomitant decrease in body temperature – as a defense mechanism in times of low caloric availability and/or decreased ambient room temperatures. Declines in circulating leptin concentrations and low ambient room temperature have both been implicated in the onset of torpor. To assess the effects of ambient room temperature and leptin concentrations on metabolic adaptation, we characterized C57BL/6J and leptin deficient (Lep^{ob}) mice following weight perturbation at both 22°C and 30°C ambients. Weight-reduced C57BL/6J mice show metabolic adaptation at both ambient temperatures and do

not enter torpor whereas weight-reduced *Lep*^{ob} animals readily enter torpor at 22°C. This suggests that sufficiently high absolute leptin concentrations may impede the onset of torpor and that torpor itself does not contribute to metabolic adaptation in mice that have an intact leptin axis.

To assess whether hyperleptinemia *per se* was capable of increasing the threshold for defended body weight, leptin was infused by minipumps into C57BL/6J mice for 18 weeks and body weight and metabolic parameters were studied following cessation of leptin infusion. Leptin infused mice did not defend elevated body weights compared to PBS infused mice suggesting that leptin alone may not be capable of setting the threshold for body weight defense implying that other changes accompanying obesity (i.e. increased free fatty acids, endoplasmic reticulum stress and/or inflammation of leptin-sensitive neural areas) are implicated. A caveat and possible confound to this study is the possibility of antibody production against the exogenous leptin that could have drastically decreased the amount of bioavailable leptin in these mice. This experiment did not assess antibody production but subsequent studies should do so.

Finally, gut microbiota have been implicated in the regulation of body weight possibly by impacting insulin resistance, inflammation, and adiposity via interactions with epithelial and endocrine cells. We assessed changes in relative abundances of cecal microbiota in mice following sustained changes in body weight and diet composition. In diet-induced obese (DIO) mice, we find that weight reduction resulted in shifts in specific bacteria abundance (*Akkermansia* and *Mucispirillum*) and that these changes were correlated with leptin concentrations. Leptin modulates mucin production in the gut possibly altering local microniches for certain bacteria providing a functional link between adiposity and gut-specific changes in bacterial populations.

Overall, the major findings of these experiments are that the threshold for body weight defense can be raised but not lowered, that metabolic adaptation observed in weight-reduced mice is not a result of torpor, and that hyperleptinemia (if no anti-bodies were produced) *per se* isolated from other obesity-related changes does not appear capable of raising the threshold.

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ABBREVIATIONS

AGRP	agouti-related protein
αMSH	alpha melanocyte-stimulating hormone
ARC	arcuate nucleus
BBB	blood brain barrier
CART	cocaine-amphetamine regulated transcript
CNS	central nervous system
CON-AL	control diet ad-libitum fed
CON-WR	control diet weight-reduced
CSF	cerebrospinal fluid
DIO-AL	diet-induced obese ad-libitum fed
DIO-WR	diet-induced obese weight-reduced
DMH	dorsomedial hypothalamus
EE	energy expenditure
EI	energy intake
HFD-AL	high fat diet (DIO) ad-libitum fed
HFD-WR	high fat diet (DIO) weight-reduced
HOMA-2	homeostasis model assessment
Jak2	janus kinase 2 tyrosine kinase
Lep^{ob}	leptin knockout
LepR	leptin receptor
$Lepr^{db}$	leptin receptor knockout
LFD-AL	low fat diet (CON) ad-libitum

LFD-WR	low fat diet (CON) weight-reduced
LH	lateral hypothalamus
MC3R	melanocortin-3 receptor
MC4R	melanocortin-4 receptor
ME	median eminence
NEAT	non-exercise activity thermogenesis
NPY	neuropeptide Y
NREE	non-resting energy expenditure
POMC	proopiomelanocortin
RMR	resting metabolic rate
SF1	steroidogenic factor-1
STAT3	signal transducer and activator of transcription-3
T3	triiodothyronine
T4	thyroxine
TEE	total energy expenditure
TEF	thermic effect of food
VMH	ventral medial hypothalamus
VTA	ventral tegmental area

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Rudy Leibel, for having convinced me in February of 2005 that I should enroll in a top-rated university to conduct my doctoral dissertation. Since joining his lab 4 years ago, he has given me the necessary tools, both intellectual and physical, to conduct the experiments described in this thesis. Also, I am indebted to his relentless and highly precise editing skills that have drastically improved all written material associated with these experiments.

I would like to thank my thesis committee members (Drs. Michael Rosenbaum, Gary Schwartz, Eric Schon, and Sharon Wardlaw) for their guidance during my PhD work. I would like to mention that Dr. Rosenbaum was highly involved in the design of many of the experiments undertaken during my thesis work and was always helpful with manuscript editing, statistics, and overall discussions about the various projects.

I would like to thank my family (Eric, Jacqueline, Jeremy, and Anthony Ravussin) for supporting me throughout my university studies and always helping out during more difficult times of scientific research. I am indebted to my wife Karoline Ravussin, whose love, support, and positive outlook on life have kept me grounded and focused during my thesis work.

I would like to thank all the members of Dr. Rudy Leibel's lab, both past and present, that have helped me in innumerable ways and are too numerous to mention here. I would especially like to thank Dr. Charles LeDuc who has been like a father figure to me in the lab. His guidance, help, and knowledge during the past four years have been instrumental in the work that has been accomplished.

AstraZeneca has provided me with financial support that has given me the opportunity of conducting such large scale mouse experiments.

Finally, I thank my PhD program (Cellular, Molecular, Structural and Genetic Studies), especially Dr. Ron Liem who helped me transfer into the program giving me the opportunity to work in Dr. Leibel's lab and Zaia Sivo who helps keep the program running smoothly for all students.

CHAPTER 1: INTRODUCTION

BACKGROUND AND SIGNIFICANCE

Homeostasis, from the Greek *hómoios* meaning "same" and *stasis* meaning "standing still", is a term first coined by Walter Bradford Cannon in 1926 based on the concept of the constancy of the *milieu intérieur* described by the French physiologist Claude Bernard at the end of the 19th century. It refers to the relative stability of open or closed biological systems attained through dynamic equilibrium adjustment and regulation mechanisms. Many physiological systems are homeostatically controlled such as body temperature [1] and blood pressure [2]. Although body weights across a population vary enormously, individuals show remarkable weight stability strongly suggesting that body weight is physiologically regulated [3,4]. Neumann, in the beginning of the 20th century, noted the relative stability of his own body weight with no conscious attempt by him to regulate energy intake or expenditure [5]. He used the term "luxuskonsumption" to describe the body's capacity to increase metabolic rate to counteract excessive energy intake. Seminal rodent studies placed the hypothalamus as a key center involved in body weight regulation [6]. In the 1940s, Hetherington and Ranson [7] showed that lesions in the region of the ventral medial hypothalamus (VMH) led to extreme obesity in rats and the VMH is often referred to as the satiety center. Lesions in the lateral hypothalamus (LH) - subsequently referred to as the hunger center - caused severe anorexia and decreased body weight [8,9]. This suggested that specific centers in the brain may be important in the physiological defense of body weight. Interestingly, once the lesioned rats reached a new stable body weight, they subsequently defended that body weight against further perturbations. For example, rats

with LH lesions that were weight reduced by 20% of their initial body weight through hypocaloric feeding and then given *ad-libitum* access to food readily regained the lost weight at rates similar to weight reduced non-lesioned rats[6]. Thermodynamic considerations dictate that changes in somatic energy content are the result of differences between energy intake and energy expenditure. The matching of energy intake (EI) to energy expenditure (EE), a requirement for weight stability, has been shown to be relatively accurate in both short term (0.57% intra-individual coefficient of variation across 24 hour measurements [10]) and longer term studies [4]. Yet a very small but persistent mismatch between energy input relative to output would result in increased body mass over time (if the mismatch is maintained). Constancy of somatic energy content ("energy balance") is achieved when input (i.e. dietary energy intake) equals output (i.e. total energy expenditure).

EI is the caloric intake of an organism minus the calories lost in stool. Total energy expenditure (TEE) is comprised of three major components: resting metabolic rate (RMR), the thermic effect of food (TEF), and non-resting energy expenditure (NREE) that includes all volitional activity and 'exercise' as well as non-exercise activity thermogenesis (NEAT) such as fidgeting. Although the molecular pathogenesis of obesity in humans is not fully understood [11,12], the disproportionate accumulation of body fat is probably caused by the interaction between environmental conditions (such as easy access to calorically dense and highly palatable foods and low requirement of physical activity to obtain this food) and genetic factors [13]. Whereas it is true that weight loss improves the health of the obese and is therefore desirable, the fact that 75-85% of people who have undergone weight loss ultimately regain the lost weight [14] indicates that

strong metabolic and environmental factors oppose the long-term maintenance of a reduced body weight.

METABOLIC ADAPTATION

The overfeeding studies conducted in a Vermont prison population further confirmed Neumann's idea of "luxuskonsumption". These studies suggested that in response to excess caloric intake, energy expenditure was increased beyond that accounted for by increased metabolic mass (fat mass, and fat-free mass) [15]. Subsequent studies in humans reported that excessive caloric intake caused subsequent decreases in hunger [16] and concomitant increases in energy expenditure [17] whereas underfeeding caused subsequent increases in hunger and decreases in energy expenditure [17]. The capacity for one to increase metabolic rate during a period of overfeeding was shown to be inversely correlated to the amount of weight gained; the higher the metabolic response to overfeeding, the less weight that person gains [18]. This suggests that there are inherent metabolic differences among people in their capacity to raise EE in response to a period of hypercaloric feeding. Weight-reduced humans, maintained at either 10% or 20% below "normal" weight (defined as maximal lifetime weight, maintained within a range of 2 kg for at least six months), show decreased EE in absolute terms, but also when expressed per unit of metabolic mass (fat and fat-free mass). This metabolic adaptation to decreased body weight seems to result mainly from increased efficiency of energy utilization in tissues and organs such as skeletal muscle [17]. More specifically, Rosenbaum *et al.* have shown that a large part of the metabolic adaptation, whether assessed by ergometry [19,20], magnetic resonance spectroscopy (MRS) [19], or physical activity logs [21], is due to increased skeletal muscle chemo-mechanical efficiency during low levels of physical activity [22]. Some of this metabolic adaptation

is related to a decrease in sympathetic nervous tone, a concomitant increase in parasympathetic nervous system tone and to decreases in concentrations of bioactive thyroid hormones [23,24]. Weight perturbation studies in weight-reduced obese and non-obese individuals show that the magnitude of metabolic adaptation is maximal at a weight loss of 10% of initial weight. In other words, subjects undergoing a sustained 20% weight reduction do not show significantly larger metabolic adaptation in total EE or in non-resting energy expenditure (NREE; both normalized for metabolic mass) when compared to subjects with a -10% weight reduction [17] (see **Figure 1.1**). These data indicate that 10% weight loss may already be beyond the threshold at which all facets of metabolic adaptation are engaged to favor weight regain. Furthermore, this metabolic adaptation persists even after 6 years of successful maintenance of a lower body weight [25,26]. Understanding the signaling pathways connecting the central nervous system to decreases in body weight is crucial if we are to produce meaningful approaches to the successful treatment of obesity. The main focus of my PhD work has been to understand the molecular and physiological mechanisms involved in metabolic adaptation to perturbations of body weight.

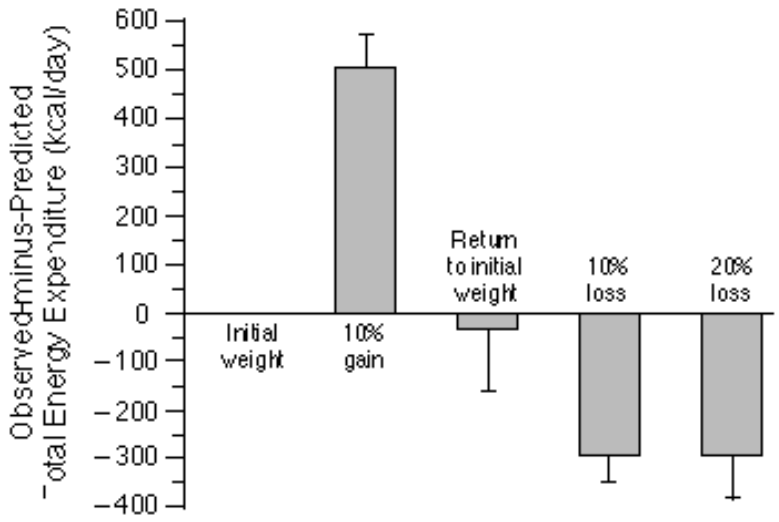


FIGURE 1.1: MEAN (±SD) OF OBSERVED – PREDICTED TOTAL ENERGY EXPENDITURE FROM HUMAN SUBJECTS

These residuals were calculated based on a regression analysis of total energy expenditure related to fat mass and fat-free mass of the human subjects before weight loss (initial). 10% gain represent subjects at 10% above the individual's customary body weight, "return to initial" are subjects that have been brought back down to initial weight following a maintenance period of +10% body weight, and "10% loss" and "20% loss" are subjects at 10% or 20% below initial body weights respectively[17].

ADIPOSTAT HYPOTHESIS

There is strong evidence that body energy stores (fat) are physiologically regulated. For most, body weight is stably maintained without requiring conscious regulation of intake and/or expenditure of calories [3,4]. An important early attempt to assign a physiological basis of weight stability was presented as the "adipostat" hypothesis by Kennedy in 1953 [27]. He presented evidence, that calorie intake in rats was correlated with total fat mass and that both were constant over time [28]. His conclusion anticipated the discovery of an adipocyte-derived signal molecule

capable of "reporting" peripheral fat content to the brain. Such a molecule would need to fulfill several requirements: 1) it must accurately reflect quantity of somatic fat stores and therefore must generate a signal in proportion to the amount of adipose tissue: 2) it needs to reach its intended site of action (presumably, but not exclusively, the central nervous system): 3) it needs to have metabolic consequences that impact energy intake and expenditure. Woods, Porte, and others suggested that insulin may play such a role in body weight regulation [29-31]. Basal insulin concentrations are positively correlated with adiposity [32,33] and insulin can reach the central nervous system (CNS) since it is present in the cerebrospinal fluid (CSF) of many species [34,35]. Subsequently, intracerebroventricular injections of insulin were shown to cause decreases in body weight and energy intake [30,36]. Another such candidate molecule arose from the study of a mouse line that contained a spontaneous mutation that caused extreme obesity. The mouse was first described at the Jackson Laboratory in the summer of 1949[37]. This mutation was designated as *ob* (for obesity) and the mice segregating for the *ob* mutation are now designated as Lep^{ob} [37] following the cloning of the gene leptin. The Lep^{ob} mice were hyperphagic - plateauing at 4x greater body weight than WT mice [37] – and were later shown to have non-insulin-dependent diabetes mellitus (this phenotype was shown to be highly strain dependent), defective thermoregulation, defective fat oxidation, and infertility [38]. A second spontaneous mutation that was known to be on a separate chromosome by virtue of linkage to somatic phenotypes, produced similar phenotypes to the Lep^{ob} animals, yet the affected mice become severely diabetic at a much younger age (was also subsequently shown to be strain dependent). This mutation was designated as *db* (for diabetes) and the mice segregating for the *db* mutation are now designated $Lepr^{db}$ following the cloning of the gene leptin receptor. Parabiosis studies between the Lep^{ob} and $Lepr^{db}$ mice were performed by Doug Coleman in the 70s. In these

experiments, the circulatory systems of two mice were connected by capillary anastomoses at the flanks resulting in small interchanges of blood between the animals. A lean wild type mouse that was pabariosed to a *Lepr*^{db} mouse quickly became hypoinsulinemic, hypoglycemic, and died of starvation while the *Lepr*^{db} mouse gained excess body weight (i.e. increased adiposity). A lean wild type mouse that was ligated to a *Lep*^{ob} mouse showed no phenotype while the *Lep*^{ob} mouse decreased food intake, insulin concentrations, and blood sugar levels. A *Lep*^{ob} mouse ligated to a *Lepr*^{db} had decreased body weight, adiposity, insulin concentrations, and blood glucose levels ultimately resulting in death while the *Lepr*^{db} mouse gained body weight and adiposity. The results suggested that the two mutations coded for proteins that worked in the same pathway [39]. From these studies, Coleman inferred that *ob* might encode a secreted molecule, for which *db* was the receptor, which was later proven to be true. The cloning of the *ob* gene (*Lep*^{ob}: 1994 [40]) and subsequently the *db* gene (*Lepr*^{db}: 1995 [41]) was the culmination of molecular cloning strategies executed by a investigators at the Rockefeller University [42].

LEPTIN AS AN ADIPOSTAT

Leptin is a strong candidate for Kennedy's proposed adipostatic signal. As the protein product of the *ob* gene [43], its plasma concentrations are highly correlated with total fat mass in weight stable rodents and humans [44-46]. Serum concentrations quickly drop in response to reduced food intake therefore reflecting both existing fat stores and acute energy balance [43]. *Lep*^{ob} mice [47], children [48], and adults [49] with congenital leptin deficiency are severely obese due to extreme hyperphagia. In both mice and humans with leptin deficiency, leptin injections result in dramatic weight loss due to decreased food intake and increased energy expenditure [47,48]. This

rapid weight loss observed following leptin administration to leptin deficient mice and humans prompted the discoverers to name this protein leptin, from the Greek *leptos* meaning thin or slim [40]. This name was assigned based upon the effects on obesity of the genetically null animals and before any of the hormone had been administered to human subjects. It was believed by some that leptin's primary role was to promote weight loss and avoid weight gain [40] and the discovery of leptin and its receptor prompted hopes that administration of leptin to obese people would result in quick and sustained weight loss. Human leptin injection studies ultimately found that even supraphysiological doses of leptin did not cause significant weight loss in non-obese or obese humans [50].

The initial hypothesis with regard to the physiology of leptin did not seem to reflect some important aspects of the relevant biology. It is probable that most hominids have evolved in regions where food was physically demanding to obtain and relatively scarce. Thus, it is not surprising that humans would have evolved more mechanisms that favor accumulation and preservation of energy stores than mechanisms that limit excess energy storage. In times of excess food availability, the capacity to store extra calories as adipose tissue would have conferred to the individual evolutionary advantages during subsequent periods of famine and these genes would have been passed down to their offspring. This hypothesis was first put forth by Neel as the "thrifty gene hypothesis" [51]. The thrifty gene hypothesis posited that alleles that conferred advantages in the past might become detrimental with progress and a changing environment. In the case of obesity, enrichment for alleles that favor weight gain over weight loss would become detrimental once caloric availability - especially of highly palatable and energy dense foods - became continuous rather than intermittent. Therefore, based on the low

energy expenditure and hyperphagia of *Lep*^{ob} and *Lepr*^{db} mice, the physiological consequences of leptin injection into *Lep*^{ob} mice, and evolutionary considerations regarding the probable positive selection for alleles favoring fat storage, others suggested that the primary role of the leptin axis was to protect adiposity, and that the *Lep*^{ob} and *Lepr*^{db} animals, due to their inability produce or respond to leptin, were in a physiological state similar to perpetual starvation [13]. Leptin's role in maintaining reproductive capacity further supports these inferences.

LEPTIN AND REPRODUCTION

Sexual maturation and fertility also require critical amounts of energy reserves to ensure survival of both the mother and the offspring[52]. Ovulation is often suppressed when a mammal is in negative energy balance, whether that state results from hypocaloric feeding, increased EE, or a combination of both [52]. Women who have low body fat mass, such as professional gymnasts, often have delayed puberty onset and post-pubertal women with low fat mass become amenorrheic [52]. As mentioned above, *Lep*^{ob} and *Lepr*^{db} mice are infertile exhibiting low gonadotropin concentrations, stunted development of reproductive organs and generally do not reach sexual maturity although these phenotypes seem to be highly strain dependent [53]. Leptin administration to *Lep*^{ob} mice induces puberty, gonadotropin secretion, gonad maturation, and restores fertility [54,55]. Humans with hypomorphic mutations in leptin or the leptin receptor genes recapitulate most of the leptin-deficient reproductive phenotype observed in mice [48]. Leptin treatment of congenitally leptin deficient subjects caused increased concentrations of gonadotropin and estradiol, gonadal enlargement and pubertal development [48]. A 48 hour fast in WT mice causes >50% decline in leptin concentrations coupled with a 90% reduction in

testosterone and 75% reduction in luteinizing hormone (LH) [43] while leptin administration blunts the fasting-induced suppression of LH and testosterone secretion and restores fertility [43,56]. Finally, leptin treatment in women with hypothalamic amenorrhea increases mean circulating LH concentrations, ovarian volume, and estradiol concentrations [57]. Allowing reproduction only to take place under favorable conditions of somatic fat stores – with high leptin concentrations reflecting sufficient adipose tissue energy stores and food availability – would have conferred evolutionary advantages to both mother and offspring. Leptin's importance in regulating energy stores to allow for reproduction may have been the initial evolutionary driving force for maintenance/development of such a hormonal system defending against decreased adiposity that has now turned into a liability in the present environment of high caloric availability with decreased requirements of EE.

LEPTIN SIGNALING

Leptin signals through a cell-surface receptor that is a member of the type I cytokine receptor family [58]. Alternative mRNA splicing produces multiple isoforms of the leptin receptor (LepR) that fall into three categories; long, short, and secreted [59]. The long leptin receptor (LepRb) form mediates physiologic leptin action through activation of the receptor associated Janus kinase 2 (Jak2) tyrosine kinase [60]. Activation of Jak2 stimulates the phosphorylation of several residues found on the intracellular portion of the LepRb including Tyr_{985}, Tyr_{1077}, and Tyr_{1138} [61]. Tyr_{1138} recruits the signal transducer and activator of transcription-3 (STAT3) that, once phosphorylated, is translocated into the nucleus regulating gene expression [62]. The majority

of leptin action on EI and EE is through signaling via the LepRb expressed in the central nervous system [63], although this isoform is also found in the immune system [64], the pancreatic β-cell [65], and within the lining of the intestinal tract [66]. Within the brain, LepRb is expressed in the hypothalamus (including the arcuate nucleus (ARC), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and lateral hypothalamus, the midbrain, as well as the hindbrain [60,67]. Circulating leptin has to reach its target cells in the brain to function yet is too large to cross the blood brain barrier (BBB) by simple diffusion. It has been suggested that a specific transport system may function to carry leptin from the periphery into the CNS [68]. Obese humans who have a 3-fold increase in serum leptin concentrations only show a 30% elevation in CSF leptin concentrations [69,70] and diet-induced obese rodents that are resistant to the effects of peripheral leptin administration still respond when leptin is administered directly into the CNS [71-73]. Taken together, these results suggest a transport mechanism for leptin between the blood and the CNS that functions in a saturable manner [74]. It has been suggested that 2 of the short form leptin receptors, *ObRa* and *ObRc*, may be involved in transporting leptin across the BBB [75,76]. Intravenous infusion of radiolabeled leptin shows more rapid and larger accumulation of the protein in the median eminence (ME) and ARC compared to other hypothalamic sites [77]. The ME is a circumventricular organ and is a specialized structure that contains fenestrated capillaries providing an important neuroendocrine connection between hypothalamic neurons and the pituitary gland [78]. Tanycytes are specialized ependymal cells found in the ME with processes extending into the hypothalamus [79]. Leptin and other BBB-impermeable substances quickly enter the ARC from the circulation, suggesting possible cellular communication between the ME and the ARC through tanycytes. Thus, circulating leptin may have direct access to the ARC. In contrast, leptin does not have such access to the VMH and DMH that are believed to be

completely behind the BBB. Decreased transport of leptin across the BBB has been implicated in what some refer to as leptin "resistance" [67], a state in which leptin signaling is attenuated (decreased STAT3 phosphorylation) in response to acute leptin administration.

LEPTIN AND ARCUATE HYPOTHALAMUS

One major and well characterized target site for leptin action on metabolism is the hypothalamus, a key CNS region involved in the control of food intake and energy expenditure. The rat hypothalamic lesion studies discussed previously suggested that the lateral hypothalamus is a "hunger center" [8,9] and the ventromedial hypothalamus is a "satiety center" [7]. The arcuate nucleus of the hypothalamus contains two distinct leptin-sensitive neuronal populations that have opposing effects on food intake and energy expenditure [60]. Leptin inhibits orexigenic (appetite stimulating) neuropeptide Y (NPY), agouti-related protein (AGRP) neurons and stimulates anorectic (appetite suppressing) proopiomelanocortin (POMC)/cocaine-amphetamine regulated transcript (CART) neurons (see **Figure 1.2**). Leptin activation of the LepRb in the ARC causes STAT3-dependent activation of the POMC/CART cell population and production of POMC. Activation/depolarization of LepRb/POMC neurons increases POMC synthesis [80] which is subsequently cleaved into multiple products, including β-endorphin and α-melanocyte stimulating hormone (α-MSH). α-MSH causes decreased EI and higher EE by activating the melanocortin-4 receptor (MC4R) and the melanocortin-3 receptor (MC3R) that are found - among other places - in the LH and PVH [81] that are downstream in the leptin signaling pathway. This causes activation of the thyroid axis, sympathetic nervous activity, and brown adipose tissue resulting in increased EE [82]. Animals and humans lacking the POMC gene or with hypomorphic

mutations in the MC4 receptor are hyperphagic and obese, recapitulating many of the phenotypes observed in *Lep*^{ob} mice [83]. NPY is an orexigenic (appetite-stimulating) peptide that also suppresses the central LepRb-mediated growth and reproductive axes [84]. Injection of this highly potent orexigenic neurotransmitter into the third ventricle of mice and rats causes obesity [85] due to marked hyperphagia [86] and decreased EE. The decrease in EE is related to inhibition of brown fat thermogenesis [87], inhibition of sympathetic nervous activity and suppressed circulating levels of thyroid hormone (T3 and T4) [88], the reciprocal of the phenotype seen in MC4R activation. AgRP, co-expressed with NPY in a special subset of ARC nucleus cells, is an inverse agonist of the α-MSH/MC4R signaling pathway thereby decreasing the anorexigenic tone of POMC-related signaling[89]. Leptin inhibits NPY/AgRP neurons via LepR mediated signaling suppressing expression of these neuropeptides [89].

In summary, LepRb signaling through the ARC stimulates the production of anorectic neuropeptides (e.g. α-MSH) and suppresses orexigenic peptide production (e.g. NPY, AgRP). Conversely, a decrease or deficiency in leptin concentrations/activity (e.g. such as during starvation/fast or in ob/ob and db/db mice) causes increases in appetite via suppressed synthesis of anorectic neuropeptides (e.g. POMC) and increased expression of orexigenic peptides (e.g. NPY and AgRP). In summary, the overall simplified effects of leptin presence on the ARC of the hypothalamus are to decrease food intake and increase EE. As will be discussed below (see Leptin and Metabolic Adaptation), leptin's true biological role may not be invoked upon its presence but rather its absence. In this scenario, it is a drop in peripheral leptin concentrations below "normal" that invokes strong physiological responses aimed at attenuating the negative energy balance and/or weight loss that could jeopardize survival and reproduction.

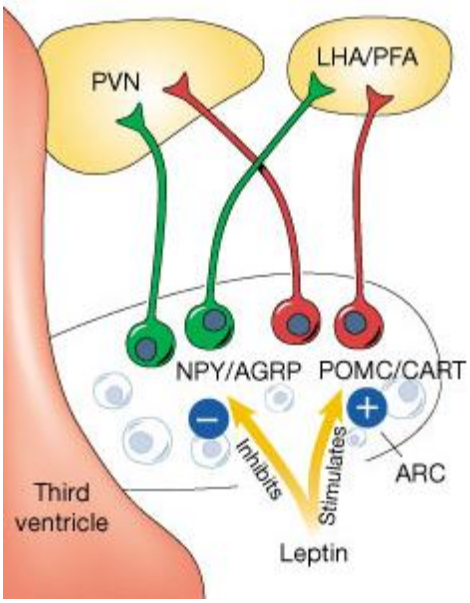


FIGURE 1.2 LEPTIN ACTION IN ARCUATE NUCLEUS AND DOWNSTREAM EFFECT
Leptin stimulates POMC/CART and inhibits NPY/AGRP causing increase EE and decreased EI [80].

LEPTIN AND EXTRA-ARC REGIONS OF CNS

LepRb neurons in the ARC make up only 15–20% of the total number of LRb-expressing neurons in the CNS [90]. VMH and ventral tegmental area (VTA) neurons that are LepRb positive clearly mediate important components of leptin action[91-93]. The role of ARC leptin-dependent effects on satiety (e.g. via LepRb/POMC and LepRb/NPY) has been well characterized and is described above. VMH neurons that are leptin sensitive, affect satiety through direct excitatory projections onto ARC POMC neurons[94,95]. Projection density of neuronal connections onto POMC cells in the arcuate were shown to be dynamically regulated by leptin concentrations (i.e. fed vs. fasted state, Lep^{ob} vs. Lep^{ob} + leptin injection). The provenance of such projections is not known at the current time. This technique, championed by Tamas Horvath and his group at Yale

University, was used in collaboration with us to explore synaptic plasticity in some of our weight-perturbed mice (chapter 2). One subpopulation of leptin-responsive VMH neurons – that co-express steroidogenic factor-1 (SF1) – is activated by leptin and is involved in leptin-induced satiety[91].

Brainstem regions, including the nucleus tractus solitarius (NTS) and the area postrema, have also been implicated in regulation of food intake[84,89,96]. The brainstem receives gut-derived inputs including vagal afferents, gut peptides such as glucagon-like peptide-1 (GLP1) and cholecystokinin (CCK). GLP-1 and CCK, which are released by the gut in response to food transit, act synergistically with leptin in neurons of the NTS resulting in increased satiety[97,98]. Direct leptin action on brainstem LepRb-positive neurons along with indirect leptin action via ARC neurons that send brainstem projections may contribute to the satiety effects of leptin.

Leptin has also been implicated in the reward aspects of food through modulations of the mesolimbic dopamine system (DA)[99]. The LH 'feeding' center is extensively connected with the mesolimbic DA system and regulates feeding motivation and food reward (Kelley & Berridge 2002, DiLeone et al. 2003, Fulton et al. 2004). Weight-reduced humans maintained at 10% below initial weight, were shown to have altered neural activity (measured by functional MRI) in specific regions known to be involved in the regulatory, emotional, and cognitive control of food intake and many of these changes were reversed upon leptin administration[100].

The neural effects of leptin are numerous and not fully understood. Dissecting the neural pathways involved in leptin-mediated changes in energy homeostasis is crucial to understand some of the homeostatic principles involved in defense of body weight. Any changes in the capacity of leptin to reach and activate the brain, may be translate to altered metabolic outcomes

that may underlie changes in apparent defended body weight. This is further discussed in the following section (Leptin and Metabolic Adaptation).

LEPTIN AND METABOLIC ADAPTATION

As mentioned above, leptin's absence may invoke its true biological role. The physiological effects of leptin reduction (induced by starvation and/or decreased body weight) on increased food intake, decreased EE, and suppressed reproductive capacity are probably very important for survival when food is scarce. In contrast to the potent effect of leptin administration to humans or rodents with leptin insufficiency (weight reduced, fasted, or congenitally leptin deficient), rodents or humans at usual or increased weights administered physiological doses of leptin show very little response [101]. In fact, in overweight and obese humans, almost 10-fold elevations of plasma leptin concentrations are required before induction of increased EE and decreased EI suggesting that leptin's major physiological role is the metabolic effects incurred in its reduction [22,50,102].

As discussed earlier, studies in humans have indicated that 10% or 20% decreases in body weight from "normal" were accompanied by disproportionate decreases in TEE, decreases in sympathetic nervous system tone (SNS) with concomitant increases in parasympathetic system nervous tone (PNS), and a downregulation of the thyroid axis (**Table 1.1**). These phenotypes are very similar to those observed in leptin deficient rodents and humans suggesting that metabolic adaptation observed in weight-reduced humans may be the result of reduced circulating leptin concentrations due to reduced body fat. It is important to point out that in these human studies the decrease in energy expenditure following weight loss did not reflect any

aspect related to the dynamic period of weight loss *per se* since measurements of energy expenditure were similar in the same people at initial weight vs. return to initial weight (subjects who had just been brought from 110% back to 100% body weight) following a maintenance of 10% elevated body weights (**Table 1.1**). This suggests that some aspect of "initial" body weight was being sensed and could actively modulate metabolism; leptin is a prime candidate to function in this manner. To validate this concept in humans, leptin was administered to the weight-reduced subjects in doses sufficient to restore circulating concentrations of leptin to those present prior to weight loss. This protocol of leptin replacement reversed nearly all of the phenotypic changes that characterize the weight-reduced state, including the normalization of the energy expenditure phenotypes as well as most of the autonomic and endocrine changes [103] (**Table 1.1**).

	Effects of 10% reduced weight maintenance	*Effects of leptin administration to weight-reduced subjects*
Energy expenditure		
24-h energy expenditure	Decreased (-15%)	Reversed
Resting energy expenditure	Decreased or unchanged	No significant change
Thermic effect of feeding	Unchanged	Unchanged
Nonresting energy expenditure	Decreased (-30%)	Reversed
Skeletal muscle work efficiency	Increased (20%)	Reversed
Autonomic function		
Sympathetic nervous system tone	Decreased (-40%)	Reversed
Parasympathetic nervous system tone	Increased (80%)	Unchanged
Neuroendocrine function		
Thyroid-stimulating hormone	Decreased (-18%)	Unchanged
Triiodothyronine	Decreased (-7%)	Reversed
Thyroxine	Decreased (-9%)	Reversed
Gonadotropins	Decreased	Reversed
Circulating leptin	Decreased (proportional to fat mass)	Reversed

TABLE 1.1: CHANGES IN ENERGY EXPENDITURE, AUTONOMIC NERVOUS SYSTEM FUNCTION AND NEUROENDOCRINE FUNCTION
Changes in subjects maintaining a reduced body weight with or without leptin 'replacement'[103]

Rodents, especially rats and mice, are commonly used models to explore leptin's role in body weight homeostasis. Energy-restricted mice fed 80% of the *ad-libitum* caloric intake of control mice are clearly capable of compensating for this decrease in energy intake by reducing energy expenditure beyond that expected by decreased body mass (metabolic adaptation)[104]. Leptin administration to calorically restricted [105] and/or fasted [43] mice restores energy expenditure to levels similar to pre weight loss and/or fast. In addition, 48-h fasted male mice show starvation-induced changes in gonadal, adrenal, and thyroid axes that are all leptin reversible [43].

Taken together, the relative absence of response to leptin injections in obese/overweight individuals even when given at supraphysiological doses in contrast to the strong physiological responses of many of the metabolic phenotypes seen in "hypoleptinemic" rodents and humans (weight-reduced, fasting, or congenically deficient) to whom leptin is administered suggests that the leptin axis may function primarily to defend body fat. Such a system might operate via a threshold mechanism; each individual having a different threshold resulting from genetic and developmental effects on the leptin signaling pathway (**Figure 1.3**) [13]. We have hypothesized that leptin provides a circulating signal that reflects adipose tissue mass, and that the metabolic responses of CNS regions to this signal depend on the integrity and function of the pathways that assimilate this and other afferent signals [13]. Variation in the genes that encode these pathways and developmental/environmental influences that may affect the expression of these genes and possibly the neuronal structural connection of the leptin signaling pathway govern the efficacy with which leptin is sensed in the CNS. If environmental changes are capable of decreasing the signaling efficacy of leptin by altering any of the subcomponents of the signaling pathway, then more leptin will be required to obtain a similar output signal. In this threshold model, higher body fat would be a rectification of low leptin signal transmission that would be achieved once sufficient circulating leptin is produced [13]. In accordance with this hypothesis, when plasma leptin concentrations fall below this preset level – due to weight loss and/or fasting that can imperil survival and reproduction – a strong physiological response ensues including increased food intake, decreased energy expenditure, and increased food seeking behavior in an attempt to protect against further weight loss. Understanding the physiological and molecular mechanisms underlying such adaptation is important to the prevention and treatment of obesity. Whether this "threshold" can be raised or lowered through environmental means – such as sustained elevated

body weight (for raising the threshold) or maintenance of a reduced body weight (for lowering the threshold) – constituted the main thrust of my PhD research.

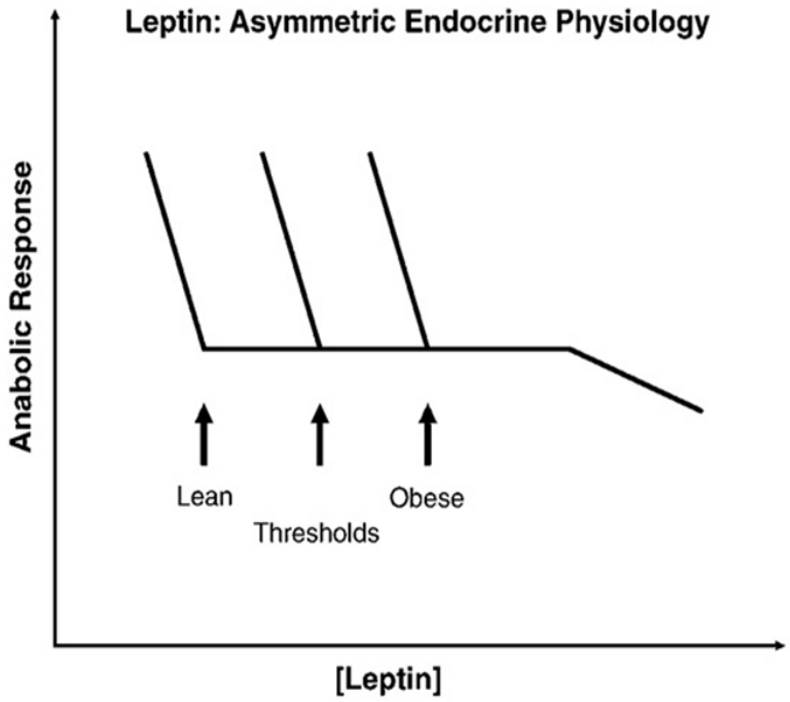


FIGURE 1.3: LEPTIN THRESHOLD MODEL
The major biologic response(s) are induced upon a decrease in circulating leptin amount below a lower threshold. During weight loss (i.e. fat loss) or decreased leptin production per unit fat mass (i.e. negative energy balance) ambient decreases in leptin below this lower threshold results in a strong anabolic response [13].

ADAPTIVE THERMOGENESIS AND METABOLISM

AMBIENT TEMPERATURE AND THERMOGENESIS

In ectotherms, body temperatures closely track ambient temperatures since they do not have endogenous mechanisms of heat production. These organisms, also called poikilotherms,

encompass reptiles, amphibians, and most fish. In contrast, homeotherms, organisms that are capable of regulating body temperature through specific means of heat production (i.e. thermogenesis) such as birds and mammals, can expend variable amounts of energy on specific mechanisms of heat production, conferring onto these organisms the capacity to live in environments that undergo larger temperature shifts. Max Kleiber, a Swiss scientist who studied energy metabolism in animals in the early part of the 20th century, showed that for most homeotherms, metabolic rate scaled allometrically to the ¾ power of body mass ($EE = M^{¾}$; **Figure 1.4**) [106]. The exact biological basis for "Kleiber's Law" is not fully understood and many hypotheses have been proposed. Some believe that it describes the consequence of the physics and geometry of the biological systems that transport, distribute, and consume energy in an organism. As the mass of an organism is increased, its surface to volume ratio is decreased with a higher proportion of the mass allocated for energy storage (e.g. fat) rather than structure (e.g. circulatory and respiratory systems). Since heat loss is directly related to surface area of an organism, larger animals are better protected from radiating heat through skin resulting in lower energy expenditure rates per unit of metabolic mass when compared to smaller animals. The allometric scaling of metabolism would seem to reflect these differences in surface to volume ratios at different masses. Others argue that the scaling is a result of the relationship between mitochondrial density and/or metabolism [107,108] [109]. Whatever the biological basis for such scaling, the fact that humans are more than 3 orders of magnitude greater in mass than mice has repercussions on the effects ambient temperature has on metabolic rate.

Mice, a model organism used for much work on the molecular physiology of energy homeostasis, have a higher surface to volume ratio than humans [110]. Furthermore, through the

use of clothing and technologies that allow for the regulation of ambient temperature energy expended for thermogenesis in most humans is very low [111]. In most rodent vivaria, the ambient temperature is set at 22º-24ºC primarily for the comfort of the personnel working in the facility. However, the thermal stress imposed by this ambient temperature – sometimes compounded by increased air flow found in ventilated cages – causes higher energy expenditure, energy intake, and sympathetic nervous system tone to maintain core body temperature [110,112,113]. The relationship of total energy expenditure (TEE) to ambient temperature for homeotherms is U-shaped, with the lowest TEE for mice occurring between 30-40°C ("thermoneutral zone")[114]. Above 40°C and below 30°C, increased metabolic rate is required to maintain stable body temperature through active cooling and thermogenic mechanisms, respectively [114]. Due to this higher requirement for thermogenesis, mice may enter torpor, a temporary physiological state characterized by a controlled decrease in metabolic rate and core body temperature (<30°C) below values considered to be normal, when confronted with low ambient temperatures and/or caloric restriction. Metabolic analyses of mice at the conventional 22°C ambient should, therefore, take into account that 40-50% of total energy expenditure is allocated to adaptive thermogenesis, resulting in reduced ability to detect subtle changes in energy homeostasis such as the effects of weight perturbation [110,114].

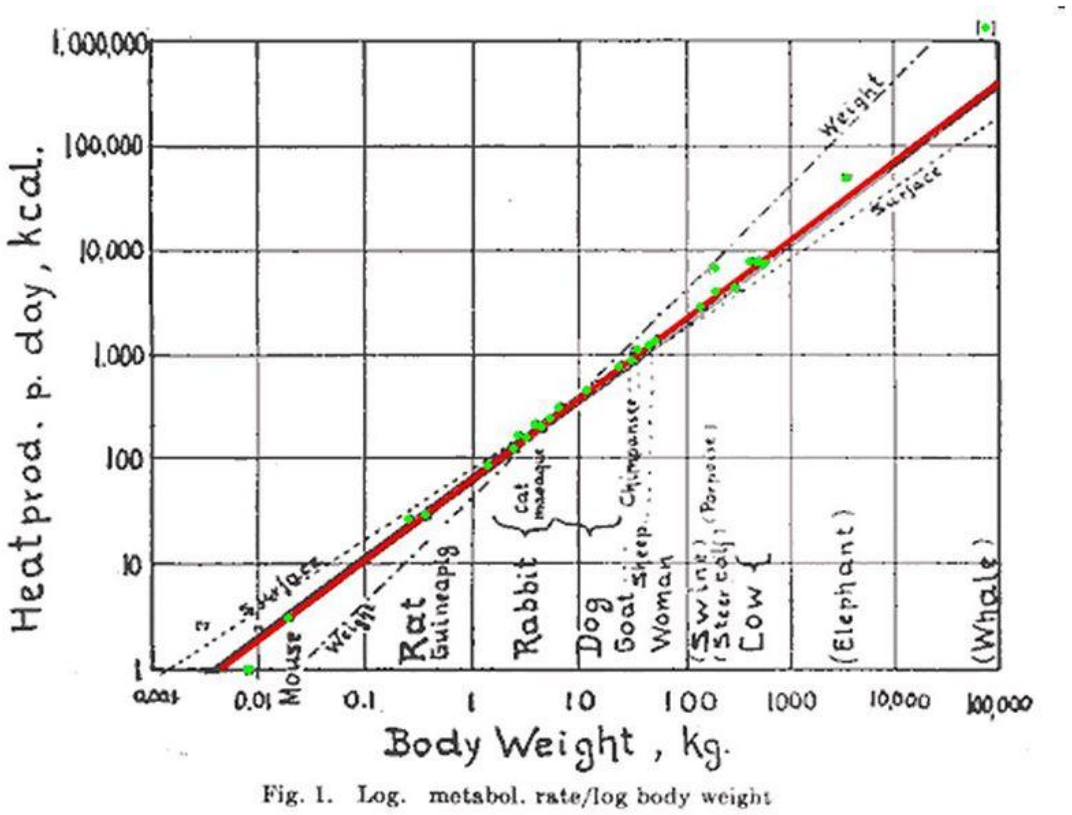


Fig. 1. Log. metabol. rate/log body weight

FIGURE 1.4: METABOLIC RATE VS. BODY WEIGHT OF DIFFERENT MAMMALS
Relationship of metabolic rate and mass across many mammal species [106].

LEPTIN AND THERMOGENESIS

Mice can undergo shallow bouts of torpor, characterized by body temperatures of 22°C lasting less than 24 hours, in response to decreased food intake, suggesting that short term hormonal cues responding to food deprivation may be required for the onset of torpor in laboratory mice [113]. The acute (≈40%) decline in circulating leptin concentrations following a 24 hour fast and leptin's known effects on energy expenditure render it a candidate hormone for triggering torpor. *Lep*^{ob} mice, both fed and fasted, become spontaneously torpid [115-117]. Leptin administration to fasted WT mice and *ob/ob* mice blunts torpor [105,115]. Yet low leptin levels

cannot be the sole signal involved in the onset of torpor since *ob/ob* mice are not always torpid. Other factors, such as nutrient availability, hormones such as ghrelin and insulin, and ambient temperature are likely integrated to determine torpor entrance in mice.

In order to further understand the role of leptin, weight reduction, ambient temperature, and energy homeostasis, we examined the bioenergetic, hormonal, and behavioral responses to maintenance of a 20% body weight reduction in singly housed C57BL/6J +/+ diet induced obese (DIO) and Lep^{ob} mice housed at both 22ºC (sub-thermoneutral) and 30ºC (thermoneutral). The results of these two studies are described in chapter 5.

MICROBIOTA AND METABOLISM

The adult human intestine is home to up to 100 trillion bacteria [118]. Experimental and computational advances have given us the tools to comprehensively characterize the nature of microbial diversity in the gut [118]. Sequencing of gut microbiota from lean and obese humans [119,120] and mice [121,122] has revealed phylum-level differences in bacterial populations suggesting a possible link between the obese phenotype and microbiota composition. Obese humans and mice are enriched in the phylum Firmicutes, and depleted in Bacteroidetes [120-122] compared to lean counterparts. Metagenomic and biochemical analyses and microbiota transplantation experiments indicate that the obesity-associated microbiota have increased ability to extract energy from a given diet [121,123]. Microbiota transplanted from genetically (Lep^{ob}) or diet-induced obese mice into germ-free WT mice caused a larger fractional increase in body adiposity compared to WT mice that received microbiota from non-obese animals (approximately 50% vs. 25% gain in fat mass). Increases in body adiposity were not related to increased caloric intake

but rather to an enhanced capacity to extract calories from the food. The increased adiposity was attributed to an increased quantity of short chain fatty acids (a by-product of bacterial catabolism of dietary fiber; non-starch polysaccharides and other plant components) in the cecum and decreased fecal gross energy content (measured by bomb calorimetry)[121].

These data suggest that shifts in the microbial community could impact metabolism and have subsequent effects on weight gain or weight loss. We therefore undertook a sequencing study of cecal bacteria from the mice studied during the first large scale mouse weight perturbation study (mice described in chapter 2). We compared the microbiota of four groups of C57BL/6J mice: diet-induced obese mice (DIO-AL) and control (10% fat) diet-fed mice (CON-AL) given *ad-libitum* access to these diets, and mice weight-reduced to 20% below initial weight (DIO-WR and CON-WR, respectively). In collaboration with Dr. Ruth Ley at Cornell University, we sequenced cecal microbiota and compared the relative abundance of specific bacterial populations with various physiological phenotypes of the mice. We hypothesized that part of the metabolic adaptation observed in weight-reduced mice might be related to changes in bacterial populations that may confer metabolic advantages to the host. The strategy, background, and results of these efforts are summarized in chapter 4. These studies were published in Obesity in May of 2011[124].

SUMMARY

I have organized this dissertation as five manuscripts (chapters 2-6). Each manuscript contains an abstract, a specific introduction, materials and methods, results, and a discussion. The Introduction above provides the historical and biological context for the studies described.

The final chapter (chapter 7) is a discussion of the experiments conducted and how they fit into the scientific literature on obesity and metabolism. This chapter also contains questions that might be explored in light of the conclusions obtained through the work reported in this thesis.

The first project I undertook during my PhD work involved the development of a mouse model in which to examine the bioenergetic and neurobiologic consequences of sustained weight perturbation [22]. In this experiment, we examined the bioenergetic, behavioral, and CNS structural responses to weight reduction of diet-induced obese (DIO) and never-obese (CON) C57BL/6J male mice (chapter 2). We tested whether the level of defended body weight could be elevated through environmental means, in this case by access to a high fat diet (60% kcal from fat) for 16 weeks. In the same mice we investigated cecal bacterial populations, by high-throughput DNA sequencing, correlating relative changes in abundance of certain species with specific physiological parameters of these mice (chapter 3). This sequencing project was undertaken to test whether some part of the metabolic adaptation observed in weight-reduced mice might be related to changes in bacterial populations that could confer metabolic advantages to the host. Finally from the same mice, we used food intake data coupled with changes in body composition to calculate energy expenditure using an energy balance technique and compared these results to EE obtained by indirect calorimetry. Those efforts are described in chapter 4. Following the first weight perturbation study – reported in chapters 2, 3, and 4 – we undertook two weight perturbation experiments aimed at further understanding the interplay between metabolic adaptation, the leptin axis, and ambient temperature on bioenergetic, endocrine, and behavioral responses. These efforts are summarized in chapter 5. Finally, chapter 6 summarizes an experiment that aimed at isolating hyperleptinemia, without the confounds of other metabolic

phenotypes that accompany obesity and the feeding of a high fat diet, through 18 weeks of leptin infusion into non-obese WT mice to determine whether elevated leptin concentrations was sufficient to raise the level of defended body weight once leptin infusion was discontinued.

CHAPTER 2: EFFECTS OF CHRONIC WEIGHT PERTURBATION ON ENERGY HOMEOSTASIS AND BRAIN STRUCTURE IN MICE

ABSTRACT

Maintenance of reduced body weight in lean and obese human subjects results in the persistent decrease in energy expenditure (EE) below what can be accounted for by changes in body mass and composition. Genetic and developmental factors may determine a CNS-mediated minimum "threshold" of somatic energy stores below which behavioral and metabolic compensations for weight loss are invoked. A critical question is whether this threshold can be altered by environmental influences, and by what mechanisms such alterations might be achieved.

We examined the bioenergetic, behavioral, and CNS structural responses to weight reduction of diet-induced obese (DIO) and never-obese (CON) C57BL/6J male mice. We found that weight-reduced DIO and CON animals showed reductions in energy expenditure – adjusted for body mass and composition - comparable (-10 -15%) to those seen in human subjects. The proportion of excitatory synapses on ARC POMC neurons was decreased by approximately 50% in both DIO and CON weight-reduced mice.

These data suggest that prolonged maintenance of an elevated body weight (fat) alters energy homeostatic systems to "defend" a higher level of body fat. The synaptic changes could provide a neural substrate for the disproportionate decline in energy expenditure in weight-reduced individuals. This response to chronic weight elevation may also occur in humans. The mouse model described here could help to identify the molecular/cellular mechanisms

underlying both the defense mechanisms against sustained weight loss and the upward re-setting of those mechanisms following sustained weight gain.

INTRODUCTION

Long-term maintenance of even modest reductions in body weight ameliorates or eliminates many of the co-morbidities of obesity [125]. The recidivism rate to obesity in formerly-obese individuals is 75-85% [14], reflecting the potent metabolic and environmental pressures opposing long-term maintenance of a reduced body weight. We have previously shown that the maintenance of a 10% or greater reduction in body weight in both lean and obese humans is associated with a decrease in energy expenditure that is 15-20% below what can be accounted for by changes in body mass and body composition. This adaptive thermogenesis does not abate over time[25], and predominantly reflects increased mechanical work efficiency of skeletal muscle, decreased circulating concentrations of bioactive thyroid hormones, and reduced sympathetic autonomic nervous system tone [17,19,25,126].

Leptin is an adipocyte-derived hormone whose circulating plasma concentrations are correlated with fat stores at usual (stable) body weight, but which rapidly decline during food restriction and/or fasting [43,127]. We have proposed that central nervous system (CNS) energy homeostasis mechanisms respond asymmetrically (in a threshold-like mechanism) to changes in circulating plasma leptin concentrations [13]. This asymmetry is evident in the demonstrations that reductions in circulating leptin concentrations in weight reduced/food restricted humans induces a strong leptin-reversible metabolic adaptation (energy expenditure reduced beyond expected per unit of metabolic mass) [22], while increases in plasma leptin concentrations as a result of weight gain do not provoke long term changes in energy expenditure [127]. Even 10-fold increases in circulating plasma leptin concentrations resulting from exogenous leptin administration do not invoke consistent increases in energy expenditure or decreases in energy intake [50]. Regulatory pathways

– constituted by specific neurons and their connections – in the hypothalamus [80] and brainstem [98,128] provide the neural substrate for the proposed threshold mechanism that uses ambient leptin as a primary afferent signal [13]. This threshold – set by genetic and developmental influences on its molecular and anatomic substrates – determines a minimum circulating concentration of leptin (hence body fat) that is "accepted" by the CNS as sufficient to ensure reproductive capacity [43] and survival in circumstances of restricted access to food calories [13]. The secular trend towards increasing prevalence of obesity, and its continued resistance to long-term successful therapy [14,129], suggest that increasing levels of body fatness are being "defended" and that structural/molecular changes in CNS regulatory regions for energy homeostasis may play a critical role in these changes. An important question in this context is whether the "threshold" for minimum adiposity can be reset upward by environmental factors, leading to physiological defense of an acquired increase in fat mass.

In rodents, weight loss due to caloric restriction results in decreased energy expenditure per unit of metabolic mass ("metabolic adaptation") consistent with the "defense" of body fat stores by CNS-mediated responses to circulating leptin and other signals (e.g. insulin) reflecting the status of somatic energy stores [130-136]. Rats selected by breeding to be predisposed to diet-induced obesity defend higher body weights than DIO resistant rats [137], readily regain lost weight following a switch back to ad-libitum food access after a period of hypocaloric feeding [138], and have increased arcuate nucleus (ARC) expression of neuropeptide Y (NPY), a key anabolic neuropeptide released from leptin-sensitive neurons that increases food intake and decreases energy expenditure [139]. Inbred mouse strains fed a high fat diet gain different amounts of body

fat that correlates with differential expression of genes in key regions of the arcuate nucleus [140,141].

The aim of the present study was to assess - in a mouse model - the physiological and molecular consequences of maintenance of increased body fat (by high fat diet) and the subsequent adaptations following caloric restriction and maintenance of a reduced body weight. Our hypothesis was that such a chronic elevation in body fat would invoke changes in the structure of the hypothalamus resulting in an upward resetting of the threshold for minimum body fat. To assess the neural substrates for changes in energy expenditure and food intake in these circumstances, we analyzed excitatory and inhibitory synapses onto the cell bodies of POMC neurons in the arcuate nucleus, a cell population that is known to play a role in body weight regulation and whose synapses are leptin-responsive. We hypothesized that prolonged maintenance of an elevated body weight by DIO followed by weight loss would result in mice that were hypometabolic compared to DIO and never-obese mice, indicating that maintenance of an elevated body weight results in long-term upward “re-setting” of a minimum threshold for body fat [13].We anticipated that the ratio of excitatory/total synapse ratios onto the POMC population would be lower in the two weight-reduced groups, a phenotype similar to the decreases characterizing congenitally leptin deficient mice [94].

MATERIALS AND METHODS

Animals and Diets: 18 week-old C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Sixteen diet-induced obese (DIO mice – fed a high fat diet starting at 6 weeks of age; Research Diets, Inc. D12492i, 60 kcal% fat), and 16 control diet-fed (CON mice – fed a low fat diet also starting at 6 weeks of age; Research Diets, Inc. D12450Bi, 10 kcal% fat) were used for these studies (**Figure 2.1A**). Upon receipt, animals were kept in a pathogen-free barrier facility maintained at 22-24 ºC with a 12-h dark-light cycle (lights on at 0700 h). The mice were individually housed in plastic pens with corn-cob based bedding, fed the same diet they had been provided at Jackson Laboratory, and given *ad libitum* access to food (diet as specified) and water during a 30 day acclimatization period. The cages were equipped with feeding baskets specially designed to minimize food spillage. During this period, body weight and food intake were monitored every 2 to 3 days.

The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Study Design: After the 30-day acclimatization period, mice in each group (DIO or CON) were paired by body weight (nearest body weight ± 0 -1.7g) and one member of each pair randomized to either an *ad-libitum* fed group (DIO-AL & CON-AL) or a weight-reduced group (DIO-WR & CON-WR). There were 8 mice in each of the 4 groups. Mice in the weight-reduced groups received 50% of their average *ad-libitum* daily food intake until their body weight reached 80% of initial value (defined as day 0, **Figure 2.1A**) at which time the mice were switched to 80% of their initial daily food intake. Subsequent adjustments in calories provided were made daily for the rest of the experimental period in order to maintain each mouse between 79-81% of initial

(pre-caloric restriction) body weight (**Figure 2.1A**). Weight-reduced mice (DIO-WR and CON-WR) had free access to water and were given 1/3 of their individual calculated food ration (± 0.1 g) in the morning (07:45-08:15h) and 2/3 of the food ration in the evening (18:30-19:00). All *ad-libitum* fed mice (DIO-AL and CON-AL) had free access to food and water throughout the day. In a subsequent study, 7 DIO-AL mice and 12 DIO-WR mice were weight perturbed in the same manner as described above and the DIO-WR were subsequently switched to *ad-libitum* access to the high fat diet. Food intake (g) and metabolizable energy intake (kcal/24h) was measured over the first twenty four hours (**Table 2.3**).

Body weight, body composition and food intake: Body weight was measured (± 0.1 g) daily before morning feeding using an Ohaus Scout Pro 200g scale (Nänikon Switzerland, between 07:45-08:15h). For *ad-libitum* fed mice (DIO-AL and CON-AL), body composition (fat mass: FM, fat-free mass: FFM, & extracellular fluid) was measured by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) [142]before the morning feeding every 2-3 weeks; before and after calorimetry measurements (see below); before start of the weight reduction protocol; and on the day prior to sacrifice. Food intake was recorded daily for the WR mice, and every 2 to 3 days for the AL mice (by weighing specially constructed feeding baskets designed to minimize spillage) during the entire weight perturbation experiment (**Table 2.1**).

Energy expenditure by indirect calorimetry: Energy expenditure was measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). O_2 and CO_2 measurements were taken every 14 minutes during a 72 hour period while mice were maintained on their respective weight maintenance feeding schedules. Because of possible initial stress

related to transfer to the chambers, only the last 48 hours of measurements were used to calculate total 24-hour energy expenditure (TEE; expressed in kcal/day) and respiratory quotient (RQ = VCO_2 / VO_2). Resting energy expenditure (REE in kcal/day) was defined as the lowest one hour period of energy expenditure, which coincided with the lowest 1 hour of total ambulatory activity during the 48-hour period and this value was extrapolated to 24 hours. Non-resting energy expenditure (NREE) was calculated as the difference between total energy expenditure (TEE) and REE. Physical activity was measured by an infrared beam system integrated with the LabMaster system. Total activity (beam breaks) in X, Y, and Z axis was stored every 14 minutes. The system is designed to differentiate between fine motor movement (defined as a single X or Y axis beam break), ambulatory movement (defined as the simultaneous breaking of two adjacent X or Y beams), and rearing, defined as the breaking of the Z axis infrared beam.

Calculations: Energy expenditure is proportional to body mass and composition [fat-free (FFM) and fat (FM mass)]. We related total energy expenditure (TEE; kcal/day) of DIO-AL and CON-AL mice to both FFM and FM by multiple regression analysis. There was no significant effect of diet composition on TEE. We therefore pooled the data from a*d-libitum* fed mice to create a baseline regression equation relating TEE (kcal/24h) to FFM and FM (grams) (TEE = 0.34 * FFM + 0.06 * FM + 5.16, R^2 = 0.66, $p < 0.01$). This equation was used to predict TEE for all mice following experimental weight perturbation, as we have done in similar studies of human subjects [17,127]. The residuals (i.e. the difference between measured and predicted values) were calculated for each animal and were tested against the null hypothesis that they were equal to zero. Baseline regression equations relating resting energy expenditure (REE – lowest one hour period of energy expenditure extrapolated to 24h) and non-resting energy expenditure (NREE = TEE - REE) to FFM and FM, predicted REE and NREE values, and residuals were also

calculated from data obtained by indirect calorimetry as described above (REE = 0.17 * FFM + 0.14 * FM + 4.96, $R^2 = 0.74$; NREE = 0.18 * FFM - 0.08 * FM + 0.20, $R^2 = 0.53$).

Serum Hormone and Metabolite Profiles: Before initiation of the weight reduction protocol, and at time of sacrifice, blood glucose (by tail bleed) and circulating leptin, insulin and bioactive thyroid hormone concentrations (by retro-orbital bleed) concentrations were determined after a 4-hour fast (see arrows on **Figure 2.1**). Blood for hormone and metabolite assays was allowed to clot for 1 hour at room temperature, spun at 4°C for 10 minutes at 1000g, and serum collected and frozen at -80°C until time of assay. Leptin was assayed using Quantikine ELISA kit (R&D Systems, Minneapolis, USA); insulin using the Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden); T3 and T4 using RIA at Hormone Assay & Analytical Services Core at Vanderbilt University (Vanderbilt University, Nashville, TN); TSH by RIA at the National Hormone and Peptide Program (UCLA Medical Center, Torrance, CA). All assays were conducted according to manufacturer's protocols. HOMA2 (calculator developed by University of Oxford - http://www.dtu.ox.ac.uk/index.php?maindoc=/HOMA/index.php based on [143]) was used to estimate insulin resistance (HOMA IR) and insulin sensitivity (HOMA S).

Synaptic quantification on POMC neurons: Animals were deeply anesthetized then transcardially perfused with 50 ml of heparinized saline followed by 200 ml of fixation solution (4% paraformaldehyde 0.195% Picric acid and 0.1% glutaraldehyde in 0.1M phosphate buffer (PB, pH 7.4) and then brains processed for immunolabeling for POMC for subsequent electron microscopic examination. Ultrathin sections were cut on a Leica ultra microtome, collected on Formvar-coated single-slot grids and analyzed with a Tecnai 12 Biotwin (FEI Company) electron

microscope. The quantitative and qualitative analysis of synapse number was performed in an unbiased fashion as described earlier [94,144]. To obtain a complementary measure of axo-somatic synaptic number, unbiased for possible changes in synaptic size, the dissector technique was used. On consecutive 90-nm-thick sections we determined the average projected height of the synapses and used about 30% of this value as the distance between the dissectors. On the basis of this calculation, the number of axo-somatic synapses was counted in two consecutive serial sections about 270 nm apart ("reference" and "look-up" sections) of 7 perikarya profiles in each animal. Synapse characterization was performed at a magnification of 20,000. Symmetric and asymmetric synapses were counted on all selected neurons only if the pre- and/or postsynaptic membrane specializations were seen and synaptic vesicles were present in the presynaptic bouton. Synapses with neither clearly symmetric nor asymmetric membrane specializations were excluded from the assessment. The plasma membranes of selected cells were outlined on photomicrographs and their length was measured with the help of Scion image software (NIH). Plasma membrane length values measured in the individual animals were added and the total length was corrected to the magnification applied. Synaptic densities were evaluated according to the formula NV=Q-/Vdis where Q- represented the number of synapses present in the "reference" section that disappeared in the "look-up" section. Vdis is the dissector volume (volume of reference) which is the area of the perikarya profile multiplied by the distance between the upper faces of the reference and look-up sections, i.e., the data are expressed as numbers of synaptic contacts per unit volume of perikaryon. The synaptic counts were expressed as numbers of synapses on a membrane length unit of 100 µm. We analyzed 6 POMC immunolabelled neurons per animal (DIO-AL n = 6, DIO-WR n = 8, CON-AL n =7, and CON-WR n = 8)

Statistical analysis: Data are expressed as means ± SEM. Statistical analyses were performed using JMP (ver. 7; SAS, North Carolina). Where applicable, 2-way ANOVA's were conducted using diet (DIO or CON) and treatment (WR or AL) as grouping variables. To determine whether the relationship between circulating leptin and fat mass differed among treatment groups, within group regressions were performed relating leptin to FM (**Figure 2.1C**) and then re-analyzed by ANCOVA using group as a covariate for all groups wherein the relationship of leptin to FM was statistically significant, i.e., all groups except CON-WR. To ascertain that circulating leptin concentrations were reduced following weight loss, comparisons of absolute leptin concentrations were made between DIO-AL and DIO-WR and between CON-AL and CON-WR. To ascertain that any metabolic differences between DIO-WR and CON-AL groups were not due to lower circulating leptin concentrations in DIO-WR, a comparison of absolute circulating leptin concentrations was made between DIO-WR and CON-AL. Statistical significance was prospectively defined as $P\alpha<0.05$.

RESULTS

Body Weight & Body Composition

At the start of the weight-reduction phase of the study (day 0, mice aged 22 weeks), DIO mice weighed 54±3% more than *ad-libitum* fed CON mice and had significantly higher fractional body fat (DIO, 29±1%; CON, 5±1% fat) (**Figures 2.1** and **2.2A**). From days 0 to 183 of the weight reduction phase, both *ad-libitum*-fed groups (DIO-AL and CON-AL) gained a significant amount of body mass (**Figures 2.1** and **2.2A**). The increase in mass of both DIO-AL and CON-AL mice was primarily the result of increased fat mass (81±4% of weight increment in DIO-AL and 79±6% CON-AL). At time of sacrifice, DIO-AL body weight was 62±3% higher than CON-AL body weight; 75±3% of this excess weight was accounted for by increased FM. By design, caloric restriction (from day 0 to day 183) resulted in a 20% decrease in body weight in both DIO-WR and CON-WR groups. DIO-WR mice lost significant amounts of FM and FFM (FM accounted for 65±4% of weight loss), whereas CON-WR mice showed a significant decrease only in FFM (FFM accounted for 87±3% of lost weight). Weight and body composition of DIO-WR and CON-AL mice were not significantly different (**Table 2.1** and **Figure 2.2A** and **B**).

Energy Expenditure (TEE, REE, and NREE)

Absolute TEE and REE of DIO-AL-fed mice were significantly higher than in CON-AL (**Table 2.1**). While DIO-AL mice were heavier and fatter than CON-AL, the relationships between TEE and REE and body composition (FM and FFM) were not significantly affected by diet composition. Residuals for 24-hour TEE and of REE of WR mice were significantly below predicted ($p<0.001$; **Figure 2.3**) indicating that TEE and REE were reduced beyond what could

be attributed to changes in body mass and composition. Residuals were calculated based on actual values minus those predicted based on FFM and FM in all AL mice. However, similar results were obtained regardless of whether residuals were calculated based on FFM alone or FFM & leptin (data not shown). In addition, the absolute values of TEE in DIO-WR mice were significantly lower than in CON-AL mice despite the near-identity of body weight and body composition in these two groups (**Table 2.1).** REE of DIO-WR mice was 7.4±2.7% lower than predicted, accounting for 67% of the reduction in total 24-hour TEE (-0.7 kcal/day); in CON-WR mice, REE was 32.8±4.5% lower than predicted. This decrease of REE (-2.5 kcal/day) in CON-WR exceeded the decrease in TEE (-2.2 kcal/day). The difference of 0.3 kcal/day is accounted for by an increase in NREE (0.3 kcal/day) due to increased locomotor activity - probably related to food seeking behavior - as reflected in the measures of physical activity (see Physical **Activity** and **Figure 2.4A** and **2.4C).**

Non-resting energy expenditure (NREE = TEE - REE) of CON-AL mice was significantly higher than the two DIO groups (**Table 2.1**). When adjusted for body mass and composition, residuals of NREE for DIO-WR mice were significantly decreased (-0.3kcal below expected when adjusted for FFM and FM; $p<0.05$), while NREE residuals were significantly increased in the CON-WR group (+0.3kcal, $p<0.05$,).

Physical Activity

Total 24 hour physical activity (ambulatory movement), measured by the TSE infrared movement system, was highest in CON-WR and lowest in DIO-AL (**Figure 2.4A**); these were the only groups that were significantly different from one another ($p<0.05$) in this regard. DIO-WR mice and CON-AL mice had nearly identical 24h total activity (**Figure 2.4A**), yet DIO-WR

group had significantly lower NREE (2.9±0.1 units vs. 3.4±0.1 respectively; **Table 2.1**) indicating that the DIO-WR were expending approximately 15% less energy per unit of movement than CON-AL although some of this decrease in NREE may be attributable to decreased thermic effect of feeding (TEF) (see **Discussion**). Cumulative ambulatory activity rhythms (sum of every 14 minute measuring period; **Figures 2.4B** for DIO & **2.4C** for CON) over 48 hours show higher peaks of movement for WR mice, irrespective of diet, in the 1 hour period prior to AM and PM feeding times (see black bars on bottom of figures). Quantification of ambulatory activity in the 1 hour periods prior to feeding of WR mice showed that WR mice have higher levels of ambulatory activity than AL mice, probably as a result of increased food seeking behavior (see **Figure 2.4D**) [145].

Leptin

The key comparison is that of absolute circulating leptin concentrations in CON-AL and DIO-WR mice. If circulating leptin concentrations were significantly reduced in the DIO-WR mice compared to CON-AL mice, then the study would be biased towards our hypothesis: i.e. that DIO-WR mice will be hypometabolic and hypothyroid compared to CON-AL. In fact, the opposite was true and circulating leptin concentrations were significantly higher in DIO-WR mice compared to CON-AL (by t test comparison: **Table 2.2B**). There was no effect of diet composition on circulating leptin concentrations since the regression equations relating leptin to fat mass are almost identical between the DIO-WR and CON-AL groups (**Figure 2.1C**), and so the inter-group differences in circulating leptin concentrations are attributable to the higher fat mass of DIO-WR.

Overall, leptin concentrations were highly correlated with total fat mass (by NMR) at the start and end of the experiment (respectively, $r = 0.97$ & 0.93, both $p<0.001$; see **Figure 2.1B**;

(Data prior to weight stabilization of all 4 groups of animals are not shown). The best fit for the relationship of leptin to fat mass was non-linear ($r = 0.96$, $p < 0.0001$), suggesting that the relationship between leptin and fat mass might differ at extremes of adiposity. To determine whether there were differences among groups in the relationship of leptin to fat mass, regressions relating leptin to FM for each of the four groups were made (see **Figure 2.1C**). As reported by others [139,146], DIO mice showed a disproportionately greater increase in circulating leptin concentrations relative to fat mass (**Figure 2.1C**). Regression equations relating leptin to FM were almost identical between CON-AL and DIO-WR, indicating no significant effect of diet composition on this relationship. There was no significant correlation of leptin and fat-mass in the CON-WR animals which is similar to what has been reported in studies of leptin in humans with extremely low FM [147,148]. The lack of significant difference in absolute circulating leptin concentrations between CON-AL and CON-WR probably reflects the non-linearity of the relationship of leptin to FM in CON-WR animals.

Other Hormones and Metabolites

Prior to the start of the weight loss protocols, circulating glucose and insulin concentrations were all significantly higher in DIO mice than CON mice (**Table 2.2**). Weight reduction resulted in significant decreases in circulating insulin, T3, and glucose concentrations in DIO-WR compared to DIO-AL mice. T3 concentrations significantly decreased in the CON-WR compared to CON-AL mice. Weight reduction, irrespective of diet, significantly decreased circulating glucose concentrations and increased insulin sensitivity (HOMA2).

Synapses onto POMC neurons in the arcuate nucleus

Figure 2.5A and **B** are examples of electron microscopy images of POMC cell bodies with either asymmetrical/excitatory synapses (**Figure 2.5A –** large white arrows) or symmetrical/inhibitory synapses (**Figure 2.5B** – large black arrows). **Figure 2.5C** is a magnified section showing both asymmetrical/excitatory (large white arrow) and symmetrical/inhibitory synapses (large black arrow). Small black arrows point to the specialization below the postsynaptic density of the asymmetrical contact (**Figure 2.5A** and **C**). **Figure 2.5D** represents consecutive serial sections of the symmetrical contact shown on in **Figure 2.5B**. CON-AL mice had the highest excitatory/total synapse ratios of the 4 groups (**Figure 2.5E**) indicating a predominance of excitatory synapses over inhibitory ones in these animals during a period (ad-libitum feeding) of relative satiety. In the weight-reduced groups of CON and DIO mice, there was a similar decrease in this ratio (-52% in DIO-WR and -53% in CON-WR when compared to CON-AL mice; $p<0.01$). In these groups of demonstrably more hungry animals, inhibitory synapses dominated over excitatory ones. DIO-AL mice also had decreased ratios of excitatory/total synapses (37% below CON-AL), revealing a predominance of inhibitory inputs on POMC perikarya at the time of relative satiety. DIO-WR mice had lower ratios than DIO-AL, although this difference did not quite reach statistical significance ($p = 0.13$).

DISCUSSION

The major findings of this study are: 1.) As in humans, mice maintaining a reduced body weight (DIO-WR and CON-WR) show decreases in REE and TEE (adjusted for FM and FFM). Most notably, the DIO-WR animals "defend" a higher body weight following an extended period of diet-induced obesity. 2.) There are no significant differences in the relationships in TEE or REE and body weight/composition between CON-AL mice on a chow diet and DIO-AL maintaining an elevated body weight; 3.) Mice maintained at a reduced body weight – regardless of initial weight - have a significantly lower ratio of excitatory/total synapses onto POMC cell bodies than CON-AL fed animals, ratios that are similar to those observed in leptin deficient *Lep*^{ob} animals [94]. These changes are accompanied by increased *ad libitum* food intake – i.e. increased hunger and food seeking behavior (**Table 2.3** and **Figure 2.4 B, C, & D**) that has been documented in weight-reduced humans [149], mice [43], and rats [133,134,145,150]. The relative hypometabolism and decreased excitatory input into hypothalamic POMC neurons in DIO-WR mice compared to never-obese animals (CON-AL) with similar body composition and circulating leptin concentrations is consistent with the hypothesis that prolonged maintenance of an elevated body weight results in an upward "resetting" of the leptin threshold.

The magnitude of the decline in energy expenditure (both TEE and REE) following weight loss observed in the DIO-WR compared to the CON-AL in this study is similar to those seen in humans (36). Interestingly, non-resting energy expenditure (NREE) was lower in DIO-WR compared to CON-AL (2.9±0.1SEM vs. 3.4±0.1 kcal/day respectively) although they had similar body weights (**Table 2.1A**; 33.3±1.2 vs. 32.3±1.4 g, respectively) and total activity counts (**Figure 2.4A**) suggesting that the DIO-WR require less energy to accomplish similar amounts of

activity (i.e. their skeletal muscles may be more efficient). Such an effect is, in fact, observed in weight-reduced human subjects [19].

In relating rodent data to human studies, it is important to consider the differences in the fractional contributions to energy expenditure, behavioral changes as a result of weight loss, and even definitions of the different components of TEE. TEE of weight-reduced obese humans (who have lost 10% or 20% of their initial body weight) and never-obese humans (who have lost 10% of their initial body weight) - adjusted for body composition - is approximately 15% below that predicted by the losses of fat-free and fat mass [17]. In humans, most of this relative decline in energy expenditure is attributable to an increase in skeletal muscle work efficiency [19,22]. In mice, our estimates of energetic cost of locomotion suggest that there may be an increase in activity-related efficiency following weight loss, but its contribution to the overall decline in TEE remains unclear. Unlike humans, the major component of decreased TEE in WR mice is decreased REE rather than NREE.

Comparisons of rodent and human TEF data are complicated by different definitions. In humans, TEF refers specifically to the energy expended during digestion in a sedentary subject [17], while in rodents this term includes postprandial changes in energy expended in physical activity [151], which may in part account for the lower NREE observed in DIO-WR vs. CON-AL mice. TEF in mice accounts for a significantly greater fraction of TEE (>15%) than in humans (<10%) [17,151]. It is possible that changes in TEF, either due to the decreased caloric intake of WR animals or to an actual decline in the fraction of caloric intake utilized in digestion, accounts for some of the observed declines in TEE and NREE. Given the significant decline in circulating concentrations of T3 in WR animals, and the report that hypothyroidism, is associated with a

decrease in TEF in rats [152,153], it is possible that WR animals would expend a lower percentage of their ingested calories in TEF. However, studies of human subjects [17] and rats [151] who are being maintained at an approximately 10-15% reduced weight have reported no changes in TEF, suggesting that maintenance of a reduced body weight is not associated with a significant decline in TEF expressed as a fraction of caloric intake.

Studies in humans suggest that there is no remission of the relative hypometabolism that accompanies the chronic maintenance of a weight-reduced state [25]. Similarly, in rats, maintenance for 16 weeks of a stable lower body weight was accompanied by a persistent hypometabolic phenotype and hyperphagia, and weight regain once ad-libitum feeding was resumed [134]. This apparent "irreversibility" of the metabolic and behavioral consequences of sustained weight loss does not seem to occur following sustained weight gain; the data presented here suggest that prolonged elevation of body weight results in an upward "resetting" defended levels of energy stores.

In the present study, long-term (16 weeks) diet-induced obese mice (DIO-AL) mice were not hypermetabolic (adjusted TEE) when compared to CON-AL mice by ANCOVA or multivariate regression. In shorter term overfeeding studies in rats [154] and humans [17], 10-15% increases in adjusted TEE are observed. Our data suggest that over longer periods of time, energy expenditure in weight-gained individuals returns to levels (adjusted for body mass and composition) that are comparable to those of individuals maintaining their usual (pre-gain) body weight. Such an inference is supported by the fact that weight-stable obese and non-obese humans have comparable adjusted energy expenditures [17]. Unlike mice that return to their usual body weight after short-term overfeeding and are then eumetabolic compared to their never-

obese littermates, long term DIO mice who were weight-reduced (DIO-WR) are hypometabolic compared to both DIO-AL and CON-AL mice, but are metabolically similar to CON-WR mice (**Figure 2.3**). The reduction in energy expenditure in the weight-reduced DIO mice – to levels less than those of age, genotype and body mass/composition-matched CON-AL mice – is consistent with our hypothesis that sustained maintenance of an increased body weight results in an upward resetting of the "threshold" for minimum body fat. It might be argued that the decline in energy expenditure of the DIO-WR is related to CNS effects that are specific to the HFD. However, the same responses are seen in the CON-WR mice being fed a low fat diet, and high fat diets are certainly prevalent among human populations. Nevertheless, it would be interesting to examine the responses of DIO-WR to a lower fat diet in terms of energy intake and expenditure.

Relevant to this issue, others have found that DIO mice "settle" at higher body weights (i.e. increased adiposity) than never-obese animals when switched from an *ad-lib* HFD to an *ad-lib* chow diet [155] (personal communication, Dr. Silvia Corvera). Chow-fed formerly DIO rats also resist weight reduction when fed a hypocaloric diet by becoming hypometabolic (like non-DIO weight- reduced rats) [155]. Furthermore, mice that are switched from a high fat diet to a low fat diet and then back to the high fat diet readily regain weight to levels similar before the diet switch [156].

Epidemiological observations of the increasing prevalence of obesity in humans [129,157,158] and long-term difficulties in sustaining even mild degrees of weight loss, suggest that the threshold for the minimal body weight that is metabolically defended may be elevated via maintenance of greater adiposity for prolonged periods of time and/or at specific time-points

during development. In both humans and rodents, weight reduction results in decreased concentrations of circulating leptin, T3, and insulin. In the present study, as expected, we detected significant effects of treatment (AL or WR) on insulin sensitivity as reflected by HOMA2 [143] : CON-WR HOMA2 was 197% higher than CON-AL, and DIO-WR was 241% higher than DIO-AL; $p<0.05$. Circulating concentrations of leptin are closely proportional to body fat mass in weight-stable mice and humans[159,160]. Leptin's capacity to reverse metabolic phenotypes seen in both rodents and humans following weight loss and/or during caloric restriction, and its effects on energy homeostatic processes in the brain, renders it a prime candidate as a mediator of metabolic adaptation under conditions of decreased somatic energy stores and/or negative energy balance [22,43,161]. The higher circulating leptin concentrations relative to fat mass in DIO-AL mice, and the loss of linearity in the relationship of leptin to fat mass in CON-WR mice are consistent with other studies of weight maintenance in rodents following overfeeding and underfeeding [146,162]. A non-linear regression analysis improved the leptin to FM ratio ($r^{2 =} 0.96$, $p < 0.0001$) when including all groups because of DIO-AL increased production of leptin per unit fat mass (see slope of linear regression in **Figure 2.1C**). The differences in the relationship of leptin to fat mass during weight maintenance following extreme weight loss or gain are less pronounced than the striking decreases or increases in the ratio of leptin to fat mass observed in humans [127] and rodents [159] during dynamic weight loss or gain, respectively. The observation that the relationship between leptin and fat mass was not different between DIO-WR and CON-AL groups in this study (i.e. that they fell on similar regression lines: **Figure 2.1C**) is an indication that these animals were, in fact, in similar states of energy balance. Since the regression of leptin on fat mass has a "non-zero Y-axis intercept", the ratios of leptin to FM, as used by some laboratories, are not appropriate for assessing "leptin

sufficiency/insufficiency" as they reflect solely the slope but not the intercept of the relationship between the variables (see **Figure 2.1C** for individual group regressions). Similar considerations dictate our use of multivariate regression to assess energy expenditure related to both FFM and FM as opposed to using ratios of TEE/FFM [163].

Thyroid hormone concentrations in blood correlate with energy expenditure by mechanisms that are not fully understood[164,165]. T3 is increased during overfeeding [23,166] and reduced during underfeeding and/or weight loss [23]. Serum T3 in the CON-WR was decreased by 51% and by 47% in DIO-WR mice compared to their respective AL controls (**Table 2.2**). These changes in T3 concentrations following weight loss are similar to those noted in humans [23].

Chronic changes in leptin signaling have been associated with structural changes in the hypothalamus[94]. These are plausible neural substrates for the consequent attenuations in energy intake and expenditure[167]. Leptin deficient *Lep*^{ob} mice had decreased ratios of excitatory/total synapses onto POMC arcuate neurons when compared to wild type mice and exogenous leptin or estrogen normalized this phenotype [94,144]. In the DIO-WR and CON-WR mice we observed ratios of excitatory/total synapses onto POMC neurons that were 52% and 53% below those in ad-lib animals and comparable to those observed in the leptin deficient *Lep*^{ob} mice[94]. We have previously shown that the excitatory/total synaptic ratio positively correlates with POMC mRNA expression[94,168]. The comparability of these changes in DIO-WR and CON-WR animals supports our inference that the DIO-WR animals are now "defending" a higher level of body fat, and that the reduced excitatory/total synapses onto POMC neurons constitute a "signature" of relative leptin deficiency. Consistent with the reduced excitatory tone in POMC neurons in the weight reduced state, POMC mRNA in the arcuate is reduced in chronically food restricted rats [150], and

restored to fed levels by exogenous leptin. It is possible that opposite changes in orexigenic/anabolic NPY/AgRP neurons contributes to the phenotype [141]. If these structural differences have functional consequences – a likely possibility given the physiology of leptin signaling in POMC neurons – the bioenergetics and endocrine profiles of the animals in this study could be accounted for[13]. The bioenergetic/neuroendocrine, behavioral, and fMRI responses of weight-reduced humans to low dose exogenous leptin are consistent with this inference[100,169]. The DIO-AL animals had lower excitatory/total synapse ratios than CON-AL, possibly reflecting effects of diet composition, the obese phenotype (i.e. increased leptin levels), or both. Feeding mice a high fat diet reduces apparent arcuate leptin sensitivity as early as 6 days after switching to high fat diet[170]. Enriori *et al.* showed that decreases in leptin responsiveness in the arcuate nucleus following diet-induced obesity could be reversed by decreasing the fat content of the diet[171]. These effects may account for the smaller difference in excitatory/total synapse ratios observed between DIO-AL and DIO-WR ratios. There may be a "floor" to this ratio.

PERSPECTIVES AND SIGNIFICANCE

These data suggest that prolonged maintenance of an acquired elevation in body weight induces changes in energy homeostatic systems that lead to "defense" of a body weight higher than that dictated by genetic/developmental status of the animal. Structural changes in the arcuate nucleus (and elsewhere) may play a role in upward resetting of defended body weight[94,167,172]. Comparable processes, if present in humans, could account for some aspects of the secular trend to increasing obesity that clearly cannot be attributed to intercurrent genetic change. Understanding the neuro-biological predicates of such an acquired upward resetting of the minimum defended level of adiposity "threshold" could provide novel approaches to the prevention and treatment of obesity. For instance, the observations presented here suggest that pharmacological elevation of melanocortinergic tone may be particularly effective in the prevention of weight regain in formerly obese subjects. These studies are also consonant with studied in humans indicating that the neurobiological responses to maintenance of a reduced body weight do not accommodate over time; i.e. that the physiology of the weight-reduced state persists.

Table 2.1.	DIO-AL (n=7)	DIO-WR (n=8)	CON-AL (n=8)	CON-WR (n=8)
	Indirect calorimetry measurements (days 132-144)			
Body Weight (g)	49.6±1.2 [a]	33.3±1.2 [b]	32.3±1.4 [b]	21.7±0.5 [c]
Fat-Free Mass (FFM, g)	23.5±0.6 [a]	20.0±0.3 [b]	20.3±0.3 [b]	15.0±0.3 [c]
Fat Mass (FM, g)	17.9±0.8 [a]	6.7±0.7 [b]	5.4±0.9 [b]	1.7±0.1 [c]
Food Intake (g/day)	3.1±0.1 [b]	2.2±0.0 [c]	3.4±0.1 [a]	2.3±0.1 [c]
MEI (kcal/day)	16.1±0.6 [a]	11.3±0.2 [c]	13.1±0.3 [b]	8.7±0.2 [d]
24-hour energy expenditure (TEE, kcal/day)	14.2±0.4 [a]	11.4±0.2 [c]	12.3±0.3 [b]	8.2±0.3 [d]
Resting energy expenditure (REE, kcal/day)	11.4±0.3[a]	8.5±0.3 [b]	9.0±0.3 [b]	5.2±0.4 [d]
Non-resting energy expenditure (NREE, kcal/day)	2.9±0.2 [b]	2.9±0.1 [b]	3.4±0.1 [a]	3.0±0.2 [a,b]
Respiratory Quotient (RQ)	0.72±0.01[b]	0.71±0.01[b]	0.87±0.02[a]	0.85±0.01[a]

TABLE 2.1: BODY WEIGHT, BODY COMPOSITION, FOOD INTAKE AND ENERGY EXPENDITURE
Mean (± S.E.). MEI is metabolizable energy intake calculated by multiplying food weight in grams by caloric density of respective diet (CON – 3.85 kcal/gram: DIO – 5.24 kcal/gram). Data for any variable not marked by same letter are significantly different by two way ANOVA with Tukey post-hoc analysis.

Table 2.2.	**A. Data obtained during initial bleed – day 0 (Figure 2.1)**			
	DIO (n=15)		**CON (n=16)**	
Leptin (ng/ml)	74.4 ± 6.5[a]		4.8 ± 1.6[b]	
Insulin (ug/l)	3.7 ± 0.5[a]		0.8 ± 0.1[b]	
Glucose (mg/dl)	153.7 ± 3.8[a]		121.7 ± 4.0[b]	
T3 (ng/dl)	60.8 ± 8.8[a]		19.9 ± 2.8[b]	
T4 (ng/ml)	55.9 ± 2.0[a]		48.2 ± 1.2[b]	
TSH (ng/ml)	205.3 ± 32.5		170.2 ± 22.9	
	B. Data obtained during terminal bleed – days 173-179 (Fig 1)			
	DIO-AL (n=7)	**DIO-WR (n=8)**	**CON-AL (n=8)**	**CON-WR (n=8)**
Leptin (ng/ml)	121.7 ± 14.9[a]	**25.1 ± 2.9[b]**	**14.0 ± 3.6[b*]**	**9.0 ± 1.3[b*]**
Insulin (ug/l)	3.7 ± 0.7[a]	1.6 ± 0.2[b]	1.2 ± 0.2[b]	0.6 ± 0.1[b]
Glucose (mg/dl)	125 ± 3[a]	105 ± 3[b]	110 ± 3[b]	94 ± 2[c]
T3 (ng/dl)	31.6 ± 5.3[a]	16.8 ± 1.8[b]	31.8 ± 8.1[a]	15.7 ± 4.4[b]
T4 (ng/ml)	45.8 ± 4	49.9 ± 2	46.1 ± 2.2	50.6 ± 2.6
TSH (ng/ml)	283.1 ± 30.5	252.8 ± 35.5	221.7 ± 52.7	226.8 ± 42.6
HOMA2 S	10.1 ± 1.2[a]	24.3 ± 4.3[ab]	30.6 ± 4.5[b]	60.2 ± 11.2[c]
HOMA2 IR	11.1 ± 1.8[a]	4.9 ± 0.7[b]	3.8 ± 0.6[bc]	2.0 ± 0.3[c]

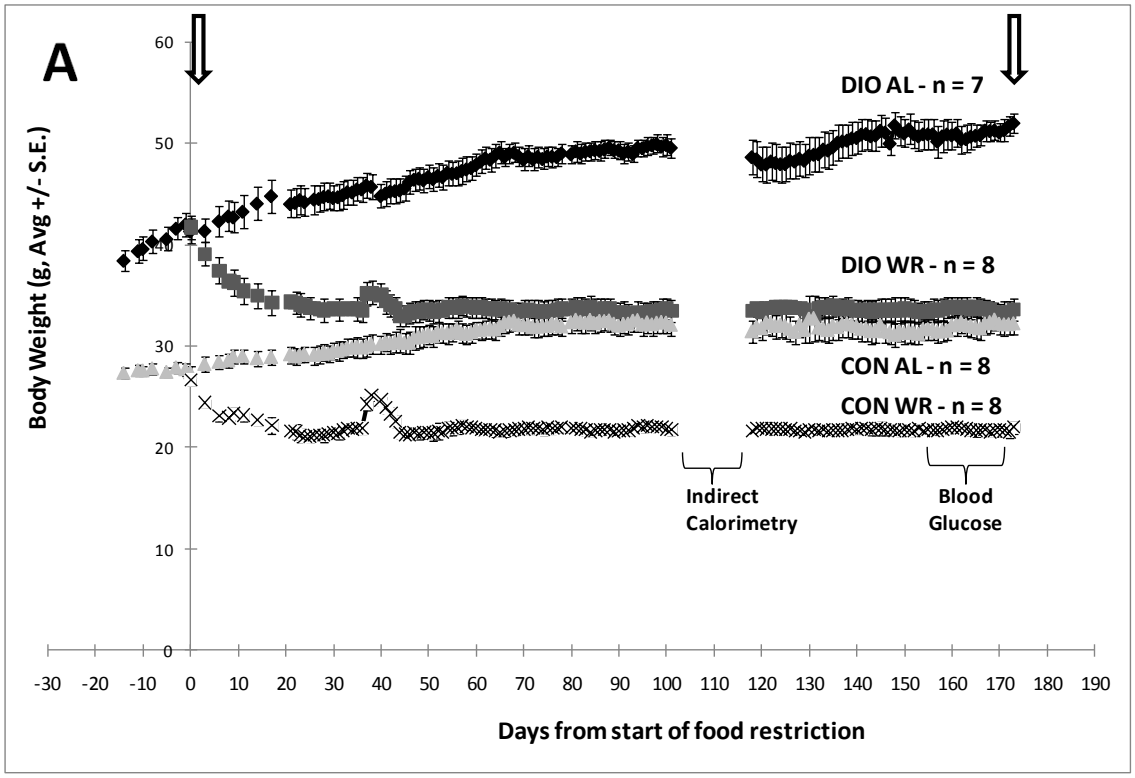
TABLE 2.2: SERUM HORMONES AND METABOLITES AT INITIAL AND SACRIFICE BLEEDS

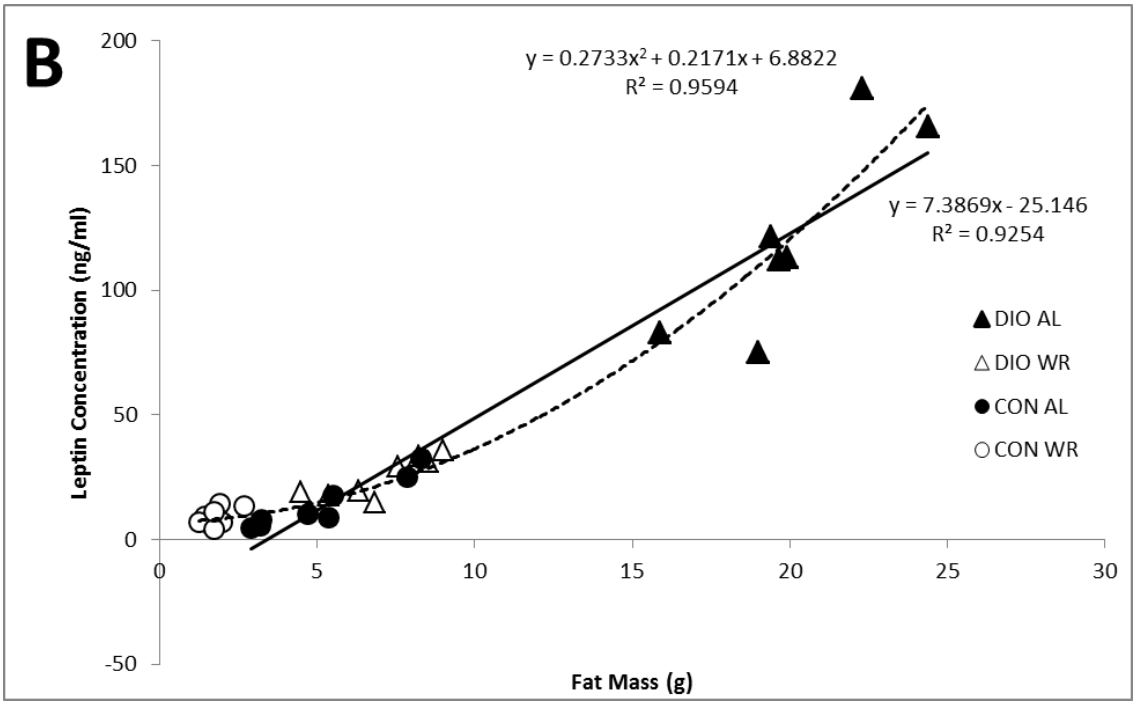
Mean (± S.E.) Data not marked by same letter are significantly different by two way ANOVA with Tukey post-hoc analysis. T-test comparisons of CON-AL and CON-WR against DIO-WR are marked by * ($p < 0.05$).

Table 2.3	Food Intake (g/24h)	Metabolizable Energy Intake (kcal/24h)
DIO-AL (n=7)	3.5±0.1*	18.5±0.6*
DIO-WR (n=12)	5.2±0.2	27.0±1.0

TABLE 2.3: FOOD INTAKE AND METABOLIZABLE ENERGY INTAKE

Food intake (g/24h) and metabolizable energy intake (kcal/24h) in DIO-AL and DIO-WR during first 24 hours after DIO-WR mice were given *ad-libitum* access to high fat diet (60% kcal from fat). Group means were compared by Student's t test (* $p < 0.001$).


A
Body Weight (g, Avg +/- S.E.)
DIO AL - n = 7
DIO WR - n = 8
CON AL - n = 8
CON WR - n = 8
Indirect Calorimetry
Blood Glucose
Days from start of food restriction


B
y = 0.2733x² + 0.2171x + 6.8822
R² = 0.9594
y = 7.3869x - 25.146
R² = 0.9254
DIO AL
DIO WR
CON AL
CON WR
Leptin Concentration (ng/ml)
Fat Mass (g)

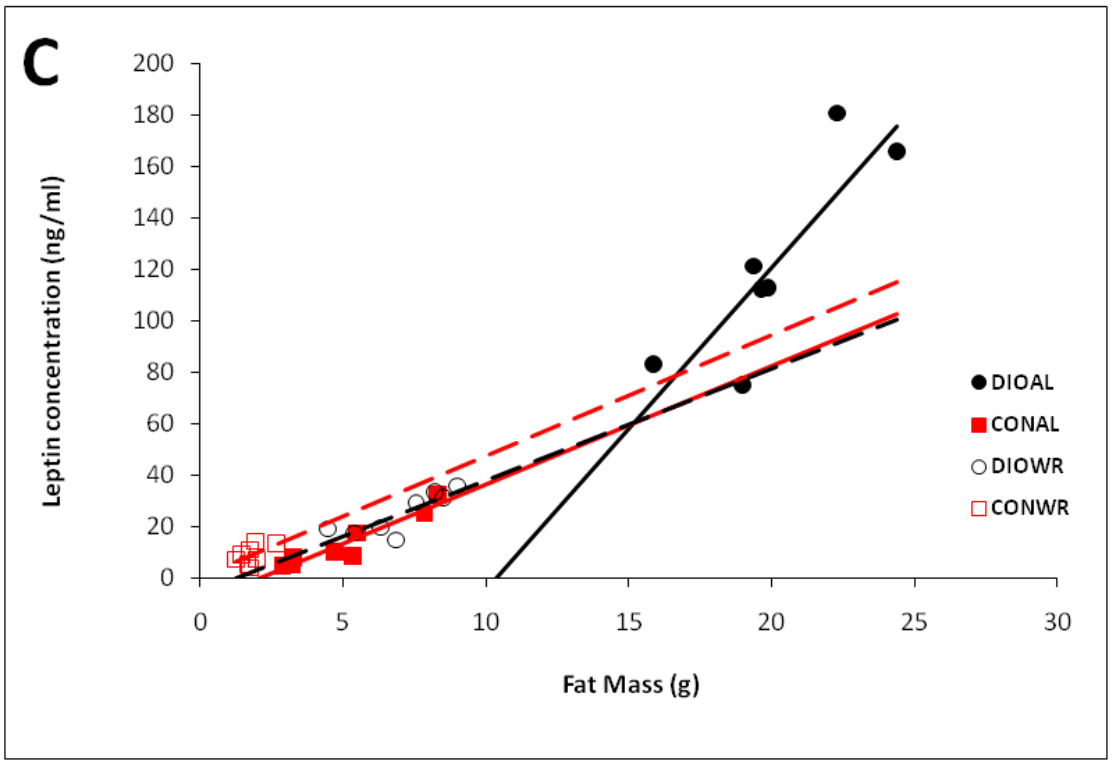


FIGURE 2.1: BODY WEIGHT, AND LEPTIN CONCENTRATIONS IN WEIGHT PERTURBED WT MICE

A. Mean (±S.E.) body weight (grams) of 4 groups of 22 week-old mice from start of food restriction protocol. Arrows represent 4 hour fasted bleeding. Small upward inflections in body weights of DIO-WR and CON-WR mice (days 36-43) was due to a batch of corn cob-based bedding that contained corn kernels that was resolved by switching mice to wood-based bedding material on day 39. **B.** Leptin (ng/ml) to fat mass (g). Linear regression using DIO-AL and CON-AL mice; solid line. Non-linear regression using all mice groups; dashed line. **C.** Relationship between leptin and fat mass in DIO-AL (●, black solid line), DIO-WR (○, black dashed line), CON-AL (■, red solid line), and CON-WR (□, red dashed line) animals. DIOAL: Leptin =12.6 (FM) - 130.3, r=0.85, p=0.014 ; DIOWR: Leptin = 4.3 (FM) - 5.4, r=0.84, p=0.009 ; CONAL: Leptin = 4.6 (FM) - 9.8, r=0.95, p=0.0003; CONWR: Leptin= 4.7 (FM) + 0.4, r=0.53, p=0.17.

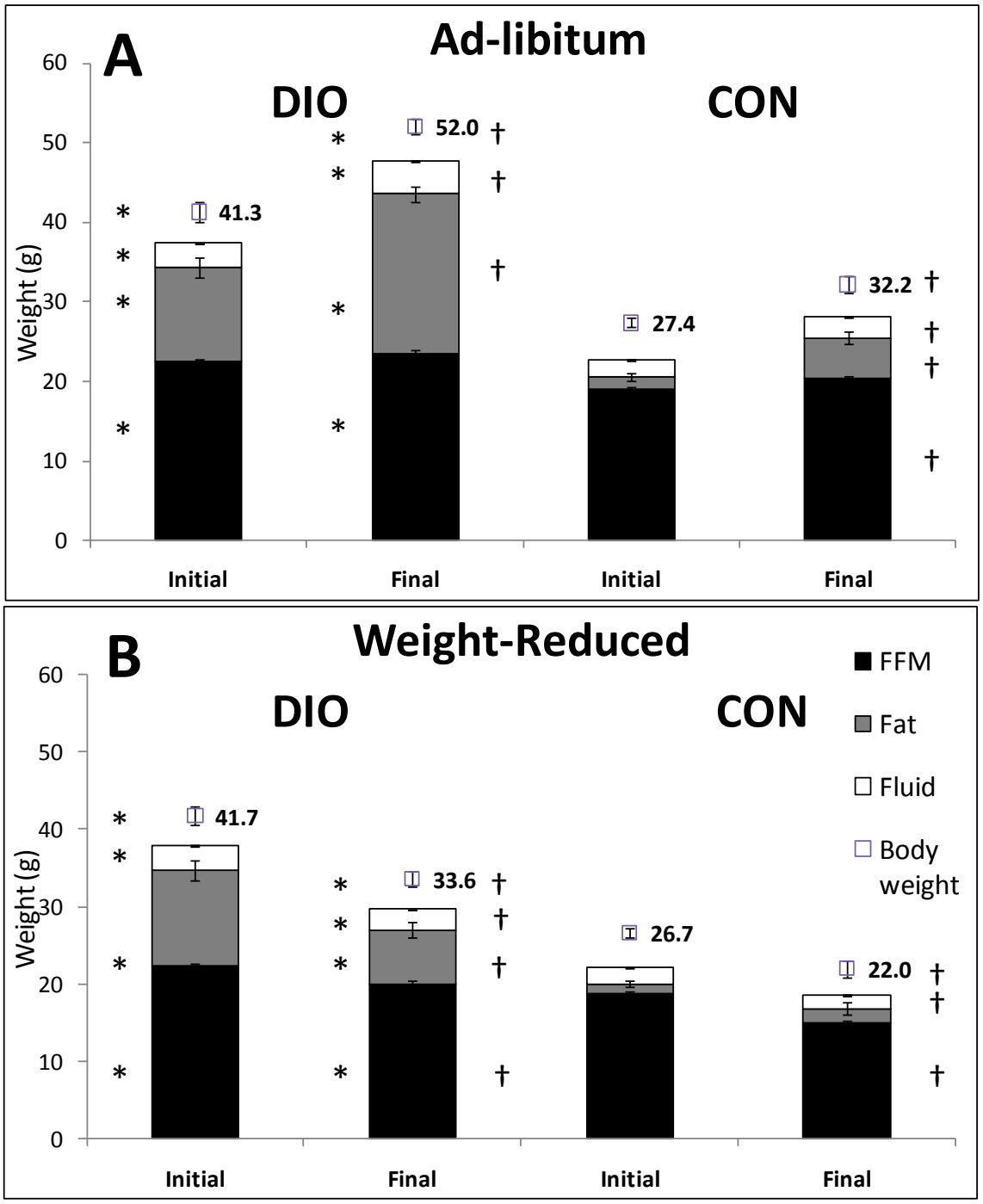


FIGURE 2.2: BODY COMPOSITION IN WEIGHT PERTURBED WT MICE

Mean (±S.E.) body weight and body composition (grams, fluid is defined as extracellular fluids) of DIO and CON mice either ad libitum fed (A) or weight-reduced mice (B) at initial (day 0) vs. termination of experiment. n=8 in all experimental groups except DIO-AL, n=7. *P < 0.001, t-test vs.CON. at same time point, † P < 0.001, paired t-test vs. initial .

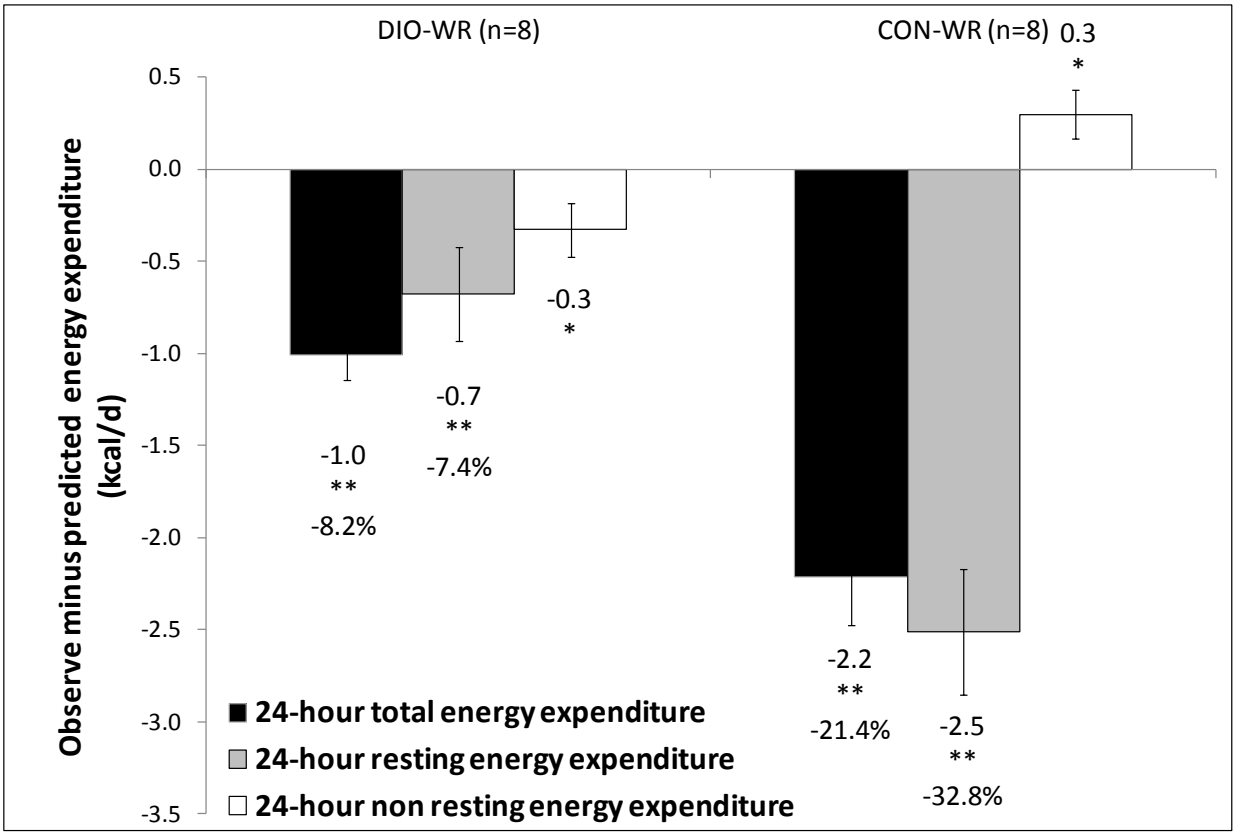
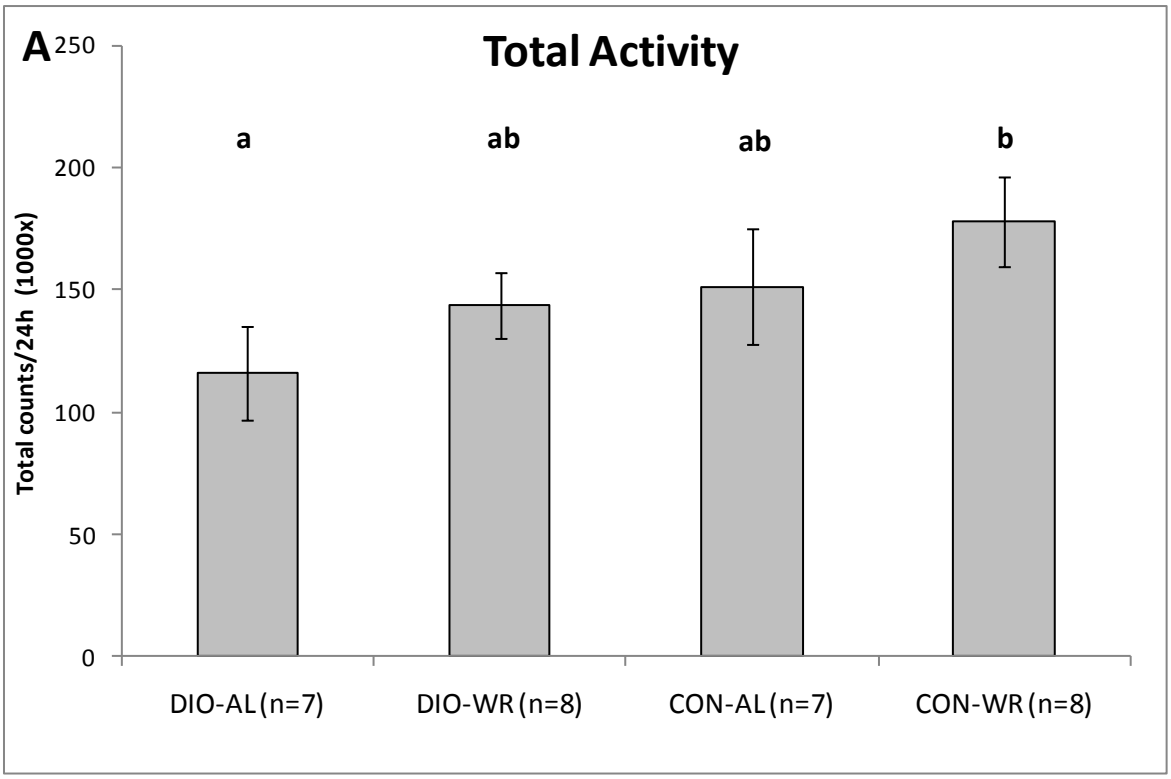
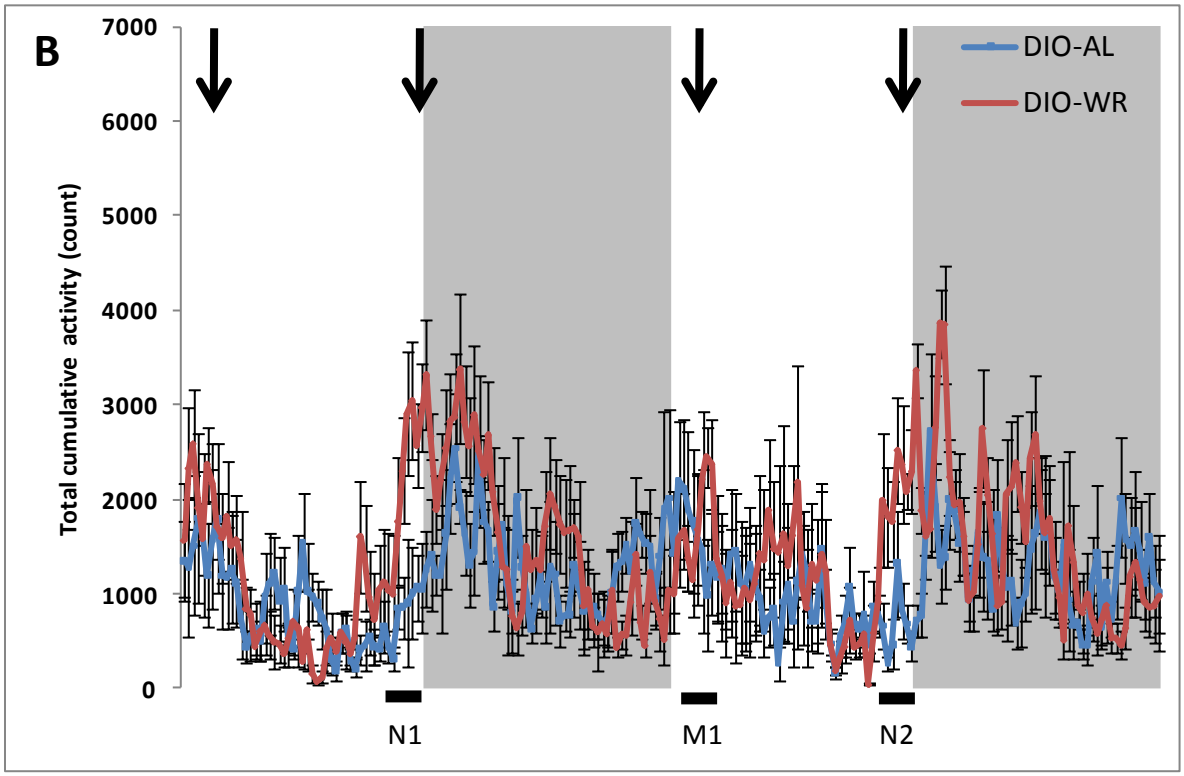
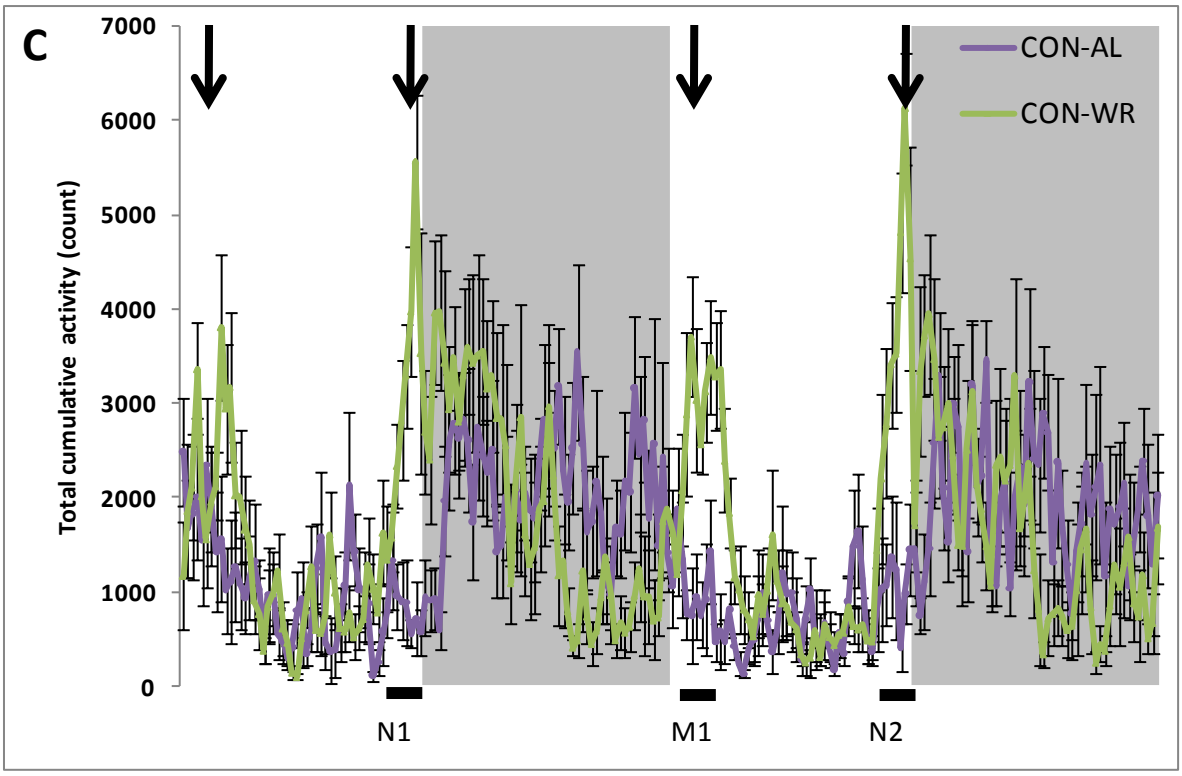


FIGURE 2.3: ENERGY EXPENDITURE PHENOTYPES IN WEIGHT PERTURBED WT MICE
Mean (±S.E.) Observed-minus-Predicted 24-hour total (black bars), 24-hour resting (grey bars) and non-resting (white bars) energy expenditure (kcal/24hr). Predicted values obtained by multivariate regressions relating 24-hour total (TEE = 5.16 + 0.34 * FFM + 0.06 * FM), 24-hour resting (REE = 4.96 + 0.17 * FFM + 0.14 * FM) or non-resting (NREE = 0.20 + 0.18 * FFM - 0.08 * FM) energy expenditure with FFM and FM of ad lib fed mice (DIO-AL + CON-AL). *$p < 0.05$, **$p < 0.01$, T-test of residuals vs. 0.

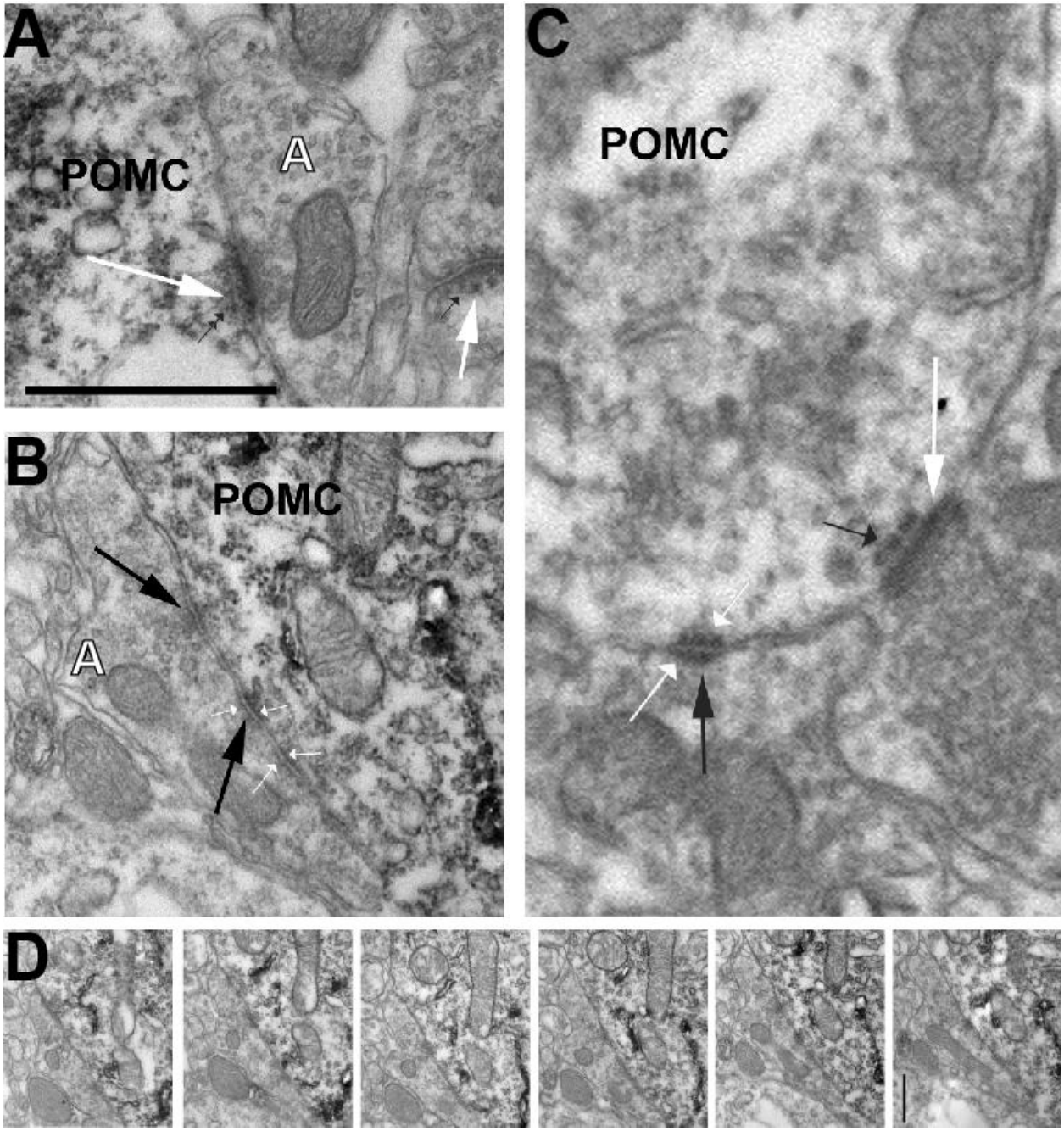

A
Total Activity
Total counts/24h (1000x)
250
200
150
100
50
0
a
ab
ab
b
DIO-AL (n=7)
DIO-WR (n=8)
CON-AL (n=7)
CON-WR (n=8)


B
Total cumulative activity (count)
7000
6000
5000
4000
3000
2000
1000
0
DIO-AL
DIO-WR
N1
M1
N2



D	DIO-AL (n=7)	DIO-WR (n=8)	CON-AL (n=8)	CON-WR (n=8)
N1 = Night feed 1	4.9±1.6 [a]	13.8±2.3 [b]	3.6±1.1 [a]	19.2±2.6 [b]
M1 = Morning feed 1	8.3±3.5 [a]	11.3±3.6 [a, b]	5.3±1.4 [a]	19.2±2.5 [b]
N2 = Night feed 2	4.5±0.7 [a]	14.4±2.1 [b]	6.9±2.2 [a]	24.1±2.1 [c]

FIGURE 2.4: MEAN ACTIVITY AND FOOD INTAKE IN WEIGHT PERTURBED WT MICE:
A. Mean (±S.E.) 24hr ambulatory movement beam breaks (sequential breaking of two adjacent x or y beams). B & C. Total cumulative ambulatory activity for each 14 minute period. N = night; M = morning. Black bars at bottom are 1h periods prior to feeding WR mice that are quantified in D. D. Cumulative ambulatory activity for 1h time period prior to feeding of WR mice (1000x beam breaks ±S.E.). Data not marked by same letter are significantly different by two way ANOVA with Tukey post-hoc analysis.


A
POMC
A
B
POMC
A
C
POMC
D

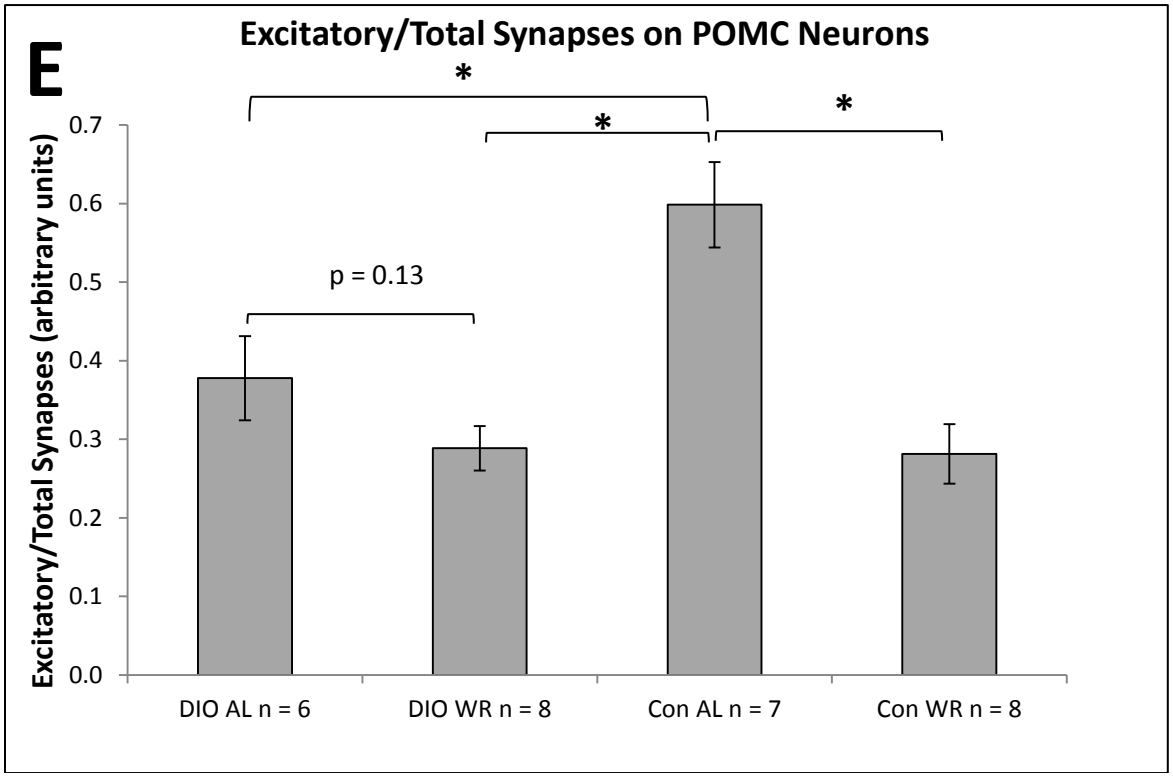


FIGURE 2.5: ELECTRON MICROSCOPY AND EXCITATORY/TOTAL SYNAPSES ON POMC NEURONS IN WEIGHT PERTURBED WT MICE

A. Sample of EM image showing asymmetrical/excitatory connections (large white arrows) onto POMC cell body and unlabeled dendritic spine. Small black arrows point to an electron dense band below the postsynaptic membrane specialization, a characteristic sign of asymmetrical, excitatory synapses. B. Sample of EM image showing a symmetrical/inhibitory connection onto POMC cell body (large black arrows). Small white arrows point to both pre-and postsynaptic thickening of the membranes typical of symmetrical synapses. C. An electron micrograph showing an asymmetrical (large white arrows) contact and a symmetrical contact (large black arrows) on a POMC-immunolabeled (light immunoperoxidase in cytosol) dendrite. Small black arrows point to the specialization below the postsynaptic density of the asymmetrical contact. D. Consecutive serial sections of the symmetrical contact shown on panel B. Note that no electron dense band (a characteristic of asymmetrical, stimulatory synapses) ever appears below the postsynaptic density. E. Mean ratio excitatory/total synapses on POMC cell bodies in the arcuate nucleus (arbitrary units±SEM): *$p<0.05$. White A in panel A and B mark axon terminals. Bar scale on A represents 1 μm for panels A and B and 0.5 μm for panel C.

CHAPTER 3: RESPONSES OF GUT MICROBIOTA TO DIET COMPOSITION AND WEIGHT LOSS IN LEAN AND OBESE MICE

ABSTRACT

Maintenance of a reduced body weight is accompanied by a decrease in energy expenditure beyond that accounted for by reduced body mass and composition, as well as by an increased drive to eat. These effects appear to be due – in part – to reductions in circulating leptin concentrations due to loss of body fat. Gut microbiota have been implicated in the regulation of body weight. The effects of weight loss on qualitative aspects of gut microbiota have been studied in humans and mice, but these studies have been confounded by concurrent changes in diet composition, which influence microbial community composition. We studied the impact of 20% weight loss on the microbiota of diet-induced obese (DIO: 60% calories fat) mice on a high-fat diet. Weight-reduced DIO (DIO-WR) mice had the same body weight and composition as control (CON) *ad-libitum* (AL) fed mice being fed a control diet (10% calories fat), allowing a direct comparison of diet and weight-perturbation effects. Microbial community composition was assessed by pyrosequencing 16S rRNA genes derived from the ceca of sacrificed animals. There was a strong effect of diet composition on the diversity and composition of the microbiota. The relative abundance of specific members of the microbiota was correlated with circulating leptin concentrations and gene expression levels of inflammation markers in subcutaneous white adipose tissue in all mice. Together, these results suggest that both host adiposity and diet composition impact microbiota

composition, possibly through leptin-mediated regulation of mucus production and/or inflammatory processes that alter the gut habitat.

INTRODUCTION

Interactions between modern environments and strong biological mechanisms favoring energy storage have contributed to a dramatic increase in the prevalence of obesity over the past three decades [173]. In humans and rodents, responses to weight reduction include reduced energy expenditure per unit of metabolic mass and increased hunger [19,100,104,134] ([124]). These responses favor recidivism to obesity [17].

Recent studies in rodents and humans implicate gut microbiota in energy homeostasis (reviewed in [118]). Sequence-based studies have highlighted differences in gut microbial community composition between obese and lean humans [119,120] and mice [121,122]. Altered gut microbial communities can impact host body weight in several ways. For example, compared to lean animals, mice rendered obese either by a high-fat diet (HFD) or by leptin deficiency (*ob/ob*), harbor a gut microbiota enriched in the phylum Firmicutes, and depleted in Bacteroidetes [120-122]. Metagenomic and biochemical analyses and microbiota transplantation experiments indicate that the obesity-associated microbiota has an enhanced ability to extract energy from a given diet [121,123]. In this context, "extraction" means an increased amount of short chain fatty acids (a bi-product of bacterial catabolism of dietary fiber; non-starch polysaccharides and other plant components) in the cecum and decreased fecal gross energy content (measured by bomb calorimetry) indicative of increased absorption of short chain fatty acids by the host [121]. Finally, specific microbiota may trigger low grade inflammation that reduces insulin sensitivity and may affect body weight by reducing neuronal (e.g. hypothalamic) sensitivity to circulating hormones such as leptin and insulin [174,175].

Turnbaugh et al. reported strong effects of a HFD on the composition of the microbiota in mice [123]. Since the switch to a HFD resulted in host weight gain, it is unclear if alterations in the gut microbiota were due to dietary changes, to host adiposity, or to interactions between diet and adiposity. To show that a HFD *per se* can cause an alteration in the microbiota, Hildebrandt and colleagues used RELMβ KO mice that become only slightly overweight when fed a HFD [176], yet still have significantly higher body weight and body fat content than low fat fed WT mice.

In the studies reported here we examined the effects of weight loss on the gut microbiota in the context of high and low fat diets (60% and 10% of calories derived from fat, respectively), while controlling for body weight. We compared the microbiotas of four groups of C57BL/6J mice: diet-induced obese mice (DIO-AL) and control (10% fat) diet-fed mice (CON-AL) given *ad-libitum* access to these diets, and mice weight-reduced to 20% below initial weight (DIO-WR and CON-WR, respectively). The DIO-WR mice had body weights and body compositions similar to those of the CON-AL mice. This design allowed us to: I. Compare diet effects on gut microbial community composition independent of body weight (DIO-WR vs. CON-AL); II. Compare the effects of weight loss in both lean and obese mice (DIO-WR vs. CON-WR); and III. Assess correlations between circulating leptin concentrations, inflammation marker expression levels in white adipose tissue, and the relative abundance of various gut bacteria.

MATERIALS AND METHODS

Animals:

The animals used in this study are described in detail in Ravussin et al. 2010 (in review [124]. Thirty-two 18-week old C57BL/6J-male mice were obtained from the Jackson Laboratory (Bar Harbor, ME); 16 (DIO) had been fed Research Diets, Inc. D12492i (60% kcal fat, 20% kcal protein), and 16 (CON) had been fed Research Diets, Inc. D12450Bi (10% kcal fat, 20% kcal protein) from 6 weeks of age. Mice were individually housed upon arrival. Animals from both diet groups were randomized to remain on the *ad-lib* diets (AL) or to be calorically restricted to decrease their body weight by 20% over a 1-2 week period by twice daily feeding of reduced (50%) quantities of their respective diets. The feeding regimen was then altered to keep each individual mouse weight stable 20% below their initial weight (WR). This reduced weight was maintained for 23 additional weeks to avoid "carryover" effects of the negative energy balance state required for weight loss, and to permit additional physiological analyses not reported here. All mice had *ad-libitum* access to water containing no bacterostatic agents throughout the entire experiment. Fat mass and fat free mass were assessed by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany). Mice were sacrificed after a 4h fast during deep anesthesia. The cecum (among other organs) was removed from each mouse. Cecal content was aseptically removed, flash frozen, and stored at -80°C until processing. The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Inflammation Markers

qRT-PCR in Inguinal White Adipose Tissue

Inguinal fat pads were removed, flash frozen in liquid N_2, and stored at -80ºC. RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) (including the DNAse purification step) and reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN) using random primers. To quantify transcript levels in the various organs, qRT-PCR was performed on a Roche 480 LightCycler using Syber green (Roche, Indianapolis IN) and normalized to cyclophilin b and presented as arbitrary units. Primers were as follows: *Saa3* forward: AGCGATGCCAGAGAGGCTGTTC, reverse: AGCAGGTCGGAAGTGGTTGG; Pai1 forward: TCCTCATCCTGCCTAAGTTCTC, reverse: GTGCCGCDCTCGTTTACCTC; *F4/80* forward: CTTTGGCTATGGGCTTCCAGTC, reverse: GCAAGGAGGACAGAGTTTATCGTG; *Slc25a* forward: GGGTGTCAAGATCTCGGAACA, reverse: GTAGTCCCTCCACTCGTTCCA; *Angptl4* forward: TTCCAACGCCACCCACTTACA, reverse: ACCAAACCACCAGCCACCAGA; *Tnfα*, forward: CCAGACCCTCACTAGATCA, reverse: CACTTGGTGGTTTGCTACGAC; *Il10*, forward: GCTCTTACTGACTGGCATGAG, reverse: CGCAGCTCTAGGAGCATGTG; *DioII*, forward: GCTGCGCTGTGTCTGGAA, reverse: TGGAATTGGGAGCATCTTCAC; *iNos*, forward: AATCTTGGAGCGAGTTGTGG, reverse: CAGGAAGTAGGTGAGGGCTTG, *Cd11c*, forward: CCTACTTTGGGGCATCTCTTTG, reverse: GCACCTCTGTTCTCCTCCTCTC.

Leptin Assay:

Following a 4h fast on the day of sacrifice, mice were bled retro-orbitally. Blood for leptin assays was allowed to clot for 1 hour at room temperature, centrifuged 10 min. at 1000 x g at 4°C, and serum was collected and frozen at -80°C until time of assay. Leptin was assayed using the Quantikine ELISA kit (R&D Systems, Minneapolis, MN).

Fecal DNA extraction

Frozen fecal samples from the cecum were ground under liquid N_2; a subsample of ~100mg was used for whole community DNA extraction [122]: A 100mg aliquot of each homogenized sample was suspended while frozen in a solution containing 500µl of DNA extraction buffer [200mM Tris (pH 8.0), 200mM NaCl, 20mM EDTA], 210µl of 20% SDS, 500µl of a mixture of phenol:chloroform:isoamyl alcohol (25:24:1)], and 500µl of a slurry of 0.1-mm-diameter zirconia/silica beads (BioSpec Products, Bartlesville, OK). Microbial cells were then lysed by mechanical disruption with a bead beater (BioSpec Products) set on high for 2 min (22°C), followed by extraction with phenol:chloroform:isoamyl alcohol, and precipitation with isopropanol. The quantity and quality of purified DNA was assessed using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Eugene, OR) and a plate reader.

16S rRNA gene PCR amplification and sequencing

16S rRNA genes were amplified from each sample using a composite forward primer and a reverse primer containing a unique 12-base barcode [177] which was used to tag PCR products from respective samples [178]. We used the forward primer 5'-

*GCCTTGCCAGCCCGCTCAGTC***AGAGTTTGATCCTGGCTCAG**-3': the italicized sequence is 454 Life Sciences® primer B, and the bold sequence is the broadly conserved bacterial primer 27F. The reverse primer used was 5'-*GCCTCCCTCGCGCCATCAGNNNNNNNNNNNNCA***TGCTGCCTCCCGTAGGAGT**-3': the italicized sequence is 454 Life Sciences' primer A, and the bold sequence is the broad- range bacterial primer 338R. *NNNNNNNNNNNN* designates the unique 12-base barcode used to tag each PCR product, with 'CA' inserted as a linker between the barcode and rRNA gene primer. PCR reactions consisted of HotMaster PCR mix (Eppendorf), 200 µM of each primer, 10-100ng template, and reaction conditions were 2 min at 95°C, followed by 30 cycles of 20s at 95°C, 20s at 52°C and 60s at 65°C on an Eppendorf thermocycler. Three independent PCRs were performed for each sample, combined and purified with Ampure magnetic purification beads (Agencourt Bioscience Corp., Beverly, MA), and products visualized by gel electrophoresis. No-template extraction controls were analyzed for absence of visible PCR products. Products were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen). A master DNA pool was generated from the purified products in equimolar ratios to a final concentration of 21.5 ng mL^{-1}. The pooled products were sequenced using a Roche 454 FLX pyrosequencer at Cornell University's Life Sciences Core Laboratories Center. Data have been deposited in GenBank under SRA022795.

Statistical analysis of mouse phenotypes

Body weights, fat mass, leptin and inflammation marker levels (Table 3.1) are expressed as arithmetic means ± SE. Statistical analyses were performed using JMP (ver.

7; SAS, North Carolina). 2-way ANOVAs were conducted using diet (DIO or CON) and treatment (WR or AL) as grouping variables with Tukey post-hoc ANOVA. T-tests were conducted when directly comparing phenotypes of DIO-WR and CON-AL mice using JMP (ver. 7; SAS, North Carolina). All statistical tests were two-tailed.

16S rRNA gene sequence analysis

Sequences generated from pyrosequencing barcoded 16S rRNA gene PCR amplicons were quality filtered. Sequences were removed if they were shorter than 200 nt, longer than 1000 nt, contained primer mismatches, ambiguous bases, uncorrectable barcodes or homopolymer runs in excess of six bases. The remaining sequences were denoised [179] and analyzed using the open source software package Quantitative Insights Into Microbial Ecology (QIIME, [180]). 16S rRNA gene sequences were assigned to operational taxonomic units (OTUs) using UCLUST with a threshold of 97% pair-wise identity, then classified taxonomically using the Ribosomal Database Project (RDP) classifier 2.0.1. Two highly abundant OTUs (716 and 303) were not classified beyond the phylum level (Firmicutes) with this method. For these two noteworthy OTUs, we used BLASTn against the NCBI non-redundant database, which yielded 98% and 97% ID matches to a 800bp 16S rRNA gene sequence of a bacterium that has not been cultured (accession number FJ836349). This matched sequence was classified as belonging to the genus *Allobaculum*, family Erysipelotrichaceae (Firmicutes) with 100% confidence in RDP (the lack of a match with the shorter fragment is likely due to the many regions of low complexity in the short fragment which, when broken into random 7bp words by the algorithm, leads to incongruent classifications).

For tree-based analyses, a single representative sequence for each OTU was aligned using PyNAST [181], then a phylogenetic tree was built using FastTree. The phylogenetic tree was used for measuring the α-diversity (phylogenetic diversity, PD) and β-diversity (using unweighted UniFrac [182]) of samples. Student's t-tests were conducted and p-values corrected for multiple comparisons. The "nearest shrunken centroid" method was used to identify OTUs that are specifically over (or under)-represented in a given category (diet, treatment or diet-treatment combinations). The amount of shrinkage was chosen in order to minimize the overall misclassification error. The analysis was performed using the PAM-R (Predictive Analysis of Microarrays) package under R software.

RESULTS

The denoised sequence library comprised 1,276 distinct operational taxonomic units OTUs (from >300,000 reads). Measures of α-diversity reflect phylogenetic richness in each sample; we measured phylogenetic diversity or PD, a phylogenetic-tree based measure of diversity calculated as the tree-branch length present in each sample (**Figure 3.1A**). The average PD of each treatment group was significantly different from the others, and DIO mice had higher PDs than the CON mice ($p < 0.05$; Student t-test, p-values corrected for multiple comparisons). Bacterial communities of DIO-WR mice had the highest PD. Interestingly, the effect of weight reduction (*i.e.*, WR vs. AL) had opposite effects on PD for the two diets: in the DIO mice the PD increased with weight reduction ($t = 3$, *p-value* = 0.004), while the PD in CON mice declined with weight reduction ($t = 6.7$, *p-value* = 7.31×10^{-10}). Finally, DIO-WR mice microbiotas had a higher PD than CON-AL mice ($t = 5.6$, *p-value* = 1.4×10^{-7}) despite equivalent body weights and body composition.

Overall effects of diet - We performed a Principal Coordinates Analysis (PCoA) on the unweighted UniFrac distances between samples to determine to what extent diet (i.e. DIO and CON) and treatment (i.e. WR or AL) affected gut microbial community diversity [182]. **Figure 3.1B** shows a clear separation between the diets when principal coordinates (PCs) 1 and 2 are plotted. In the DIO mice, the AL (blue dots) and WR (red dots) weight states can be distinguished, but such differences cannot be appreciated in the control mice between AL (purple dots) and WR (green dots) weight states. Globally, these results indicate that different diets promote different bacterial community diversity,

and that weight reduction affects the gut community composition of DIO (60% fat) mice but not that of mice fed a 10% fat control diet (CON-WR).

Figure 3.1C summarizes the relative abundances of bacterial phyla in the different mouse groups. CON mice have greater abundance of Firmicutes than DIO mice: this difference reflects the dominance of two OTUs classified as the genus *Allobaculum*. Mice eating a HFD (DIO-AL and DIO-WR) have greater abundances of Firmicutes (excluding *Allobaculum* OTUs), and lower abundances of *Allobaculum* OTUs, when compared to animals fed the control diet (CON-AL and CON-WR). Bacteroidetes levels are elevated in all mice ingesting the high fat diet (DIO-AL and DIO-WR) when compared to CON-AL and CON-WR mice. DIO-AL and DIO-WR mice also have a higher abundance of Defferibacteres due to the presence of *Mucispirillum*.

We performed a nearest shrunken centroid classification analysis to determine which OTUs account for differences in composition of the gut microbial community [183]. In addition, this analysis assesses how well a mouse microbiota is assigned to its treatment group based on its composition. In this analysis as well, the two diets are very well separated: the class error rate between the two diets is very low (p=0.08, 2 mice out of 25 are misclassified). However, when analyzing diet in the context of treatment, it is not possible to distinguish between CON-AL and CON-WR (misclassification error rate = 1) while DIO-AL and DIO-WR are readily distinguishable (only 1 out of 5 and 1 out of 6 respectively, were misclassified). The "classifying OTUs" (*i.e.*, those driving the community differences) were retrieved from this analysis and an unsupervised hierarchical clustering was performed on their abundances (**Figure 3.2A**). The resulting

heatmap (**Figure 3.2B**) shows an OTU (Firmicutes; *Allobaculum*) that is almost absent from all of the DIO samples and is present in all but one of the CON samples.

Nearest shrunken centroid classification revealed 8 OTUs that discriminated between the two diets. Seven of the eight OTUs are under-represented in CON mice and over-represented in DIO. The OTU with the greatest contrast between CON and DIO was a member of the Lachnospiracaea family of the Firmicutes phylum: this OTU is underrepresented in CON (score of -0.82) and over-represented in the DIO mice (score of +1.04). An OTU classified to the genus *Allobaculum* was over-represented in CON (score of +0.61) and under-represented in DIO (score of -0.78). Members of an OTU classified as the genus *Mucispirillum* were also positively correlated with the DIO mice (score of +0.51) and negatively correlated with the CON mice (scores of -0.4).

Effect of weight reduction on composition of the microbial community - The DIO-WR mice form a separate cluster from the DIO-AL (*ad-libitum)* group and are intermediate between the DIO-AL and the CON animals in the PCoA plot of unweighted UniFrac distances (**Figure 3.1B**). There is no significant difference in mean unweighted UniFrac distances within and between treatments (WR vs. AL, $t = 0.82$, *p-value* = 0.41). The average unweighted UniFrac distances within and between diets is significantly different, indicating that diet type is a strong factor in bacterial diversity regardless of the abundances of specific types of bacteria ($t = 9.47$, p-value = 9.13×10^{-19}).

Nearest shrunken centroid analysis, which takes into account OTU abundances, indicated that 4 of the 5 DIO-AL mice, and 5 out of the 6 DIO-WR mice, could be correctly classified (overall error rate = 0.176). Five OTUs discriminated between the DIO-WR and DIO-AL. *Allobaculum* was enriched in the weight reduced mice and

contributed most to the separation of these communities. Others, listed in order of effect size, are OTUs classified as members of the Ruminococcaceae and Lachnospiraceae families, and a member of the genus *Lactococcus*, all of which were enriched in AL; and an OTU classified as a Firmicute that was enriched in DIO-WR.

Unlike DIO mice, CON-AL and CON-WR microbiotas did not segregate in the unweighted UniFrac PCoA nor on the basis of shared OTU abundances. When comparing the relative OTU abundances and the effect of the weight reduction in DIO mice (**Figure 3.1C**), there was an increase in the abundance of *Allobaculum* in leaner mice, but the overall abundance of Firmicutes was constant. In the CON mice, the relative abundances of Firmicutes and *Allobaculum* stayed approximately the same between the AL and WR, but in the CON-WR mice there was an increase in the abundance of *Allobaculum* OTU_303, and a decrease in the abundance of *Allobaculum* OTU_716. We also noted a decrease in the relative abundance of members of the Proteobacteria phylum.

Mice of the same weight but ingesting different diets: comparison of DIO-WR and CON-AL - In the overall analysis using unweighted UniFrac, the DIO-WR formed an intermediate cluster between the DIO-AL and the CON. PAM-R analysis comparing the microbiotas of mice of same body weights and body composition, but ingesting different diets (DIO-WR vs. CON-AL), identifies 5 OTUs (**Figure 3.2A**) that accounted for differences between these two groups of mice: OTUs classified as members of the Lachnospiraceae family, the Firmicutes phylum, and the genera *Bacteroides* and *Mucispirillum* were found to be enriched in the DIO-WR mice, and *Allobaculum* was found to be enriched in the CON-AL mice. Abundances of OTUs

belonging to the Lachnospiraceae and the Deferribacteraceae accounted for the majority of the differences between the DIO-WR and CON-AL mice. At the phylum level (**Figure 3.1C**), the DIO-WR mice harbored higher abundances of Bacteroidetes than the CON mice. Although the CON-AL mice had higher relative abundances of Firmicutes, this trend was driven exclusively by *Allobaculum* OTUs: when *Allobaculum* OTUs were excluded, the CON-AL mice showed lower Firmicutes abundance than the DIO-WR. The Deferribacteres (e.g., genus *Mucispirillum*), although present in low percentage in the DIO-WR, were absent from the CON-AL.

Circulating leptin, inflammation markers in inguinal fat, and bacterial community composition - I) Circulating *Leptin*. As expected, serum leptin concentrations were highly correlated with total fat mass (by NMR) (**Figure 3.3A**; $r^2 = 0.92$, $p < 0.0001$), and there was no effect of weight loss *per se* on this relationship. DIO-WR mice lost significant amounts of fat mass (FM) and fat free mass (FFM) (FM accounted for 65±4% of weight loss), whereas CON-WR mice showed a significant decrease only in FFM that accounted for 87±3% of lost weight. As a result, circulating leptin concentrations in DIO-WR mice were reduced about 80% compared to initial concentrations in DIO; whereas in CON animals, weight loss reduced leptin concentrations by only 12%. Consequently, DIO-WR mice had significantly higher circulating leptin concentrations, and slightly but not significantly higher fat mass, than CON-AL mice when these phenotypes are compared by direct t-test. These differences in absolute circulating concentrations of leptin reflected differences in fat mass only, i.e. were not due to differences in circulating leptin normalized to fat mass (**Figure 3.3A**). **Figure 3.3** shows that circulating leptin concentration is positively correlated with OTU

abundance of the genera of *Mucispirillum*, ρ_s = 0.61 p=0.002, *Lactococcus* ρ_s = 0.52 p=0.008, and Lachnospiraceae, ρ_s = 0.63 p<0.001, respectively (**Figure 3.3b-d).** *Allobaculum* abundance was negatively correlated (**Figure 3.3e**) with leptin concentration (ρ_s = -0.73 p=0.001). No patterns were detected when comparing circulating concentrations of T3, T4, insulin, or adiponectin to relative abundances of the microbiota (data not shown).

II) *Inflammation markers.* Expression levels of selected inflammatory markers and the solute carrier *Slc25a25* were examined in inguinal fat pads (**Table 3.1**). *Slc25a25* is a mitochondrial transporter that is believed to be involved in energy expenditure homeostasis; its gene expression in WAT correlates positively with diet composition and cold stress (personal communication Dr. Kozak). Weight reduction was associated with significant decreases in *Pai1* and *Saa3* mRNA levels in DIO-WR compared to DIO-AL mice. *Slc25a25* levels were higher in DIO mice than CON mice, regardless of weight reduction. *F4/80* levels were significantly lower in CON-WR mice compared to all three other groups. *Cd11c* expression was significantly higher in DIO-AL than all other groups, although weight reduction *per se*, showed (by 2-way ANOVA) near significance (p=0.07). No significant differences in levels were seen for *Il10* and *Tnfa* across all groups. Expression level of *DioII*, a gene that influences energy expenditure by peripheral tissue conversion of thyroxine (T4) to the more physiologically active triiodothyronine (T3), was decreased in DIO-WR but increased in CON-WR animals. *iNos* expression was significantly decreased in DIO-WR animals but significantly increased in CON-WR animals. **Figure 3.4** is a heatmap showing the correlations between the inflammation markers and the abundances of selected OTUs. Expression

levels of *Slc25a25* were strongly positively correlated with relative abundance of *Bacteroides*, *Mucispirillum*, and an unclassified *Lachnospiraceae*, and negatively correlated with *Allobaculum*. *Saa3* and *Pai1* were positively correlated with Lactococcus and a Lachnospiraceae. *Allobaculum* OTUs showed the opposite trend.

We also measured expression levels in adipose tissue of *Angtpl4* (also known as *Fiaf*) and found the WR mice to have lower levels of expression than the AL mice (**Table 3.1**). **Figure 3.4** shows a very strong correlation between *Angtpl4* levels and an unclassified member of the Clostridiales. *Angtpl4* levels also correlated with relative abundance of *Lactococcus* and unclassified Ruminococcaceae and Lachnospiraceae OTUs.

DISCUSSION

In this study, diet composition *per se* had the biggest effect on the gut microbiota. Our DIO-WR and CON-AL groups had similar body composition and weights, allowing a comparison of their gut microbiotas without the confounding effects of weight/adiposity. The differences in relative bacterial abundances between DIO-WR and CON-AL corroborate those of Hildebrandt et al., who reported an effect of diet composition independent of host body weight, although the body weights in those experiments were not as closely matched as those in the present study and the mice in our study are not segregating for a monogenic mutation (RELMβ) [176]. Significant differences have been demonstrated in the diversity of the microbiotas of rodents and humans ingesting high fat and low fat diets [123,176,184], but these studies do not adequately control for differences in body mass or body composition. In addition, these studies do not distinguish whether effects on gut microbiota are a result of increased caloric intake *per se* or the fact that the composition of the diet was higher in fat content. Our results confirm unambiguously that dietary fat content, and not increased caloric intake, affects gut microbiotas in animals of similar weights.

Weight reduction affects the composition of the gut microbial community in mice and humans [118-120,122,123]. However, the weight loss in these studies resulted from changes in diet composition and/or changes in number of calories ingested, potentially confounding the respective contributions of diet composition, weight loss, and their interactions. We show here that, in mice fed a high fat (60%) diet, maintenance of a 20% reduced body weight affects the composition of the gut microbiota. This effect is not

seen in weight-reduced mice fed a low fat (10%) diet. The different effects of weight reduction on the gut microbial community composition between these two groups of mice may reflect effects of diet, initial body weight/composition (and attendant biological consequences), and/or their interactions.

The changes in gut microbiota observed in weight-reduced mice on the DIO diet but not on the CON diet is intriguing. This difference may be attributable to the differential effects that weight loss has on absolute changes in leptin concentrations between the DIO and CON mice. Leptin concentrations are linearly correlated with fat mass - **Figure 3.3A**. DIO-WR mice lost significant amounts of fat mass (FM) (accounting for 65±4% of weight loss), whereas CON-WR mice showed a significant decrease only in fat-free mass (accounting for 87±3% of lost weight). As a result, leptin concentrations in DIO-WR mice were reduced about 80% compared to initial concentrations in DIO, whereas in CON animals, weight reduction lowered leptin levels by only 12%. Our results suggest that the effects of body weight change on the gut microbiota may be mediated, in part, by changes in circulating leptin concentrations.

A connection between circulating leptin concentrations and the composition of the microbiota is suggested by the following observations: (1) several operational taxonomic units (OTUs) have abundances that are correlated with circulating leptin concentrations, and (2), these OTUs have been shown to interact with intestinal mucin, an important component of the intestinal milieu made up of heavily glycosylated proteins produced by endothelial cells. Mucin is important in creating micro-niches that are favored by some bacterial populations. For instance, *Akkermansia* and *Allobaculum* abundances and circulating leptin concentrations were negatively correlated, whereas *Mucispirillum*

abundance was positively correlated with circulating leptin concentrations and showed highest relative abundance in the obese mice. These OTUs are also noteworthy because *Akkermansia* can subsist on mucin [185], and *Mucispirillum* is known to colonize the mucus layer [186]. These relationships raise the question of whether leptin concentration affects mucin production and/or composition in the gut, which then in turn could influence the preponderance of specific populations of bacteria [187]. Administration of leptin into the colon of rats strongly stimulates mucin production; and leptin stimulates mucous production *in vitro* in human intestinal mucin-producing cells (HT29-MTX) [187,188]. Humans segregating for a single nucleotide polymorphism (Q223R) in the leptin receptor (LEPR) are more susceptible to infection by *Entamoeba histolytica* (in press JCI [189]). Mice segregating for this same mutation, also showed increased susceptibility to *Entamoeba histolytica* infection, and increased apoptosis of cecal epithelium cells, suggesting that there is a direct link between leptin biology and mucosal immunity. Together, these results suggest that circulating leptin concentrations may affect the composition of the gut microbiota by affecting mucin production in the intestine. A decline in circulating leptin concentrations, such as those seen in the DIO-WR mice could have a larger impact on the microbiota than a relatively small decrease in CON-WR mice.

The OTUs that account for the differences between DIO (WR & AL) and CON (WR & AL) mice, and are also negatively correlated to circulating leptin concentrations, belong to the genus *Allobaculum,* a member of the Firmicute family Erysipelotrichaceae (formerly Mollicutes). Interestingly, members of this family have been shown in several independent studies to change in abundance in response to changes in relative amounts of

dietary fat intake [123,190]. Furthermore, *Allobaculum* relative abundance has been reported to be positively correlated with plasma HDL concentrations in hamsters fed a diet supplemented with grain sorghum lipid extract (GSL) [191]. In our study, both diet composition (i.e. relative amount of dietary fat; DIO vs. CON) and body weight status (AL vs. WR) correlated with *Allobaculum* abundances, indicating that diet composition alone cannot account for changes in relative abundance, and that some metabolic or phenotypic change caused by maintenance of lower body weight must also be involved (see **Figure 3.3E**).

Does the composition of the microbiota itself contribute causally to host adiposity? Several studies suggest that the absence of micriobiota (gnotobiotic mice raised in a germ-free environment) is protective against diet-induced obesity [192-194], although perhaps not in all mouse strain/diet combinations [190]. There are several ways in which the specific composition of microbiota might influence host adiposity[118,195]. One is via the increased availability of short chain fatty acids produced by microbial breakdown of complex polysaccharides, giving the host access to more of the ingested calories [121]. Another is by inducing inflammation, which can lead to insulin resistance and hyperphagia [175]. A change in microbiota induced by a high fat diet can trigger metabolic inflammation when increased gut permeability allows lipopolysaccharides to enter the circulation [174,196]. Specific changes in microbiota preponderance that are either increased (*Allobaculum*) or decreased (Lachnospiraceae) following maintenance of a WR state (irrelevant of diet composition), and correlated with hormones known to influence energy homeostasis (e.g. leptin), suggest that the specific composition of the microbiota may play a role in host energy balance in weight-perturbed individuals.

We observed a correlation between certain gut microbiota (e.g. *Lactococcus* and Lachnospiraceae*)* and gene expression levels in inguinal fat of inflammation markers (*Saa3* and *Pai1*) and a mitochondrial transporter (*Slc25a25*). Contrary to what we anticipated, certain inflammation markers, such as *Tnf-α* and *F4/80*, were not significantly elevated in DIO-AL mice when compared to CON-AL mice (**Table 3.1**). These discrepancies may be related to the fat pad (inguinal) in which gene expression was tested. Koren *et al* (2010) have reported correlations between relative abundance of specific members of the gut microbiota (e.g. Lachnospiraceae) and circulating markers (e.g. LDL concentrations) known to correlate with inflammation [197]. Specific gut microbial communities induce low-grade inflammation in white adipose tissue: mice deficient in toll-like receptor 5 developed increased visceral fat, hyperlipidemia, hypertension, and decreased insulin sensitivity, an aggregate phenotype similar to that seen in humans with "metabolic syndrome"; this constellation of phenotypes can be transferred to germfree wild-type recipients by microbial transplantation from affected animals [175]. Thus, specific phylotypes observed in our study could be drivers of inflammation, although establishing a causative role will require further testing.

Host adiposity, diet composition, and gut microbiotas interact in complex, probably reciprocal ways. **Figure 3.5** is a schematic of these possible interactions. Leptin concentration, both circulating and within the gut, and dietary fat, may interact to affect gut mucous production, the microbiota, and barrier integrity in ways that ultimately influence adiposity. The studies described here begin to disarticulate the effects of diet and weight perturbation, *per se*, on relative abundances of gut microbiota. The molecular mechanisms underlying these effects on gut microbiota, and the consequent roles of these

bacteria in energy homeostasis and “metabolic inflammation” are clearly areas of clinical importance. Establishing the strength and direction of the relevant arrows of causality will require some relatively straightforward extensions of the studies and techniques reported here (**Figure 3.5**).

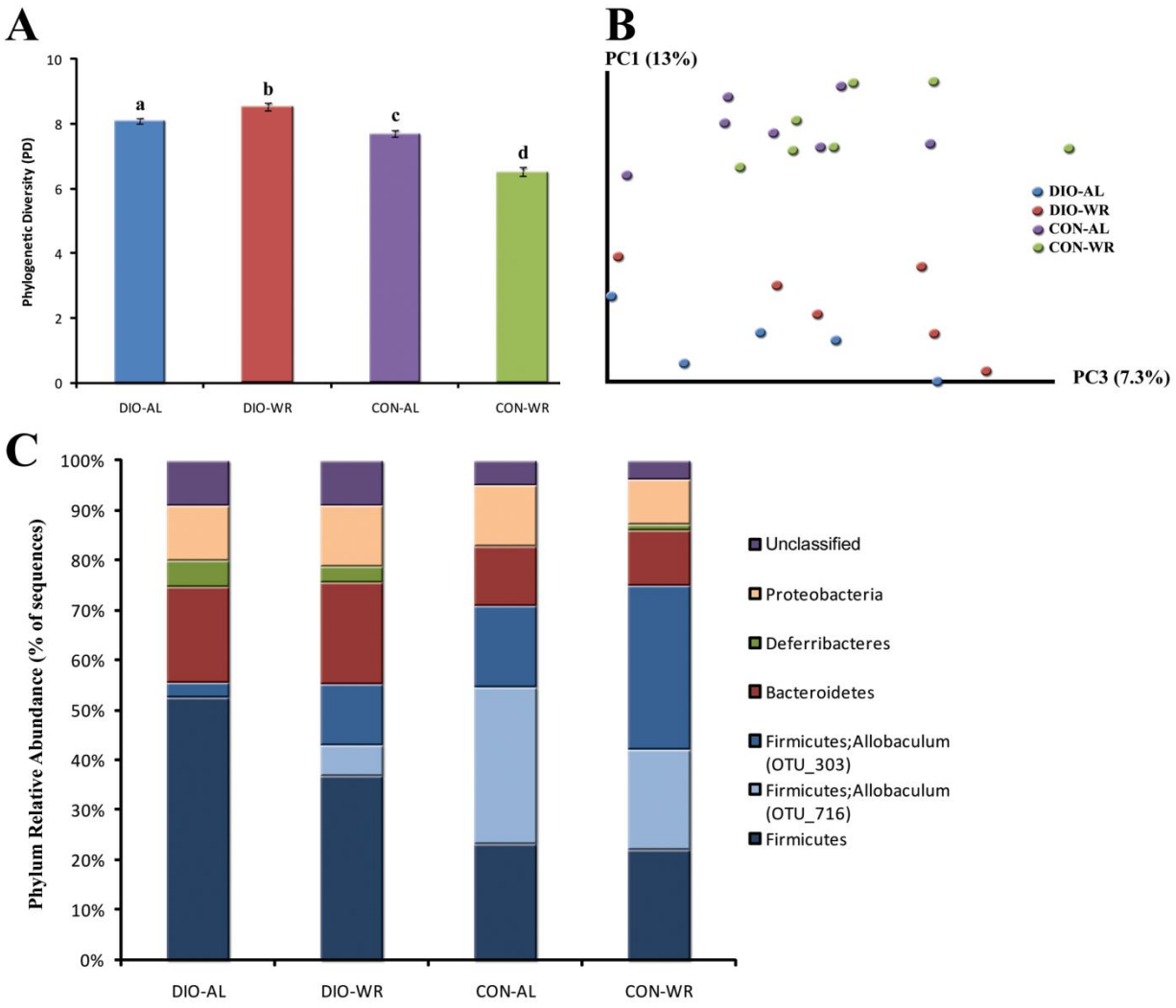


FIGURE 3.1: EFFECTS OF DIET AND WEIGHT REDUCTION ON THE GUT MICROBIOTA

(A) Phylogenetic Diversity (PD) of the cecal samples from the 4 groups of mice (mean±SEM compared by two-way ANOVA) and (B) PCoA plot of the unweighted UniFrac distances. PC1 and PC3 values for each mouse sample are plotted; percent variation explained by each PC is shown in parentheses: DIO-AL: blue; DIO-WR: red; CON-AL: purple; CON-WR: green. (C) Relative operational taxonomic unit (OTU) abundances of the different phyla in each of the mice. The phylum Firmicutes was broken down into OTU 303, OTU 716 (which are both classified as *Allobaculum*), and all other Firmicutes that did not fall into these two OTUs.

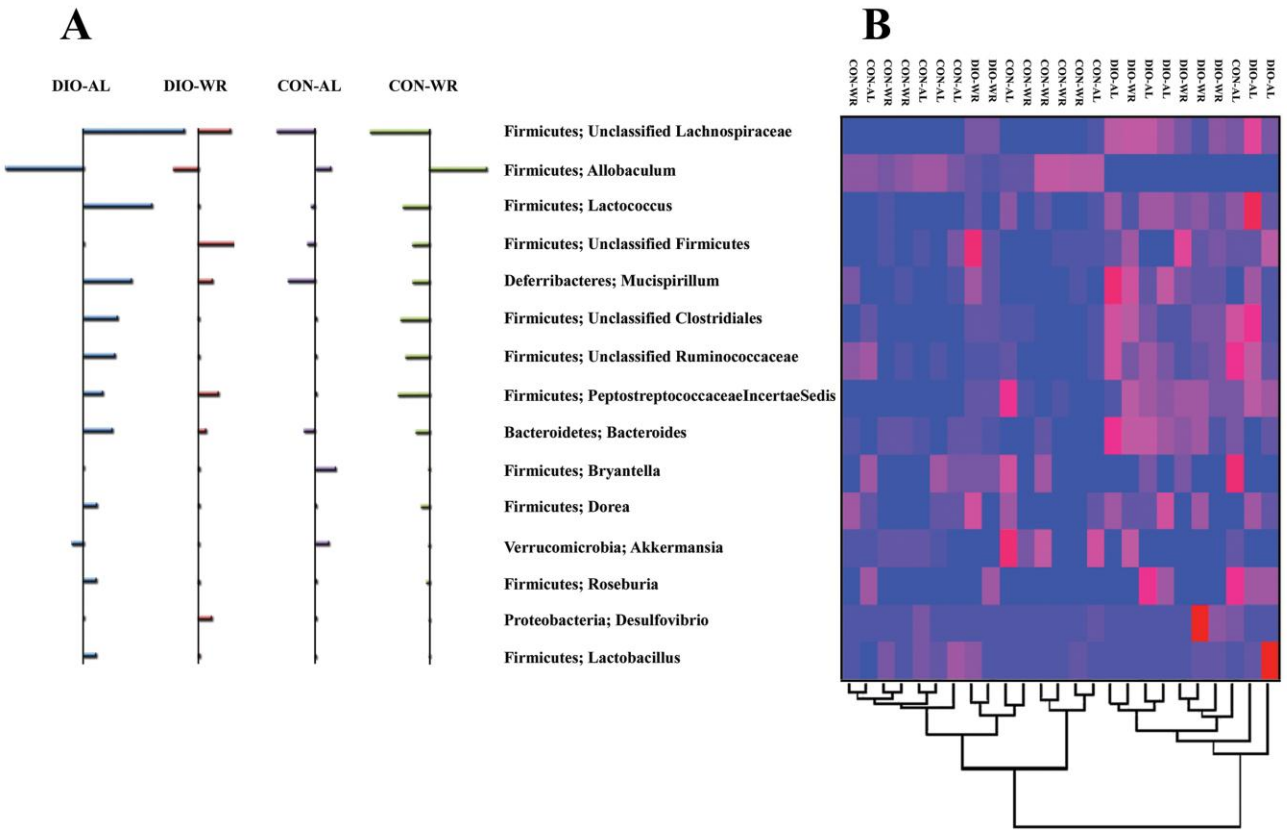


FIGURE 3.2: MEMBERS OF THE MICROBIOTA THAT DIFFER IN ABUNDANCE BY DIET COMPOSITION AND TREATMENT (WR VS. AL)

(A) Nearest shrunken centroid analysis of the 15 OTUs accounting for the differences among the four groups of mice. For each OTU listed at right, direction of the horizontal bars indicates relatively over-represented (right) and under-represented (left); the length of the bar indicates the strength of the effect. (B) Heat Map of the "classifying" OTUs. Columns show, for each mouse, the abundance data of OTUs listed at left. The abundances of the OTUs were clustered using unsupervised hierarchical clustering (Blue=low abundance, Red=high abundance). The Phylum;Genus of each of the classifying OTUs is noted.

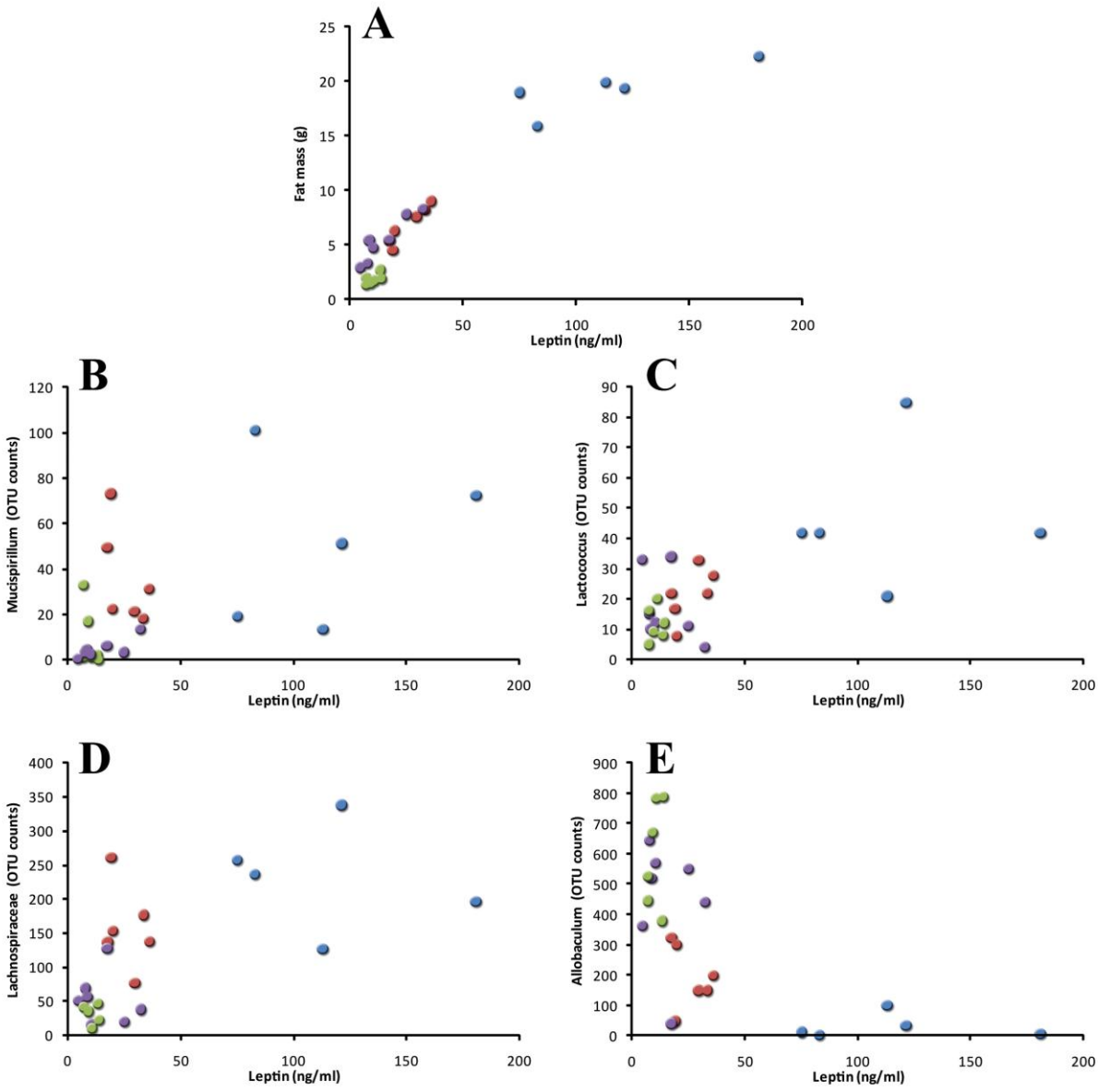


FIGURE 3.3: ASSOCIATIONS BETWEEN HOST SERUM LEPTIN CONCENTRATIONS AND GUT MICROBIOTA

(A) Correlations of fat mass content (by NMR) with circulating leptin concentrations. (B, C, D, E). Correlations between leptin concentrations and the abundance of OTUs of interest. The colors of the points correspond to a given diet- treatment combination (red: CON-WR, dark blue: CON-AL, light blue: DIO-WR and green: DIO-AL).

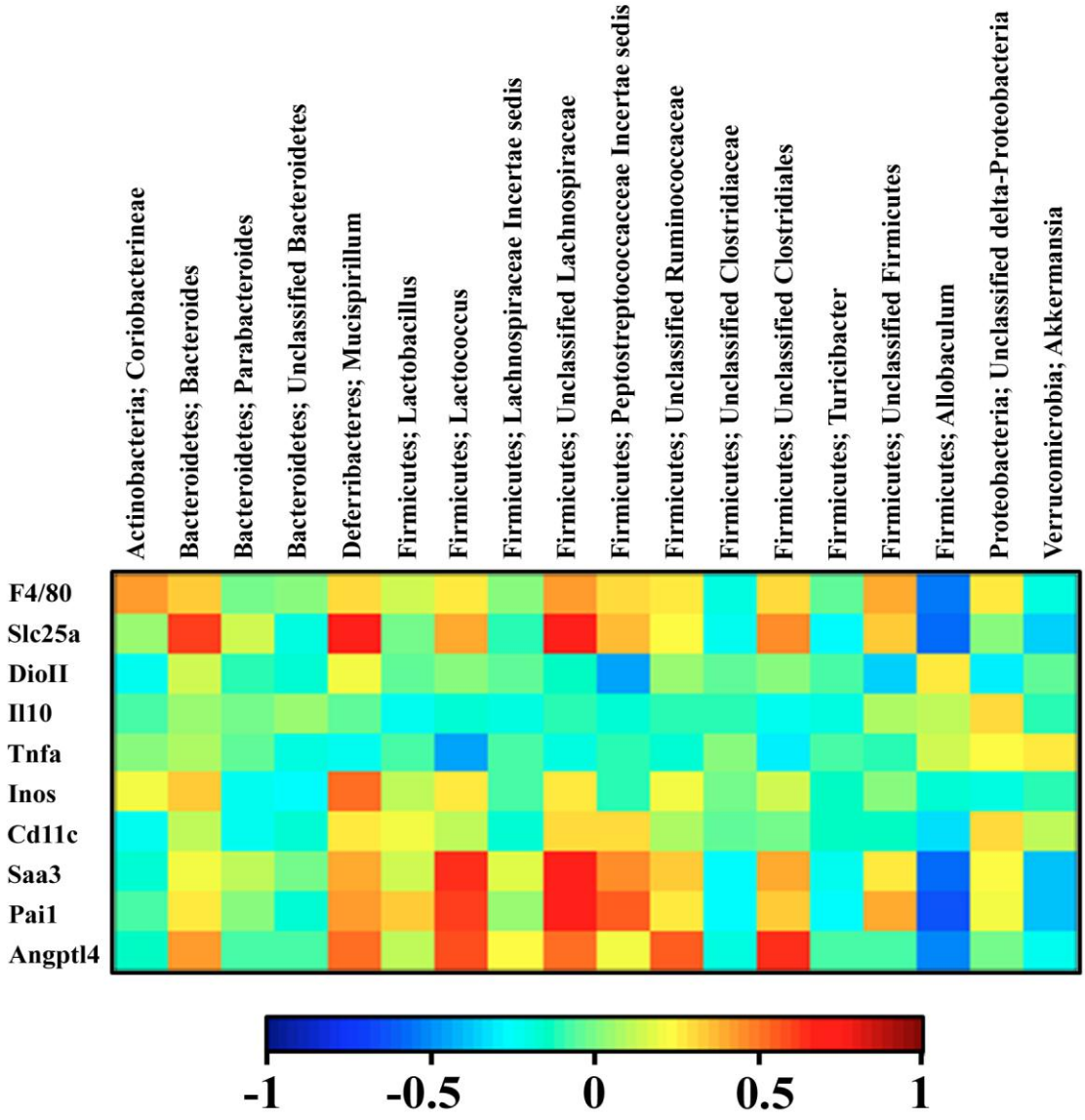


FIGURE 3.4: HEATMAP DESCRIBING THE CORRELATION OF THE ABUNDANCES OF DIFFERENT OTUS AND TRANSCRIPTION LEVELS OF INFLAMMATION-RELATED GENES IN INGUINAL ADIPOSE TISSUE

The colors range from Blue (Negative correlation; -1) to Red (Positive correlation; 1). Significant correlations are noted by *P<0.05 and **P<0.01(The computed false discovery rate is about 0.25 using the Benjamini Hochberg procedure [198])

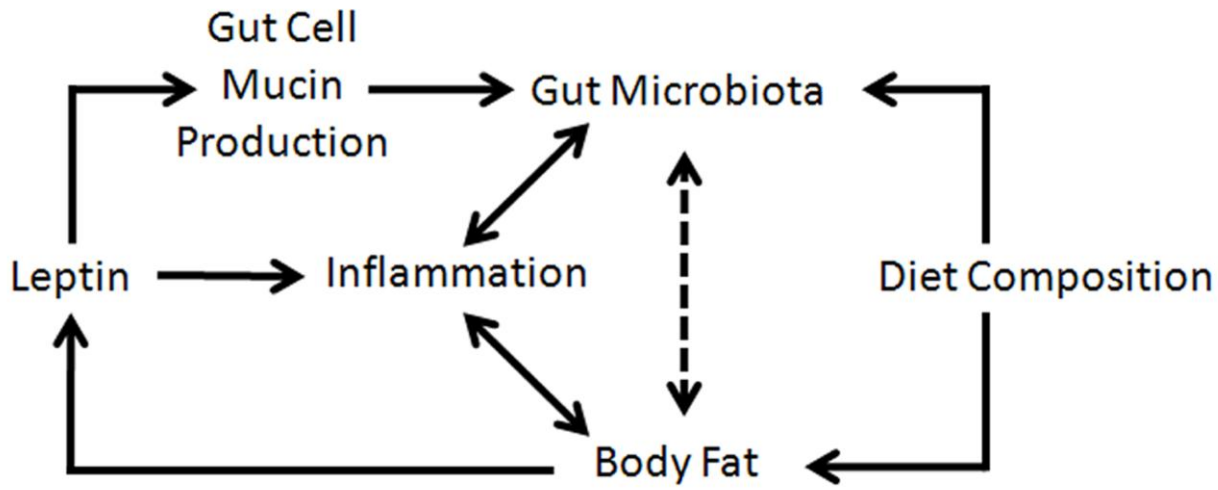


FIGURE 3.5: SCHEMATIC DEPICTING POSSIBLE INTERRELATIONSHIPS AMONG DIET COMPOSITION, GUT MICROBIOTA, CIRCULATING LEPTIN, BODY FAT, MARKERS OF INFLAMMATION, AND GUT MUCIN

Body fat directly determines leptin production and elevated body fat increases macrophage infiltration (with associated production/release of inflammatory molecules such as TNFα, SAA3, and CCL2 (MCP-1) in adipose tissue). The results presented here suggest that diet composition (fractional fat content) directly affects gut microbiota independent of effects mediated by body weight and body composition. Leptin promotes proliferation, differentiation, and survival of immune cells. Leptin also stimulates mucin production in mouse and human intestinal cells [187,188]. Mucin affects local bacterial "microniches" in the gut by favoring the growth of some bacteria [185,186]. Leptin can affect intestinal barrier function by inhibiting apoptosis and promoting regeneration of intestinal epithelium [199,200]. These changes in epithelial composition may in turn affect microbiota populations in the gut. The dashed line between body fat and gut microbiota suggests biologically possible connection(s) that might be mediated by adipocytokines or other molecules secreted from adipose tissue.

Body weights, fat mass, leptin and inflammation marker levels

	DIO Diet		CON Diet				
*	AL (n=7)	WR (n=8)	AL (n=8)	WR (n=8)	Diet	Treatment	Diet * Treatment
Body Weight (g)	52.0±1.0 [a]	33.6±1.0 [b]	32.2±1.1 [b]	22.0±0.6 [c]	F = 292.5 **	F = 243.9 **	F = 20.2 **
Fat-Free Mass (FFM, g)	23.5±0.5 [a]	20.0±0.3 [b]	20.3±0.3 [b]	15.1±0.4 [c]	F = 111.4**	F = 135.4**	F = 5.4*
Fat Mass (FM, g)	20.1±1.0 [a]	7.0±0.6 [b]	5.1±0.7 [b]	1.8±0.1 [c]	F = 230.6**	F = 151.8**	F = 53.3**
Leptin (ng/ml)	121.7 ± 14.9[a]	25.1 ± 2.9[b$]	14.0 ± 3.6[b]	9.0 ± 1.3[b]	F = 75.8**	F = 51.0**	F = 41.4**
#							
F4/80	1.06 ± 0.06 [a]	1.06 ± 0.09 [a]	1.0 ± 0.08 [a]	0.58 ± 0.05 [b]	F = 14.4**	F = 8.9**	F = 9.2**
Slc25a	1.08 ± 0.06 [a]	1.01 ± 0.06 [a]	0.63 ± 0.06 [b]	0.60 ± 0.04 [b]	F = 40.9**	F = 0.6	F = 0.1
DioII	1.34 ± 0.46 [a]	0.06 ± 0.03 [b]	0.87 ± 0.26 [a b]	1.64 ± 0.19 [a]	F = 4.1	F = 0.9	F = 14.3**
IL 10	0.87 ± 0.14	1.63 ± 0.23	1.17 ± 0.24	0.99 ± 0.23	F = 0.6	F = 1.9	F = 4.7*
Tnfa	1.07 ± 0.23	1.64 ± 0.15	1.58 ± 0.14	1.36 ± 0.19	F = 0.5	F = 1.0	F = 5.1*
INOS	1.29 ± 0.10 [a]	0.79 ± 0.08 [b c]	0.72 ± 0.03[c]	1.06 ± 0.10 [a b]	F = 3.6	F = 1.0	F = 28.7**
Cd11c	1.11 ± 0.16 [a]	0.86 ± 0.09 [b]	0.86 ± 0.08 [b]	0.71 ± 0.08 [b]	F = 3.7	F = 3.6	F = 0.2
Saa3	2.2 ± 0.55 [a]	0.89 ± 0.34 [b]	0.03 ± 0.02 [c]	0.02 ± 0.02 [c]	F = 23.5**	F = 4.4*	F = 4.3*
Pai 1	1.81 ± 0.29 [a]	1.25 ± 0.24 [a]	0.1 ± 0.05 [c]	0.18 ± 0.03 [c]	F = 54.8**	F = 1.6	F = 2.8
Angptl4	1.80 ± 0.23 [a]	0.72 ± 0.08 [bc]	1.07 ± 0.09 [b]	0.38 ± 0.12 [c]	F = 15.6**	F = 42.6**	F = 2.1

TABLE 3.1: BODY WEIGHT, FAT MASS, LEPTIN AND INFLAMMATION MARKER

*Body weight, body composition and circulating leptin concentrations (mean±SEM): previously published data. $ denotes a significant difference between DIO-AL and CON-WR mice by t-test ($p < 0.05$)[124].

mRNA levels (normalized to cyclophilin b and presented as arbitrary units) for inflammation markers measured by qRT-PCR in inguinal fat pads normalized to cyclophyllin b (mean±SEM). Data not marked by same letter are significantly different by two-way ANOVA with Tukey HSD post-hoc analysis. Direct t-tests were conducted for body weight and body composition of DIO-WR and CON-AL mice. Significant differences are indicated by $ ($p < 0.05$).

CHAPTER 4: ESTIMATING ENERGY EXPENDITURE IN MICE USING AN ENERGY BALANCE TECHNIQUE

ABSTRACT

Assessment of energy expenditure in laboratory rodents is critical in understanding the metabolic consequences of environmental (i.e. calorie restriction, exercise paradigms) or genetic (i.e. knockout & overexpressing models) manipulations. Indirect calorimetry is the "gold standard" for such assessments. However the cost and maintenance of the relevant equipment, the stress resulting from moving animals into these devices, and the generally limited periods of residence limit the use of these instruments. Here we describe an energy balance method using food intake and changes in body weight and composition (TEE_{bal}) in mice over 37 days to determine mean 24 hour energy expenditure; the results obtained are compared with those obtained by indirect calorimetry (TEE_{IC}). The two methods are highly correlated (TEE_{IC}; r^2 = 0.88: $TEE_{bal} = 1.04 * TEE_{IC} - 0.06$, $p < 0.0001$). TEE_{bal} estimates are slightly higher than those obtained by TEE_{IC} (+0.45 kcal/24h), probably due to small losses of chow into the cage bedding. TEE_{bal} can be performed in "home cages" and provides integrated long term measurement of energy expenditure.

Introduction

Changes in body mass and chemical composition in response to over- or under- feeding must conform to a biological restatement of the first law of thermodynamics:

$$\Delta \text{ Somatic Energy Content} = \text{Total Energy Intake} - \text{Total Energy Expenditure}$$

[**Equation 1**]

Measurement of any two components allows calculation of the third. Each component can be directly measured, but experimental circumstances may favor indirect estimates because of a desire to obtain prolonged measures, the relative accuracies of the respective measurements, and the availability of suitable instrumentation.

The energy balance method (EBM) has been used in human studies to estimate energy expenditure (EE) by titrating energy intake (EI) to achieve stability of body weight and composition [17,201]. Conversely, measures of EE, by the "doubly labeled water" technique, have been used in conjunction with changes in body composition to estimate EI in human subjects [202]. Here we describe the use of an energy balance technique that can be used to measure long term energy expenditure (or intake) in mice. We measured energy intake and changes in body mass and composition over a 37-day period, and used these measurements to estimate total energy expenditure (TEE_{bal}) in mice being fed high and low fat diets. These estimates of TEE_{bal} were highly correlated with those obtained by indirect calorimetry (TEE_{IC}; $r^2 = 0.88$: $TEE_{bal} = 1.04 * TEE_{IC} - 0.06$, $p < 0.0001$). This technique is particularly useful for long term measures of energy expenditure, and does not require a calorimeter.

MATERIALS & METHODS

Experimental Design:

Sixteen diet-induced obese (DIO – fed Research Diets, Inc. D12492i, 60% kcal as fat = HFD), and sixteen control diet fed (CON – fed Research Diets, Inc. D12450Bi, 10 % kcal as fat = CON) C57BL/6J-male mice were obtained at 18 weeks of age from the Jackson Laboratory (Bar Harbor, ME). These animals had been fed these respective diets since 6 weeks of age. Individually housed animals from both diet groups were randomized to remain on the *ad-lib* diets (DIO-AL and CON-AL) or to be calorically restricted to decrease their body weight by ~20% over a 1-2 week period by twice daily feeding of reduced quantities (50% of *ad-libitum* intake) of their respective diets. After a 20 ±1% weight reduction, weight-reduced (DIO-WR and CON-WR) mice were provided calories sufficient to stabilize their weights for an additional 23 weeks. Nine weeks after initiation of the weight reduction protocol (**Figure 4.1A**, denoted day 0), body weights (daily for all mice) and food intake (FI; daily for WR and every two days for AL mice) were recorded for the next 93 days, except on days when mice were in the calorimeter. The first day of this 93 day period is designated as day 0 (**Figure 4.1A)**. The 93 days following day 0 are divided into 3 measurement periods (**Figure 4.1A**):

1) TEE_{bal}: A 37-day period used to estimate TEE using an energy balance method (days 0 – 37)

2) TEE_{IC}: A 28-day period during which mice underwent 48 hour indirect calorimetry to estimate TEE (days 37 – 65)

3) All days excluding indirect calorimetry days (when MEI was not recorded) were used to determine – by autocorrelation and power analyses - the minimum number of days of FI required to estimate MEI to within various levels of accuracy.

One DIO-AL mouse died during the study and data from one CON-AL mouse was not included due to a malfunction in one of the calorimetry chambers. Aspects of this study have been described earlier [203].

On day 0, day 37, and following each 72-hour calorimetry period, body composition was determined using a Bruker Minispec mouse TD-NMR analyzer (Bruker Inc, Billerica MA) that had been calibrated with 30 mouse carcasses also subjected to chemical analysis [142]. All body composition measurements were made at 8-9 am, before weight reduced animals were fed.

Measures of Metabolizable Energy Intake (MEI) and Energy Expenditure (TEE):

Metabolizable energy intake (MEI), defined as grams of food ingested per 24h (weighed using custom-made stainless steel feeding baskets that minimized spillage - Dieter Wenzel; Detmold, Germany) multiplied by the metabolizable energy for the respective diets (5.24 Kcal/g for high fat (HFD) and 3.85 Kcal/g for the control (CON) diet), was recorded every two days for the *ad libitum*-fed mice. For WR mice, MEI was recorded daily and 1/3 and 2/3 of the daily food ration was provided at 8-8:30 am and 6:30-7 pm, respectively.

Energy expenditure was measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). Calibration of the system was performed as per the manufacturer's guidelines. The rates of O_2 consumption and CO_2 production were measured every 14 minutes during a 72 hour period. In calculating TEE, potential confounds due to stress of being placed in calorimetry chambers was minimized by using data from only the final 48 hours of the 72 hour period [203].

Estimates of TEE using energy balance (TEE_{bal}):

MEI and interim changes in body mass and composition were used to estimate TEE over a 37-day period (TEE_{bal}) [17]. Energy expenditure was calculated as MEI +/- the change in somatic energy stores [204,205] (see **equation 2** below). The energy cost of depositing dietary calories as somatic tissue is greater than that released by their oxidation. We assigned 13.2 kcal for each gram of fat mass gained, and 9.0 kcal for each gram of fat mass lost; 2.2 kcal for each gram of fat-free mass gained and 1.0 kcal for each gram lost [204,205].

Equation 2 (below) was used to calculate TEE_{bal}:

$$TEE_{bal} = MEI + (\Delta somatic\ Fat\ Energy + \Delta somatic\ Fat\text{-}Free\ Energy) \quad \textbf{[Equation 2]}$$

TEE_{bal} was compared to TEE_{IC} – expressed as kcal per 24 hrs – on a per mouse basis (**Figure 4.1B**).

Determination of minimal duration of FI to accurately estimate MEI

In order to test the day-to-day independence of the MEI data collected over days 0 – 37 and days 67 – 104 (total of 65 days: **Figure 4.1A**), a condition that must be fulfilled in order to perform a power calculation estimating the minimum duration of FI measurements required to obtain a specific level of accuracy of MEI, autocorrelation analyses were conducted on all mice [206].

To determine the minimum number of days that are required to estimate an individual animal's 24 hour food intake to within 5% and 10% of the mean MEI for all animals as a group, we used the relationship:

$$\bar{x} * y\% \geq \frac{1.96\sigma}{\sqrt{n}} \quad \textbf{[Equation 3]}$$

Where $\bar{x}$ is the mean 24 hour food intake (over 65 days) of each of the 15 AL mice, and y is a specified degree of error expressed as a percentage. The product of mean MEI for a mouse and the selected level of error (5% and 10% for each mouse) is related to an estimate of the 95 % confidence interval for the SEM ($\frac{1.96\sigma}{\sqrt{n}}$) of the mean MEI for each AL-fed mouse where σ is the standard deviation for MEI and n is the number of days of measured energy intake. $\bar{x}$ and σ were estimated for each mouse using the entire 65 day period during which MEI was measured. Here we arbitrarily stipulate that the SEM for any mouse should be less than or equal to 5% or 10% of the mean (i.e. 0.05 or 0.10 * $\bar{x}$) and then solved for the requisite n (number of days of food intake measurement) for each mouse. Mean ± sem and confidence intervals were constructed for the estimates of n obtained for the 15 AL mice using both 5% and 10% error in equation above. By using equation 3 and solving for all y% (assigning n in unit integers from $n_1 \ldots n_{67}$) a plot was constructed for 15 AL mice, indicating the number of days of energy intake needed to obtain a given level of accuracy of energy expenditure: **Figure 4.1E**).

Statistical Analysis

Data analysis was performed using JMP 8.0.2 Statistical Discovery Software (SAS Institute, Cary, NC). Student's t-test for paired samples was used to assess differences in estimates of TEE_{bal} and TEE_{IC}.

RESULTS

TEE_{IC} vs. TEE_{bal}: Correlation Analysis

Estimates of TEE_{IC} and TEE_{bal} (37 day measurement) were highly correlated (TEE_{bal} = 1.04 * TEE_{IC} – 0.06, r^2 = 0.88; p < 0.0001 **Figure 4.1B**). No significant differences between TEE_{IC} and TEE_{bal} were identified by direct t-test comparison (p = 0.60) and by paired t-test analysis using only WR mice, whose food intake is the most precisely known since rations – that were completely consumed – were provided by us twice daily (p = 0.65).

TEE_{IC} vs. TEE_{bal}: Bland-Altman and Relative mean differences plots

A Bland-Altman plot (aka Tukey difference mean plot [207]), a method used to assess the concordance between two methods measuring the same variable, indicates that TEE_{bal} estimates are slightly but significantly higher (3.8±1.7%; p < 0.05) than TEE_{IC} estimates (Bias = 0.45 kcal/24h; see solid black line **Figure 4.1C**) when including all groups of animals. A relative mean difference value for each mouse was calculated by dividing the difference between the methods (TEE_{bal} - TEE_{IC}) by TEE_{IC} for each pair of measures (**Figure 4.1D**). Close agreement was found between estimates of TEE_{bal} and TEE_{IC}, in both absolute (mean difference of 0.47±0.17 kcal/24h; 95% CI: 0.1 to 0.8 kcal/24h; **Figure 4.1C**) and relative (mean difference of 0.6±3.0%; 95% CI: −5.3 to 6.5%; **Figure 4.1D**) terms.

Duration of MEI required

Temporal analyses revealed strong and significant (p<0.01) autocorrelation of daily energy intake in the WR but not the AL mice, presumably because, in the WR, food rations were adjusted daily in 0.1 g units based on the previous days ration and intercurrent changes in body weight. No autocorrelation was detected for AL mice (n = 15), establishing the independence and

randomness of the FI measurements in mice from these groups and so power calculations were conducted using equation 3 on AL mice only. The average minimal days required for AL mice to fall within 10% and 5% of the mean was 4.3±0.5 (CI: 3.3 – 5.3) and 17.2±1.9 (CI: 13.1 – 21.4) days, respectively. Twenty one days of continuous energy intake measurements provide 95% confidence that the surrogate measure of energy intake will be within 5% of "true" mean energy intake for that animal and can be used in conjunction with intercurrent changes in body composition over the time period MEI was measured (using equation 2) to estimate TEE_{bal} (**Figure 4.1E**).

Body weight and body composition used in TEE_{bal}

Body weight increased slightly in DIO-AL (+0.6g), DIO-WR (+0.5g), and CON-AL (+0.4) but was unchanged in CON-WR mice during the 37-day TEE_{bal} measurement. Most of the increased body weight was explained by higher fat mass. The changes in body composition fell within the range of the sensitivity of the NMR device. We have previously shown that NMR estimates of FFM and FM are virtually identical to chemical composition analysis ($r^2 = 0.99$ for both FM and FFM; $p < 0.05$) and intra-individual within-day CV (4 measurements per mouse) were 2.8±2.7% (CI: 0.0-10.0) for FM and 2.2±1.0 (CI: 0.6-5.0)% for FFM [142].

DISCUSSION

The strong correlation between TEE_{bal} and TEE_{IC} ($r^2 = 0.88$; $p < 0.0001$) indicates that the TEE_{bal} method – using energy intake and changes in somatic mass and composition - can provide a valid estimate of long term TEE (**Figure 4.1B**). The Bland-Altman plot - used to assess the degree of agreement between the two measures [208] - indicates that the methods are closely concordant. The two methods differ by only 0.45 kcal/24h (3.8±1.7%) (see bias; **Figure 4.1C**). 4 out of 7 DIOAL mice showed a difference between TEE_{bal} and TEE_{IC} greater than 10%, whereas only 2 mice from all other groups combined showed such a difference (**Figure 4.1D**). The larger differences seen in the DIO-AL group may be the result of the fragility of the high fat diet, which is easily concealed in the bedding, leading potentially to overestimates of *ad-libitum* food intake measurements. This problem is minimized when using less friable diets, and is reflected in the smaller range of relative mean differences seen in the CON diet fed groups of mice (**Figure 4.1D**). When the four DIO-AL mice that showed a greater than 10% difference between estimates of TEE are excluded from the analysis, the correlation between methods is improved ($TEE_{bal} = 0.92 * TEE_{IC} + 1.04$, $r^2 = 0.94$; $p < 0.0001$) and the difference between methods is decreased (bias = 0.17 kcal/24h).

TEE_{bal} has advantages in comparison to TEE_{IC}. Indirect calorimetry systems require that mice be transferred from home cages to specialized units whose novelty can create stress and/or behavioral modification. Logistical considerations frequently limit the duration of calorimetry studies to 48-72 hours. Balance measurements can be conducted over extended periods of time with animals in their home cages. Long duration studies conducted in this way will detect subtle

differences in energy expenditure not detectable by short term calorimetry. However, the balance technique cannot assess diurnal variations in energy expenditure or directly determine RQ (which could, however, be estimated from diet composition and changes in body composition).

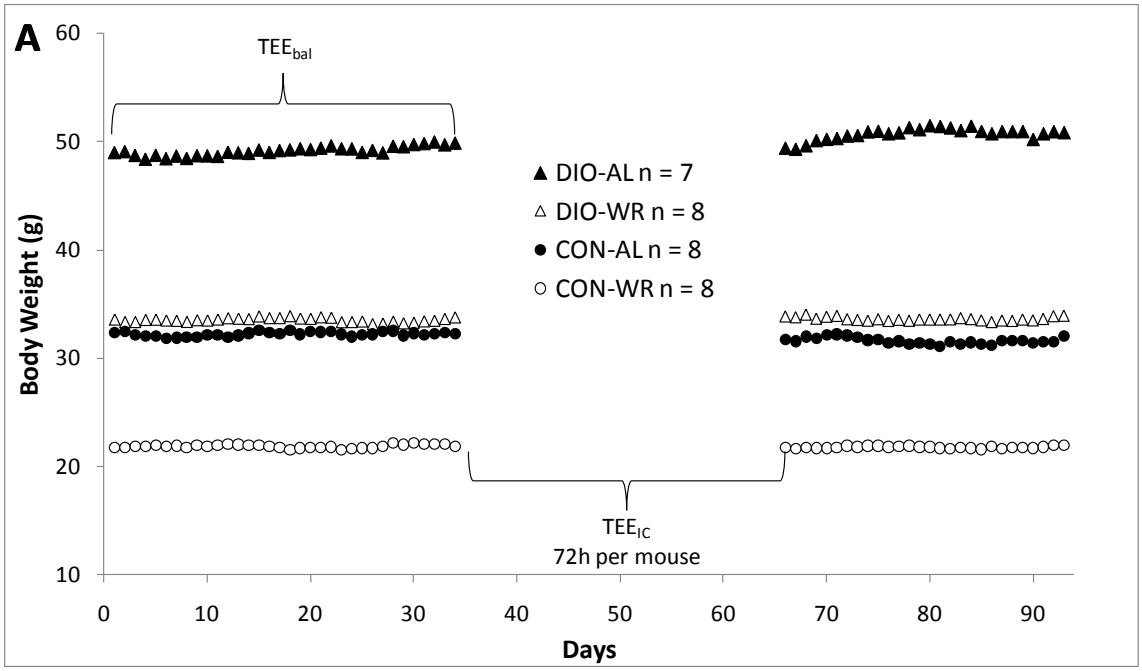
The power analyses using AL fed mice only suggests that 21 days (upper CI limit; see end of results section) of FI measurements will provide a measure of MEI to within +/- 5% of true mean 95% of the time (**Figure 4.1E**). This estimate is quite conservative and therefore shorter time periods may be acceptable depending on the accuracy of the FI measurements.

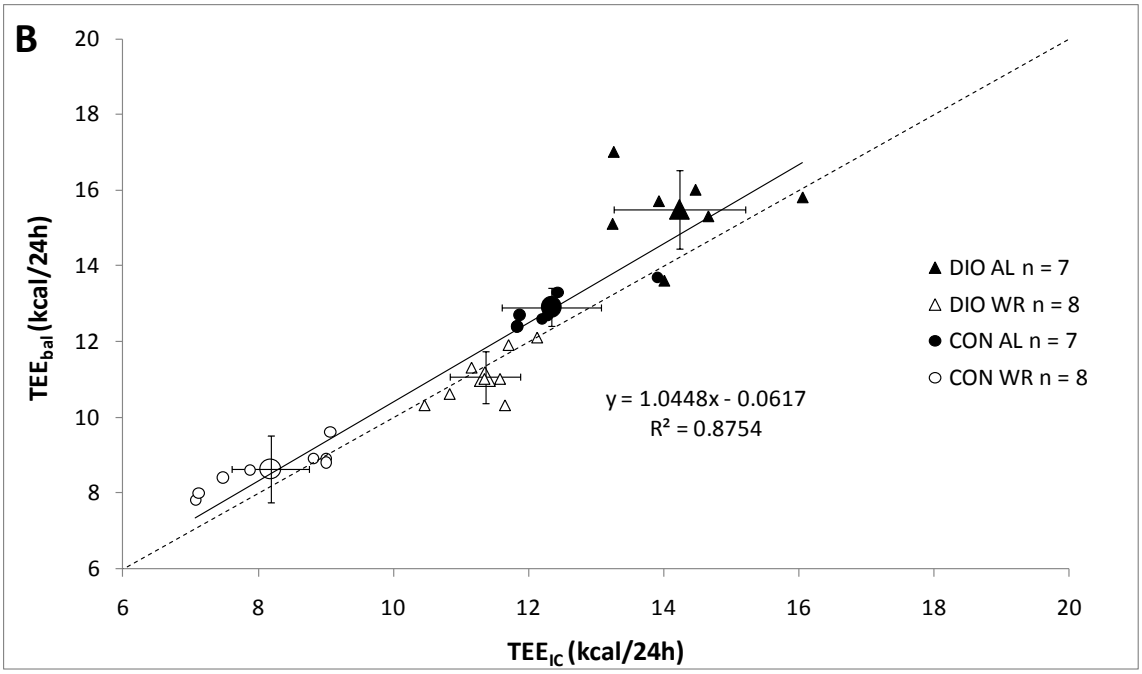
Additional considerations

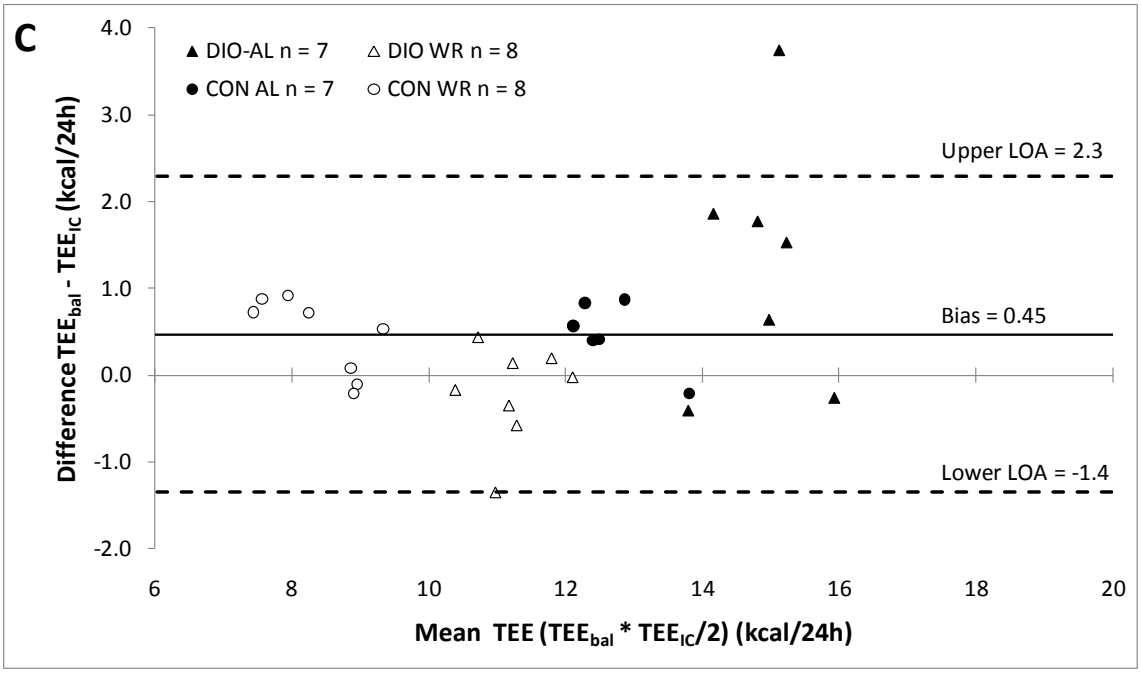
Accuracy of body composition measures are critical for correctly estimating energy expenditure using TEE_{bal} technique with these errors being amplified as the number of days of food intake measured decreases [142]. The accuracy and precision of the Bruker Minispec TD NMR that we use in the laboratory has been shown to be able to detect changes in body composition observed in our cohort of mice (**Table 4.1**) [142]. Since we used 37 days for the TEE_{bal} calculation and we estimated that 21 days was sufficient to be within 5% of mean MEI (**Figure 4.1E**), and that some of the discrepancies between TEE_{bal} and TEE_{IC}, especially in the DIO-AL group, may be a result of the overestimation of MEI due to the fragility of the HFD as mentioned above. Finally, indirect calorimetry used with both humans and rodents - the nominal "gold standard" – has technical limitations affecting both the sensitivity and accuracy of the instrument [209]. Differences in estimated TEE observed between TEE_{bal} and TEE_{IC} are necessarily the product of aggregate errors in all measurements obtained to permit the comparison.

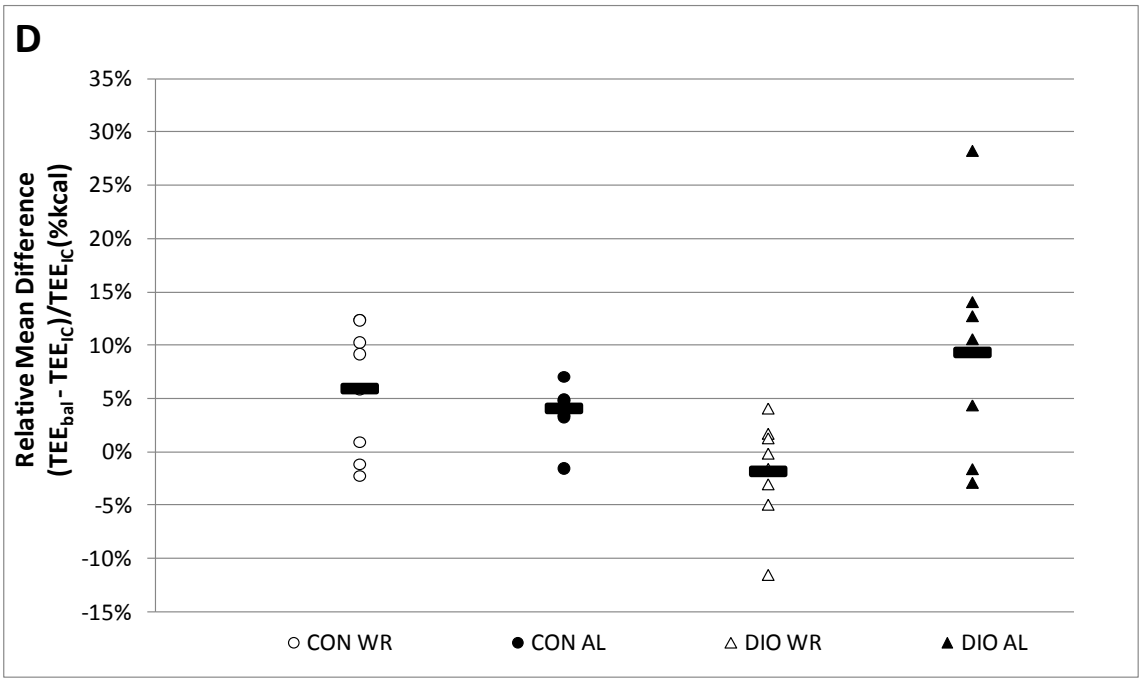
The approach described here can also be used to estimate long term spontaneous energy intake by solving **equation 2** for MEI, using direct measures of TEE and body composition.

Serial measurements of TEE and body composition would be best, to control for intercurrent changes in both parameters due to growth. In humans, the determination of *ad-libitum* energy intake to the level of accuracy required to assess the relative contributions of energy intake and expenditure to weight change using currently available methods is often inaccurate, sometimes by up to 50% [210]. By combining long term measurements of energy expenditure (differential rates of excretion of $^{2}H_2O$ and $H_2{}^{18}O$) with precise measurements of body composition, the energy balance equation can be "solved" for energy intake [202,211]. This approach could be tested for accuracy by conducting such a study in a room calorimeter in conjunction with bomb calorimetry of a weighed diet.


A
TEE$_{bal}$
DIO-AL n = 7
DIO-WR n = 8
CON-AL n = 8
CON-WR n = 8
TEE$_{IC}$
72h per mouse
Body Weight (g)
Days
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
90


B
TEE$_{bal}$ (kcal/24h)
TEE$_{IC}$ (kcal/24h)
DIO AL n = 7
DIO WR n = 8
CON AL n = 7
CON WR n = 8
y = 1.0448x - 0.0617
R² = 0.8754
20
18
16
14
12
10
8
6
6
8
10
12
14
16
18
20


C
▲ DIO-AL n = 7
△ DIO WR n = 8
● CON AL n = 7
○ CON WR n = 8
Upper LOA = 2.3
Bias = 0.45
Lower LOA = -1.4
Difference TEE$_{bal}$ - TEE$_{IC}$ (kcal/24h)
Mean TEE (TEE$_{bal}$ * TEE$_{IC}$/2) (kcal/24h)
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
6
8
10
12
14
16
18
20


D
Relative Mean Difference (TEE$_{bal}$ - TEE$_{IC}$)/TEE$_{IC}$(%kcal)
35%
30%
25%
20%
15%
10%
5%
0%
-5%
-10%
-15%
○ CON WR
● CON AL
△ DIO WR
▲ DIO AL

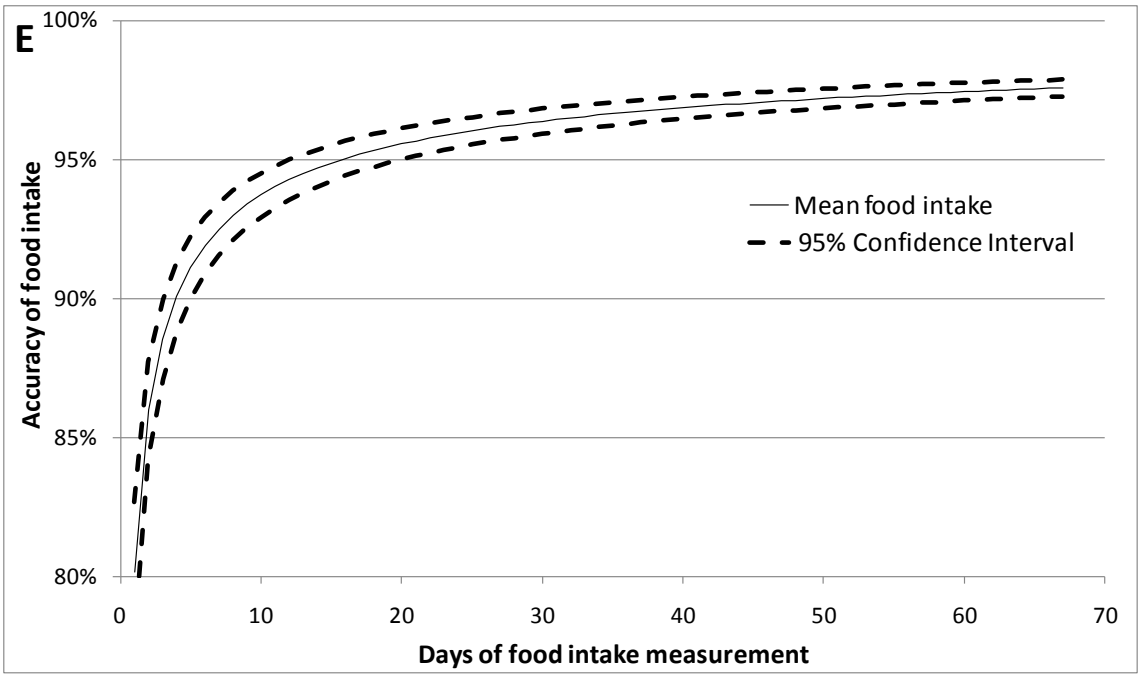


FIGURE 4.1: BODY WEIGHTS, TEE_{BAL} AND TEE_{IC} COMPARISON, AND FOOD INTAKE ACCURACY

A. Body weights during experimental periods. TEE_{bal} estimated using energy balance method and TEE_{IC} determined using indirect calorimetry. During the 30 day interval shown, each mouse spent 72 hours in the chamber; the last 48 hours of this period were used to estimate TEE_{IC}. **B.** Regression analysis of total energy expenditure (TEE) by energy balance method (TEE_{bal}) and indirect calorimetry (TEE_{IC}). Dashed line is y = x. 4 large symbols represent group means and error bars are SEM for both TEE_{bal} (ordinate) and TEE_{IC} (abscissa). **C.** Bland-Altman plot (aka "Tukey difference-mean plot") of both methods. Solid black line represents "bias" (arithmetic mean of differences: TEE_{bal} - TEE_{IC}) and dashed black lines represent "bias" ± 1.96SD of differences ="limits of agreement" (LOA). **D.** Relative mean difference plot: (TEE_{bal} - TEE_{IC})/TEE_{IC}).Solid black bars are mean values for each group. **E.** Plot showing number of days of energy intake needed to achieve a specified accuracy true mean food intake.

	Initial - Day 0			Final - Day 37		
	BW (g)	FFM (g)	FM (g)	BW (g)	FFM (g)	FM (g)
DIO AL	49.0±0.9	23.5±0.4	16.1±1.0	49.6±1.2	23.5±0.6	16.7±0.9
DIO WR	33.6±1.1	19.7±0.2	6.6±0.7	34.1±1.1	20.1±0.3	7.1±0.7
CON AL	32.4±1.0	20.3±0.3	4.7±0.7	32.8±1.0	20.2±0.2	5.1±0.8
CON WR	21.7±0.5	14.7±0.3	1.6±0.1	21.7±0.5	14.8±0.3	1.7±0.1

TABLE 4.1: BODY WEIGHT AND BODY COMPOSITION

Mean body weight and body composition (g±s.e.m) at initial and final days of TEE_{bal} period.

CHAPTER 5: EFFECTS OF AMBIENT TEMPERATURE ON ADAPTIVE THERMOGENESIS DURING CHRONIC WEIGHT PERTURBATION.

ABSTRACT

Food-restricted rodents, may become torpid, a state of decreased metabolic activity and core body temperature. The rapid suppression of leptin production during fasting may play an integral role in the induction of torpor. We previously showed that, at ambient room temperature (22°C), mice maintained at 20% below their initial body weights (WR) by calorie restriction expend energy at a rate lower than that which can be accounted for by the decrease of fat and fat-free mass. The relevance of these studies in rodents to the decreased energy expenditure that characterizes weight-reduced humans could be confounded if there is a contribution of torpor in the hypometabolic phenotype observed in the WR mice (at 22°C). We examined the bioenergetic, hormonal, and behavioral responses to maintenance of a 20% body weight reduction in singly housed C57BL/6J +/+ HFD and *Lep*^{ob} mice housed at both 22ºC (sub-thermoneutral) and 30ºC (thermoneutral). Weight-reduced high fat-fed +/+ mice (HFD-WR) showed similar quantitative reductions in energy expenditure – adjusted for body mass and composition – at both 22ºC and 30ºC: -1.4kcal/24h and -1.6kcal/24h below predicted, respectively. Even though they did not enter torpor (defined as core body temperature below 30°C) there were small but significant declines in core body temperature in +/+ HFD-WR mice at 1400h and 0500h when compared to *ad-libitum* fed mice at both ambient temperatures. In contrast, WR mice lacking circulating leptin (OB-WR) housed at 22ºC entered torpor in the late lights off period (0200h – 0500h) with core body temperatures reaching 23ºC in some of the animals. The similarity in absolute calories conserved in HFD-WR mice whether housed at 22 ºC

or 30 ºC suggests that torpor does not account for the hypometabolic phenotype. In contrast, weight-reduced mice lacking functional leptin become torpid when housed at 22 ºC but not at 30 ºC. These studies confirm that mice with an intact leptin axis display metabolic adaptations to weight reduction similar to those seen in weight-reduced humans. Awareness of the effects of ambient temperature on metabolic homeostasis in mice should inform the design of a wide variety of experiments aimed at defining the molecular physiology of weight regulation.

INTRODUCTION

We are interested in the physiology of the weight-reduced state [17,203]. Understanding the neurobiologic bases of the reduced energy expenditure that accompanies maintenance of a reduced body weight is important to devising effective long term treatments of obesity [103]. While the mouse is a useful model for many aspects of this problem, their bioenergetics are, of course, not entirely comparable to those in humans. One important difference – in part a consequence of their higher somatic surface-to-volume ratio - is the mouse's higher zone of thermoneutrality (30°- 40°C) relative to that of a clothed human (22° - 25°) [111]. This difference contributes to the animal's use of torpor (see below) to conserve energy in the face of metabolic/thermal stress [110,113]. To the extent that such changes are invoked by weight reduction, they constitute confounds to the use of mice as models in this context. In the studies described here, we have attempted to characterize and quantify the contribution of such potential confounds to studies of energy homeostasis in weight-reduced mice.

In response to restricted access to food, mice can adapt by decreasing energy expenditure and lowering core body temperature – torpor - , a phenomenon that is not observed in humans [212-214]. Daily torpor, usually defined as a hypometabolic state (> 50% decline in total energy expenditure) followed by hypothermia (core body temperature < 30°C), is a commonly used adaptation in many small mammals under conditions of low food availability and/or decreased ambient temperature [113]. The initiation of daily torpor in mice is thought to result from decreased availability of calories in conjunction with sub-thermoneutral ambient temperatures.

The pre-optic/anterior hypothalamus (POAH) contains temperature sensitive neurons. Direct manipulation of POAH temperature produces reciprocal changes in metabolic rates (i.e.

cooling causes increased energy expenditure and heating causes decreased energy expenditure) [215,216]. Caloric deprivation results in the up-regulation of the sympathetic nervous system tone to white adipose tissue leading to decreased leptin production, a signal that seems to be intricately linked to the onset of torpor during acute responses to caloric reduction [217]. The decline in leptin and increase in ghrelin concentrations accompanying negative energy balance have been proposed to jointly act by increasing NPY activity in hypothalamus [218]. Increased NPY activity causes a decrease in metabolic rate leading to bouts of torpor that are interspersed with periods of increased food-foraging based activity [219]. Prolonged caloric restriction with sub-thermoneutral ambient temperature can result in a subsequent decrease in metabolic rate and core body temperature (torpor) [113,214]. Due to their low mass and high surface-to-volume ratio, the body temperature of mice is very responsive to food deprivation and sub-thermoneutral ambient temperature (<30°C). In these circumstances, their body temperature can decline to near ambient temperature following a drop in metabolic rate [110]. In mice, brown fat thermogenesis is an important source of heat production, resulting from UCP1-mediated mitochondrial uncoupling of the synthesis of ATP from the proton gradient through uncoupling protein 1[220]. Heat production, including brown fat thermogenesis, is primarily under the control of the sympathetic nervous system (SNS) and thyroid hormones, both of which are suppressed at reduced body weights [103,112] and are implicated in torpor [113].

In most rodent vivaria, the ambient temperature (22º-24ºC) is set for comfort of personnel working in the facility. This ambient, however, constitutes a constant thermal stress on these animals, requiring higher energy expenditure, energy intake, and sympathetic nervous system tone to maintain core body temperature [110,212]. The relationship of total energy expenditure (TEE) to ambient temperature is U-shaped, with the lowest TEE for mice occurring between 30-40°C

("thermoneutral zone"). Above 40°C and below 30°C, increased metabolic rate is required to maintain stable 5.body temperature through active cooling and thermogenic mechanisms, respectively [114]. These responses are frequently inadequately controlled for, or ignored, in metabolic studies of mice [110,114]. For example, mice deficient in UCP1 protein (*Ucp1*-/-) are resistant to diet-induced obesity (DIO) at 22 ºC [221] but highly susceptible to the same diet when housed at 30 º C [222], a reflection of the greater thermogenic stress imposed at 22 ºC. Likewise, mice lacking type 2 deiodinase (*DioII-/-*), a protein involved in the conversion of T4 to T3 in BAT and other tissues, are susceptible to DIO at 30ºC but not at 22ºC [223]. Thus, ambient room temperature can clearly affect inferences reached with regard to energy homeostasis in rodents [110,114,222,224].

The leptin axis is implicated in fasting-related phenotypes including torpor [43,113]. At 22 ºC ambient, in comparison to +/+ animals, *Lep*^{ob} mice maintain a 2 – 2.5ºC lower body temperature and have suppressed SNS tone [225]. At ambients 12°C or lower, administration of leptin protects mice null for both *Ucp1* and *Lep* from hypothermia and death [226]. Leptin administration also increases energy expenditure in food-restricted lean mice [105] and inhibits daily torpor in a 25g marsupial (*Sminthopsis macroura*) [227].

To further investigate the interplay of ambient room temperature, leptin, and metabolic adaptation to weight reduction, we examined the bioenergetics, hormonal and behavioral responses to weight reduction of +/+ (WT) DIO and *Lep*^{ob} mice housed at both 22ºC and 30ºC. We studied energy expenditure phenotypes (TEE, REE, and NREE), ambulatory movement and core body temperatures in 5 groups of animals: 10 low-fat (10% kcal from fat) diet-fed WT mice (LFD-AL); 7 DIO WT (60% kcal from fat, HFD-AL); 5 high fat diet fed *Lep*^{ob} (OB-AL) mice; 12 weight-reduced high fat diet fed WT (HFD-WR); and 9 weight-reduced high fat diet fed *Lep*^{ob}

(OB-WR) mice housed at both sub-thermoneutral (22ºC) and thermoneutral (30ºC) ambient temperatures. We also measured serum T3, glucose, and insulin concentrations in the WT mice. Finally, we assessed the same parameters in the weight reduced (HFD-WR) and low fat diet fed never-obese (LFD-AL) WT mice following a switch to *ad-libitum* access to a high fat diet. We hypothesized that torpor would not account for a significant portion of the hypometabolic phenotype anticipated in our weight-reduced WT animals [203], but would play a major role in *Lep*^{ob} mice.

MATERIALS AND METHODS

To assess the role of ambient temperature, caloric restriction/diet composition, and leptin deficiency on bioenergetic, behavioral, and hormonal responses to weight perturbation in mice, we examined wild type (WT) C57BL/6J (**Experiment 1**) and *Lep*^{ob} (**Experiment 2**) mice before and during maintenance of a 20% weight loss at both thermoneutral (30°C) and standard housing sub-thermoneutral (22°C) ambient temperatures.

Animals and Diets:

Experiment 1 – Wild type mice: 18 week-old C57BL/6J male mice were obtained from Jackson Laboratory (Bar Harbor, ME): 20 diet-induced obese (HFD mice – fed a high fat diet starting at 6 weeks of age; Research Diets, Inc. D12492i, 60% kcal as fat), and 12 low fat diet-fed (LFD mice – fed a low fat diet also starting at 6 weeks of age; Research Diets, Inc. D12450Bi, 10% kcal as fat) were used for these studies (**Figure 5.1**). Upon receipt, animals were housed in a pathogen-free barrier facility maintained at 22 ºC with a 12-h dark-light cycle (lights on at 0700 h). Mice were individually housed in plastic pens with wood-based bedding, given *ad libitum* access to diet identical to that provided at Jackson Laboratory, and water, throughout a 60 day acclimatization period. During this period, body weight was measured every 2-3 days. One HFD mouse and two LFD mice died during this period of unknown causes.

Experiment 2 – *Lep*^{ob}: Sixteen 5 week-old B6.V *Lep*^{ob} /J- male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Upon receipt, animals were housed 2 per cage in plastic pens with wood-based bedding in a pathogen-free barrier facility maintained at 22 ºC with a 12-h dark-light cycle (lights on at 0700 h). The mice were acclimatized for 1 week on standard breeder chow (Purina PicoLab 5058) and then switched to a high fat diet (identical to that used in

Experiment 1) starting at 6 weeks of age (Research Diets, Inc. D12492i, 60% kcal as fat). Mice were given *ad-libitum* access to food and water. Two mice died during this period of unknown causes.

All protocols were approved by the Columbia University Institutional Animal Care and Use Committee.

Study Design:

Experiment 1: After the 60-day acclimatization period, 27 week old mice in each group (HFD or LFD) were placed individually for 72 hours in indirect calorimetry chambers to measure baseline energy expenditure (TSE Systems, Bad Homburg, Germany – see Energy Expenditure by Indirect Calorimetry section below). Mice in the HFD group were rank-ordered by body weight, and grouped in triads. The heaviest mouse of each triad was assigned to the *ad-libitum* fed group (HFD-AL, final n = 7); the next two animals were assigned to the weight-reduced group (HFD-WR, final n = 12) until all mice were assigned to a treatment group. All mice receiving *ad-libitum* LFD were maintained on this feeding regimen until switched to HFD (day 89, **Figure 5.1A**). Mice in the weight-reduced (WR) group received 50% of their mean *ad-libitum* daily food intake until their body weights reached 80% of initial value (in mean period of 17 days) (day 27: **Figure 5.1A),** at which time the mice were fed 80% of their initial daily caloric intake. Mice were weighed daily and subsequent adjustments in calories provided were made for the remainder of the experimental period in order to maintain each mouse between 79-81% of initial body weight (**Figure 5.1A**). All mice had free access to water. HFD-WR mice were given 1/3 of their individually calculated food ration (± 0.1 g) in the morning (0745-0815h) and 2/3 of their food ration in the evening (1830-1900h). All *ad-libitum*-fed mice (HFD-AL and

LFD-AL) had free access to food continuously throughout the study. Food intake was recorded daily for the WR mice. The ambient room temperature was raised from 22°C to 30°C for 34 days following the start of the weight reduction period (days 10 – 44: **Figure 5.1A**), and then brought back down to 22°C for the remainder of the experiment. The number of calories required to maintain body weight in the HFD-WR mice was approximately twice as high at 22°C as at 30°C. Mice were maintained on their respective diet regimens for 78 days (see black arrow at day 89: **Figure 5.1A**), at which time the HFD-WR and LFD-AL were given *ad-libitum* access to the HFD for the remainder of the experiment. Energy expenditure was determined by indirect calorimetry at 4 different time-points throughout the experiment: 1) at 22°C before weight reduction; 2) at 30°C 7 days post cessation of weight reduction; 3) at 22°C 46 days post cessation of weight reduction; 4) after HFD-WR and LFD-AL mice had been given *ad-libitum* access to the high fat diet for 38 days (**Figure 5.1A**). 4-hour fasting blood was obtained by retro-orbital bleeding on the days on which mice were removed from the calorimeter (mice removed at 0800h and bled at 1200h), and at time of sacrifice (see Serum Hormone and Metabolite Profiles for details on blood handling).

Experiment 2: At 17 weeks of age, mice were individually housed in cages equipped with feeding baskets designed to minimize food spillage (**Figure 5.1B**). At 19 weeks of age, baseline energy expenditure of all 16 mice was measured by indirect calorimetry (TSE Systems, Bad Homburg, Germany). Baseline food intake was recorded over 7 days. Mouse triads were rank-ordered by body weight on the day before the start of the weight reduction protocol and the heaviest mouse assigned to the *ad-libitum* fed group (OB-AL, n = 5) while the next two lower in weight were assigned to the weight-reduced group (OB-WR, n = 9) until all mice were assigned to a treatment group. Mice in the weight-reduced (WR) groups received 50% of their average *ad-*

libitum daily food intake until their body weight reached 80% of initial value (mean period = 22 days) (**Figure 5.1B**) at which time the mice were fed 80% of their initial daily food intake. Subsequent adjustments in calories provided were made daily in order to maintain each mouse between 79-81% of initial body weight for the following 65 days (**Figure 5.1B**). Following 65 days of maintenance at the reduced body weight, the ambient room temperature was increased to 30°C (thermoneutrality) and the mice housed in the indirect calorimetry chambers for the ensuing 14 days. Food intake for the OB-WR mice during these two weeks was "clamped" to their intake at 22°C, on which regimen at 30 °C ambient, as anticipated, they gained weight (see OB-WR, **Figure 5.1B**). Total calories provided to OB-WR were decreased at the end of this two week period to match total energy expenditure by calorimetry, halting their weight gain at 90±0.8% of initial pre-weight loss body weight. Mice were again placed in the calorimeters at 30°C to assess total energy expenditure at thermoneutrality while ~10% below initial pre-weight loss weight (day 35, **Figure 5.1B**).

Body weight, body composition and food intake: Body weight was measured (± 0.1 g) before the morning feeding using an Ohaus Scout Pro 200g scale (Nänikon, Switzerland, between 0800-0830h). Body composition (fat mass: FM, fat-free mass: FFM, & extracellular fluid) was estimated by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) [142] before the morning feeding: every 2-3 weeks for both **Experiments 1 and 2**; before and after calorimetry measurements; before start of the weight reduction protocol; and on the day prior to sacrifice. Food intake (±0.1g) was recorded daily for WR animals (HFD-WR and OB-WR).

Energy expenditure by indirect calorimetry: Energy expenditure was measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany). O_2 and CO_2 measurements were taken every 26 minutes during a 72 hour period while mice were maintained on their respective weight-maintenance feeding schedules. Because of possible stress related to transfer to the chambers, only the last 48 hours of measurements were used to calculate total 24-hour energy expenditure (TEE; expressed in kcal/day) and respiratory quotient (RQ = VCO_2 / VO_2). Resting energy expenditure (REE in kcal/day) was defined as the lowest one hour period of energy expenditure; this coincided with the lowest 1 hour of total ambulatory activity (generally 1300-1400), during the 48-hour period; this value was extrapolated to 24 hours. Non-resting energy expenditure (NREE) was calculated as the difference between TEE and REE (NREE = TEE – REE). Physical activity was measured by an infrared beams integrated with the LabMaster system. Total activity (beam breaks) in X, Y, and Z axes was stored every 26 minutes. The system is designed to differentiate between fine motor movement (defined as a single X or Y axis beam break), ambulatory movement (defined as the simultaneous breaking of two adjacent X or Y beams), and rearing, defined as the breaking of the Z axis infrared beam.

Core body temperature: Rectal core body temperature (±0.1°C) of mice was measured every 3 hours for 24 hours using a Thermalert Monitoring Thermometer (TH5 model: Raytek Santa Cruz, California USA). Measurements were started at 0800 and completed the following day at 0800 in both experiments. Temperature measurements (at 22°C and 30°C ambient) were obtained at time points at least 5 days away from any indirect calorimetry measurements or bleeds.

Calculations: Energy expenditure is proportional to body mass and composition [fat-free (FFM) and fat mass (FM) [163,203]. For **Experiment 1** (WT mice), total energy expenditure (TEE; kcal/day) of HFD-AL and LFD-AL mice were related to both FFM and FM by multiple regression analysis for all four calorimetry periods [17,228]. There was no significant effect of diet composition on TEE in any of the four calorimetry periods. We therefore pooled the data from *ad-libitum* fed mice to create separate baseline regression equations relating **TEE** (kcal/24h) to FFM and FM (grams) using the calorimetry data timepoints 2 (**Figure 5.1A**: 30°C ambient temperature) and 3 (**Figure 5.1A**: 22°C ambient temperature): at 30°C, TEE = 4.3 + 0.13 * FFM + 0.09 * FM; $R^2 = 0.79$, $p < 0.0001$; at 22°C, TEE = 1.2 + 0.45 * FFM + 0.15 * FM; $R^2 = 0.87$, $p < 0.0001$. These equations were used to predict TEE for all mice following experimental weight perturbation (at the respective ambient temperatures), as we have done in similar studies of human subjects [17,127] and mice [203]. The residuals (i.e. the difference between predicted and measured values) were calculated for each animal and were tested against the null hypothesis that they were equal to zero. Baseline regression equations relating resting energy expenditure to FFM and FM, predicted **REE** values and residuals were also calculated from data obtained by indirect calorimetry as described above (**Figure 5.2A** and **B**). REE = lowest one hour period of energy expenditure extrapolated to 24h; at 30°C, REE = 3.6 - 0.01 * FFM + 0.11 * FM; $R^2 = 0.78$, $p < 0.0001$; at 22°C, REE = 1.02 + 0.28 * FFM + 0.21 * FM; $R^2 = 0.93$, $p < 0.0001$). Non-resting energy expenditure (at 30°C, NREE = TEE - REE) (NREE = 0.60 + 0.13 * FFM - 0.02 * FM; $R^2 = 0.08$, $p = 0.55$, and at 22°C, NREE = 0.17 + 0.18 * FFM – 0.06 * FM; $R^2 = 0.21$, $p = 0.19$. Since FFM and FM did not significantly predict NREE ($p = 0.19$), residuals were not calculated using the equations above but rather determined arithmetically at each calorimetry timepoint by subtracting REE from TEE (NREE = TEE – REE).

Serum Hormone and Metabolite Profiles: For **Experiment 1,** blood glucose (by tail bleed) and circulating serum insulin and bioactive thyroid hormone concentrations (by retro-orbital bleed) were determined after a 4-hour fast following each of the calorimetry experiments, and at time of sacrifice (see circles on **Figure 5.1A**). Blood for hormone and metabolite assays was allowed to clot for 2 hours at room temperature, spun at 4°C for 20 minutes at 1000g, and serum collected and frozen at -80°C until time of assay. Insulin was assayed using the Mercodia Mouse Insulin ELISA (Mercodia AB, Uppsala, Sweden); T3 using RIA at Hormone Assay & Analytical Services Core at Vanderbilt University (Vanderbilt University, Nashville, TN). All assays were conducted according to manufacturer's protocols. HOMA2 (calculator developed by University of Oxford - based on [143]) was used to estimate insulin resistance (HOMA IR) and insulin sensitivity (HOMA% S).

RESULTS

Effects of Weight Perturbation and Ambient Room Temperature on Body Weight/Composition

Experiment 1: Mice were made obese by *ad-libitum* exposure to the high fat diet for 27 weeks. These HFD mice weighed 45±3% more than *ad-libitum* LFD-fed mice, and had significantly higher fractional body fat (HFD, 36±1%; LFD, 17±1% fat) at the time of initiation of caloric restriction (HFD-WR, **Figure 5.1A**). On the first day of calorie restriction, ambient room temperature was elevated to 30°C and maintained at 30°C for the next 34 days. During this dynamic weight loss phase, HFD-WR mice were fed 50% of their ad libitum caloric intake until reaching 80±1% initial body weight (weight loss period = 17±2 days, HFD-WR mice lost 9.8±0.3g (72% of decrease accounted for by FM). HFD-AL mice gained 4.9±0.3g (100% accounted for by increased FM); LFD-AL showed no significant changes in body weight or composition during this time period (Days 10-27, **Figure 5.1A**). When the ambient room temperature was lowered back to 22ºC (day 44, **Figure 5.1A**), calories required to stabilize body weight of the HFD-WR mice were initially ~2x those required at 30° ambient; at 2 weeks, calories required were approximately 66% higher at 22ºC than at 30ºC.

Experiment 2: By design, caloric restriction (from days 34 to 98) resulted in a 20% decrease in body weight in OB-WR mice (time to achieve 20% weight reduction = 19±2 days). When ambient room temperature was raised to 30°C (day 99) and food intake of OB-WR was "clamped" to their intake at 22°C, these animals gained weight at a rate of 0.7g per day for the following 14 days. By 14 days, the mice weighed 90% of their initial body weight, meaning that they had regained 10% of the original weight lost. Total calories provided to OB-WR were then

decreased to match total energy expenditure (calculated from indirect calorimetry), and body weight was maintained at 90±0.6% of initial until time of sacrifice (day 126).

Effects of Weight Perturbation and Ambient Room Temperature on Energy Expenditure:

Experiment 1: TEE (40-45% lower) and REE (55% lower) was significantly decreased in all mice housed at 30°C (**Calo 2**) compared to 22°C (**Calo 3**: **Figure 5.1A** & **Table 5.2**). Residuals for 24-hour TEE and REE of HFD-WR mice were significantly and comparably below predicted at both 22°C (-1.4 kcal/24h, $p<0.01$ and -1.0 kcal/24h, $p<0.01$, respectively; **Figure 5.2A**) and at 30°C (-1.6 kcal/24h, $p<0.01$ and -0.8 kcal/24h, $p<0.01$, respectively; **Figure 5.2B**), indicating that – irrespective of ambient temperature - these components of EE were reduced beyond what could be attributed to changes in body mass and composition. The residual calculations were made from predicted values obtained from the multiple regression analysis using HFD-AL and LFD-AL together and adjusting for FM and FFM. Non-adjusted (for body mass and composition) TEE was significantly lower in HFD-WR and LFD-AL compared to HFD-AL at both 22°C (**Figure 5.2C**) and 30°C (**Figure 5.2D**) ambient for most measurement periods. Adjusted TEE and REE (using ANCOVA with FM and FFM as covariates) showed that while HFD-WR mice had significantly lower TEE compared to HFD-AL mice, this difference was no longer significant when mice were placed at 22°C (**Table 5.2**). Adjusted TEE of HFD-WR was significantly lower at both ambient temperatures when compared to LFD-AL (**Table 5.2**). HFD-WR mice weighed on average 14% more than LFD-AL and had 1 gram (5%) higher FFM (**Table 5.1**), yet had significantly lower non-adjusted (for FFM and FM) TEE and similar REE at 30°C, differences that are abolished when measured at 22°C. After HFD-WR and LFD-AL mice had extended (38 days) *ad-libitum* access to HFD (**Calo 4** - 22°C), both groups had significantly

increased body weights (**Table 5.1** & **Figure 5.4**) reflecting increases in both FFM and FM. FFM, the major contributor to metabolic rate, was no longer significantly different between groups at the **Calo 4** timepoint which was reflected in significant increases in absolute TEE and REE compared to the pre-weight gain measurements; these measures of EE were no longer significantly lower than those obtained in HFD-AL (**Table 5.2**) indicating a normalization of energy expenditure parameters.

Experiment 2: OB-WR mice had significantly lower absolute TEE at both 22°C (**Figure 5.2E**) and 30°C (**Figure 5.2F**) ambient temperatures compared to OB-AL mice. OB-WR TEE was decreased to approximately 30% of maximal TEE during the late hours (0400-0600) of the lights off period at 22°C (**Figure 5.2E**), coinciding with a decline in core body temperature from approximately 34°C to 26°C (**Figure 5.3B**). Absolute TEE was also significantly lower in OB-WR mice at 30°C, except during some feeding periods (black arrows: **Figure 5.2F**).

Effects of Weight Perturbation and Ambient Room Temperature on 24h Core Body Temperature:

Experiment 1: Core body temperature was significantly lower in HFD-WR mice compared to AL fed groups at the 1400h and 0500h measurement times at both 22°C and 30°C ambient temperatures (**Figure 5.3A**). No effect of ambient temperature *per se* on core body temperature of HFD-WR was noted.

Experiment 2: When housed at 30°C, OB-WR mice had significantly lower core body temperatures than OB-AL at all time points except at 2000h (**Figure 5.3B**). Interestingly, OB-WR mice maintained at 22°C ambient temperature showed decreased body temperature only at 0200h and 0500h compared to OB-AL mice housed at the same ambient temperature. However,

the differences (≈10°C) in body temperature between OB-AL and OB-WR at these two time points were much greater than those observed when mice were maintained at 30°C (≈ 3°C). Calorically restricted OB-WR mice maintained at 22°C become torpid during the latter part of the dark cycle as indicated by the striking 10 degree drop in body temperature observed at 0200h and 0500h. However, body temperatures of the OB-AL and OB-WR were identical near feeding times (0800h and 2000h).

Effects of Refeeding and Diet Switch (High Fat Diet) on Body Weight Gain in HFD-WR and LFD-AL mice:

Experiment 1:

HFD-WR and LFD-AL mice were switched to HFD on day 89 (**Figure 5.1A**) to evaluate effects of ad-libitum access to a high fat diet on food intake, weight gain, and metabolic efficiency (as reflected by the ratio of weight gain to kcal of food eaten). Food intake was measured daily for 22 days following the switch to high fat diet. Following 90 days of ad-libitum access to HFD, both HFD-WR and LFD-AL mice gained similar amounts of weight (19.0±1.0 and 20.9±0.3 grams, respectively) with FM accounting for 64±2% and 66±2% of the weight increments (**Figure 5.4A**). Body weights and body composition at time of sacrifice were not significantly different between groups (i.e. HFD-AL, HFD-WR, and LFD-AL). For the first five days following the diet switch, 24 hour food intake was significantly increased in HFD-WR and LFD-AL mice compared to HFD-AL mice (**Figure 5.4B**). During the first 24h, HFD-WR ingested 25% more calories than LFD-AL. After day 5, there was no significant difference in 24 hour food intake among the three groups. Since HFD-WR and LFD-AL mice gained similar absolute amounts of body weight, and 64% and 66% of that weight gain was fat, respectively, we

estimated feed efficiency by dividing the weight gain (g) by the cumulative food intake (g) for each 24 hour period. Feed efficiency was significantly greater in HFD-WR and LFD-AL mice than the HFD_AL during the first seven days post diet switch (**Figure 5.4C**). HFD-WR mice showed more than a two-fold higher feed efficiency during the first 24 hours compared to LFD-AL.

Effects of Weight Perturbation and Ambient Room Temperature on Blood Hormone and Metabolites:

Experiment 1:

At both 30°C and 22°C ambient temperatures, serum insulin (**Table 5.3**) and leptin (**Figure 5.5**) concentrations were highest in the most obese mice (HFD-AL) and lowest in the leanest mice (LFD-AL). Leptin correlations were highly correlated with total FM (by NMR) with near identical regression equations at both 22°C (r^2 = 0.96; leptin concentration = 4.6 * FM – 14.9) and 30°C (r^2 = 0.92; leptin concentration = 4.6 * FM – 14.5). Insulin sensitivity, measured by HOMA%S, was lowest in HFD-AL mice and highest in LFD-AL mice, and weight reduction significantly improved insulin sensitivity in the HFD-WR group at Calo2 and Calo3 time points (**Table 5.3**). At 30°C ambient (Calo2), circulating T3 concentrations were significantly lower in the LFD-AL group compared to HFD-AL (2.8±0.1 ng/ml vs. 3.3±0.1 ng/ml, respectively). At 22°C ambient, T3 concentrations in all three groups were higher than at 30ºC, with the greatest relative increase observed in HFD-AL: HFD-AL +57%; HFD-WR +26%, LFD-AL +18%. HFD-WR and LFD-AL were subsequently given *ad-libitum* access to HFD (day 89, **Figure 5.1A**). Following 40 days of HFD feeding, insulin concentrations doubled in the HFD-WR mice and quadrupled in the LFD-AL mice while glucose concentrations rose significantly in both

groups to concentrations comparable to those of HFD-AL mice (Calo 4; **Table 5.3**). These changes in insulin and glucose concentrations seen in HFD-fed animals who were previously HFD-WR and LFD-AL are reflected in 2 fold and 4 fold increases in insulin resistance, respectively, as reflected by HOMA IR (**Table 5.3**).

DISCUSSION

We recently reported that maintenance of a 20% reduction in body weight in diet induced obese (DIO) and never-obese C57BL/6J mice resulted in decreased energy expenditure per unit of metabolic mass [203]. That study was conducted at 22°C ambient and 24 hour core body temperatures were not ascertained. Laboratory mice can become torpid when food-deprived; low (relative to thermoneutrality) ambient temperatures and reduced circulating leptin concentrations facilitate the initiation of torpor [110,115]. *Lep*^{ob} mice, both fed and fasted, can spontaneously become torpid [115-117]. Leptin administration to fasted WT mice and *ob/ob* mice blunts torpor [105,115]. Under circumstances of caloric deprivation and weight loss, humans conserve energy by changes in resting energy expenditure and skeletal muscle physiology, but do not become torpid [17,42]. The studies reported here were designed to assess whether torpor plays a role in the observed metabolic adaptation seen in mice maintained at 20% below initial body weight in a standard (22°C) ambient [203]. By including ad-libitum fed (AL) and weight-reduced WT and *ob/ob* mice at both thermoneutrality (30°C) and sub-thermoneutral temperatures (22°C), we were able to investigate the interactions of weight status, leptin axis, and ambient temperature on body temperature and energy expenditure.

Torpor and the leptin axis

We find that torpor is not invoked - at either 22°C (sub-thermoneutral) or 30°C (thermoneutral) ambient temperatures - in metabolic adaptations of calorically restricted WT mice (HFD-WR) that have a functionally intact leptin axis. When WT mice were calorically restricted and maintained at 80% of their initial body weight, they reduced their metabolic rates (adjusted for FM and FFM) by similar absolute amounts at both ambient temperatures (-1.4

kcal/day at 22°C and 1.6 kcal/day at 30°C below predicted) but did not become torpid (**Figure 5.2 C&D** and **Figure 3.A**). Mice housed at 30°C require 40-45% fewer calories to maintain a stable 5.body weight than when housed at 22°C (**Table 5.2**). The 1.6 kcal/day reduction in energy expenditure in HFD-WR mice housed at 30°C constitutes a 19% decrease of 24 hour TEE and the 1.4kcal/day reduction in energy expenditure at 22°C constitutes a 9.6% decrease of 24 hour TEE in the HFD-WR mice vs. the HFD-AL. The similarity of absolute decreases in the energy expenditure of HFD-WR mice maintained at 22° and 30° ambients suggests that the adaptation to weight reduction is dictated by the change in body mass and composition *per se* and is not related to differences in thermogenic demand and/or torpid behavior. A secondary analysis of the TEE and REE data was conducted using an ANCOVA with covariates FM and FFM (**Table 5.2**). This analysis revealed that the HFD-WR mice had significantly lower adjusted TEE at 30°C (≈30% lower) but not at 22°C. The discrepancy between results obtained by either using multiple regression analysis or ANCOVA may lie in how the two analytical approaches modify the data. In the multiple regression analysis, only the *ad-libitum* mice are used to create the baseline relationship of TEE to FM and FFM. The weight-perturbed mice are then compared to this regression that defines a eumetabolic relationship between TEE and metabolic mass. On the contrary, the ANCOVA uses a mean obtained from all of the mice in order to calculate the adjusted (for FM and FFM) values. This mathematical approach has a tendency to pull values closer together possibly obscuring real differences in metabolism. A second possible explanation is that the thermogenic stress that mice are subjected to at 22°C may obscure real metabolic changes that can only be observed once mice are at thermoneutrality.

When mice with congenital leptin deficiency are weight reduced (OB-WR) at 30° C ambient , they display a 3°C decrease in core body temperature between 0000h to 0500h (lights

off from 1900h to 0700h). These animals become torpid during those hours (core body temperature < 30°C) when housed at 22°C ambient. Circulating leptin concentrations in both humans and rodents are highest from 0000h to 0600h, the period when the OB-WR mice enter torpor at 22°C ambient [229,230]. If REE is defined as the lowest one hour of TEE in an awake post-digestive state when a mouse is not in torpor (similar to definition of REE in humans), OB-WR mice have an REE of ≈10kcal/24h/mouse at 22°C ambient (**Figure 5.2E**) and ≈5 kcal/24h at 30°C (**Figure 5.2F**) between 1100h and 1600h. The two-fold higher REE observed in OB-WR mice housed at 22°C vs. at 30°C reflects the increased thermogenic demand for maintainenance of normal body temperature at the lower ambient. Interestingly, TEE in OB-WR mice housed at 22°C is similar to that in animals housed at 30°C (≈5 kcal/24h) between 0200h and 0800h. (**Figure 5.2E** and **Figure 5.3B**). The similarity in TEE values in the OB-WR mice at 22°C and 30°C ambient temperatures at 0200-0800h suggests that in the absence of a functional leptin axis, sub-thermoneutral temperatures and the maintenance at a reduced body weight results in metabolic rate decreases a drop in core body temperature when housed at 22°C. In WT HFD-WR mice, TEE at 0200h to 0800h is approximately 50% lower at 22°C (5.5kcal/24h) than at 30°C (11kcal/24h). The OB-WR mice are shutting down adaptive thermogenesis at an ambient temperature that constitutes a constant thermogenic stress (i.e. 22°C). The WT HFD-WR mice with intact leptin axis (**Figure 5.5**) are capable of maintaining near normal body temperatures at both 30° and 22°C ambient temperatures, which is reflected in the 2-fold higher TEE at the latter ambient temperature (i.e. 22°C). On the contrary, exposure to 22°C ambient in the weight-reduced *Lep*^{ob} animals (OB-WR) that lack an intact leptin axis results in a decrease in both metabolic rate and core body temperature (i.e. torpor).

Effects of high fat diet feeding on never-obese and calorically restricted formerly obese mice

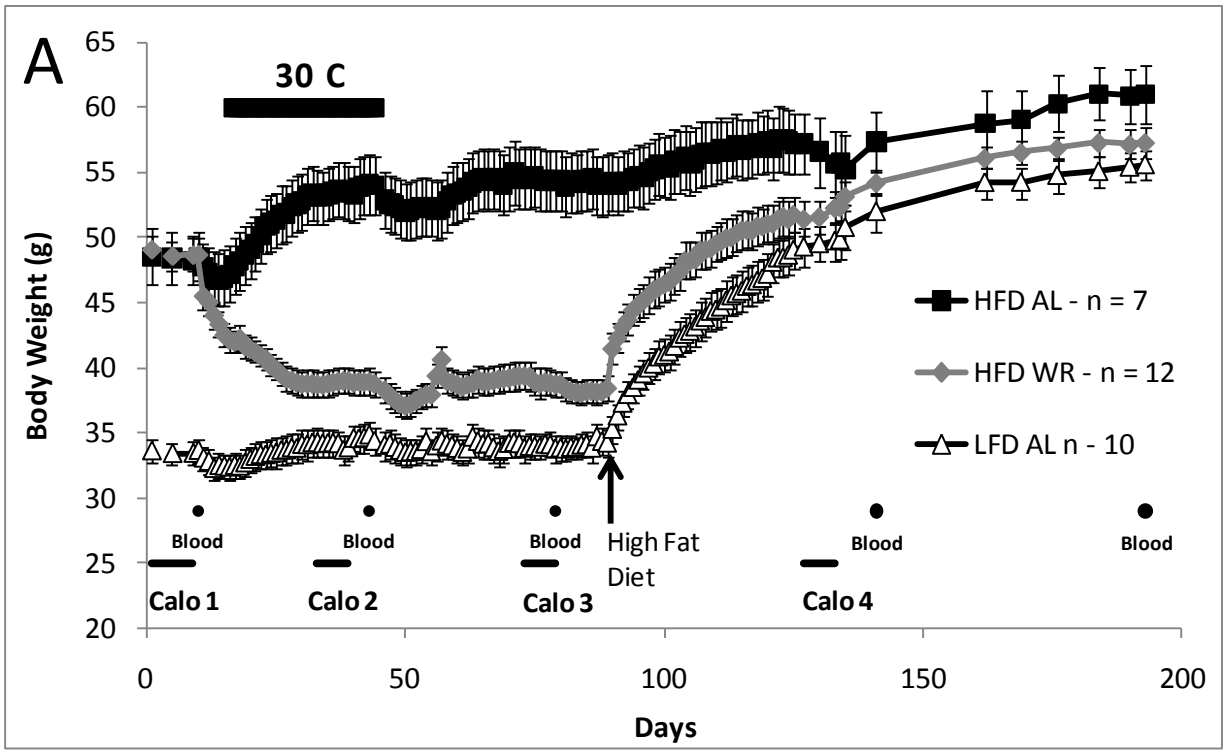
When non-obese control-diet fed mice (LFD-AL) and formerly-obese weight-reduced mice (HFD-WR) were given *ad-libitum* access to the HFD, both groups ingested significantly more calories - despite lower body weights - than the obese HFD-AL for the first 7 consecutive days (**Figure 5.4B:** see arrow **Figure 5.1A**). This increased caloric intake per unit body mass resulted in rapid gains of body weight in both groups and probably reflects hedonic drive to eat the highly palatable 5.food [231]. The higher feed efficiency observed in HFD-WR mice on the first day most likely reflects combined effects of: 1) increased metabolic efficiency (i.e. increased ratio of weight gained per calorie consumed); 2) lower initial day 0 body weight of the HFD-WR mice since they would have been without food in the gut as opposed to the LFD-AL which already had *ad-libitum* food access and would therefore have had some residual weight from non-digested food in the digestive tract.

These data suggest that torpor is not involved in the metabolic adaptation seen in weight-reduced WT animals at either 22°C or 30°C. Torpor was observed only in weight reduced mice housed at sub-thermoneutral temperatures (i.e. 22°C) that lacked circulating leptin (OB-WR), suggesting that a functional leptin axis in weight-reduced conditions is sufficient to maintain core body temperature and inhibit the onset of torpor even at 22°C ambient temperature (**Table 5.4**). Under fasting conditions, the sympathetic nervous system tone is upregulated to white adipose tissue, resulting in increased lipolysis and decreased leptin production. Dopamine β hydroxylase -/- mice that are incapable of sympathetically activating WAT due to the lack of epinephrine and norepinephrine production do not suppress leptin production and do not enter torpor during a fast [232]. The normal relationship of circulating leptin concentration to FM

observed at both ambient temperatures (**Figure 5.5**) suggests that mice are; 1) not in negative energy balance; and 2) have an intact leptin axis that should preclude these mice from entering torpor. In the absence of photic clues, twice daily feeding alters the circadian rhythm of mice [233]. In 6-month calorically restricted humans (fed 25% fewer calories than their baseline requirement), mean 24 hour leptin concentrations were decreased (in proportion to the decrease in fat mass) but showed normal 24 hour cycling with a slight increase in amplitude [234]. In the mice of this study, circadian entrainment resulting from the feeding schedule may have kept the 24 hour leptin rhythm intact. In C57BL/6J female mice, a 24 hour fast caused only 10% of the mice to enter torpor [235]. The mice in this study were without food at most 14 hours between the night (1830-1900h) and the morning feeding (0745 – 815h). In male C57BL/6J, an acute 14 hour fast results in a maximum drop of 20-25% in circulating leptin concentrations [236]. Since our mice have been entrained for more than 2 months to receive food at specific times each day, this drop – related to an acute fasting period – may be abrogated. A leptin time course on twice daily fed mice could answer this question.

As noted in the Introduction, we are interested in the physiology of the weight-reduced state. The weight-reduced mouse shows reductions in energy expenditure (corrected for metabolic mass) that are comparable to those in humans [203]. Because the studies were conducted at the conventional ambient of 22°C, we wanted to assess the possibility that some of this decline might be an artifact of the induction of torpor in these animals. We found no evidence of torpor in WT wt-reduced animals. However, the increased metabolic demand imposed by the weight reduced state at 22°C minimizes the degree of hypometabolism when compared to animals at 30deg C ambient. In studies such as these, and others in which intercurrent metabolic stress

could be a confound, an ambient of 30°C will provide bioenergetic data most comparable to those in human subjects.


A
30 C
Body Weight (g)
65
60
55
50
45
40
35
30
25
20
HFD AL - n = 7
HFD WR - n = 12
LFD AL n - 10
Blood
Blood
Blood
High Fat Diet
Blood
Blood
Calo 1
Calo 2
Calo 3
Calo 4
0
50
100
150
200
Days

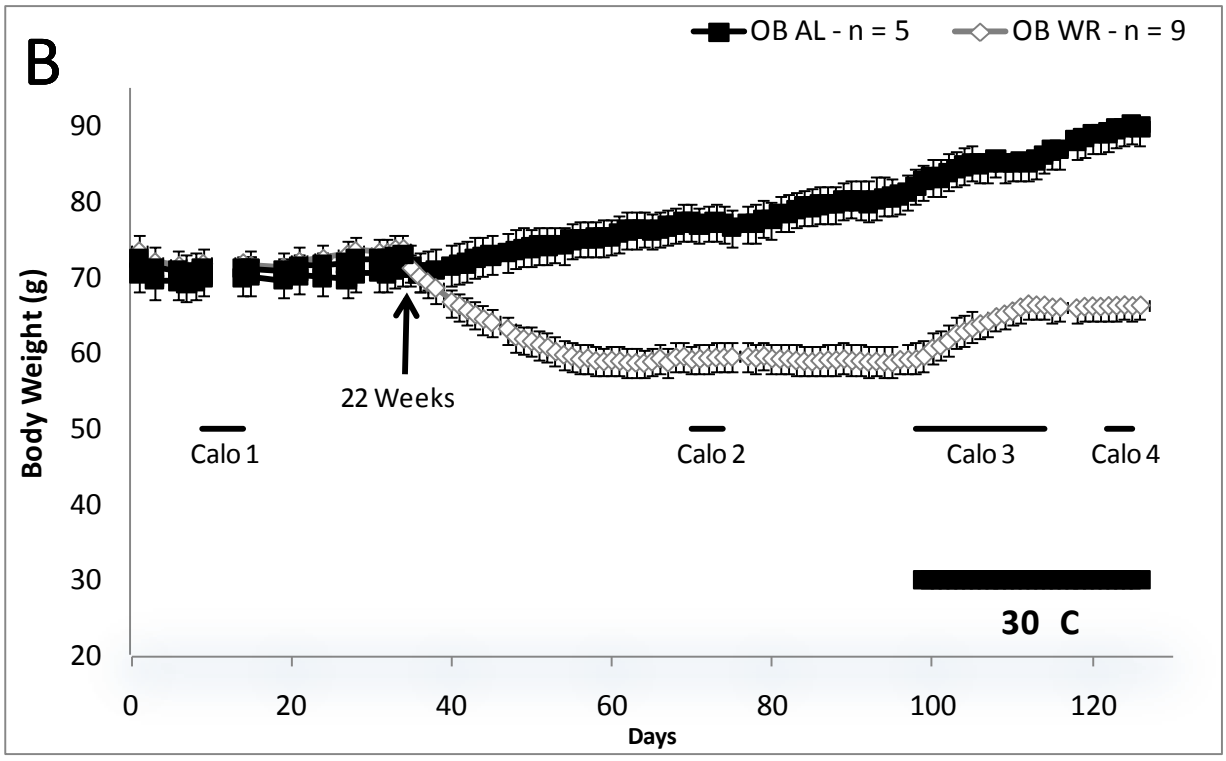
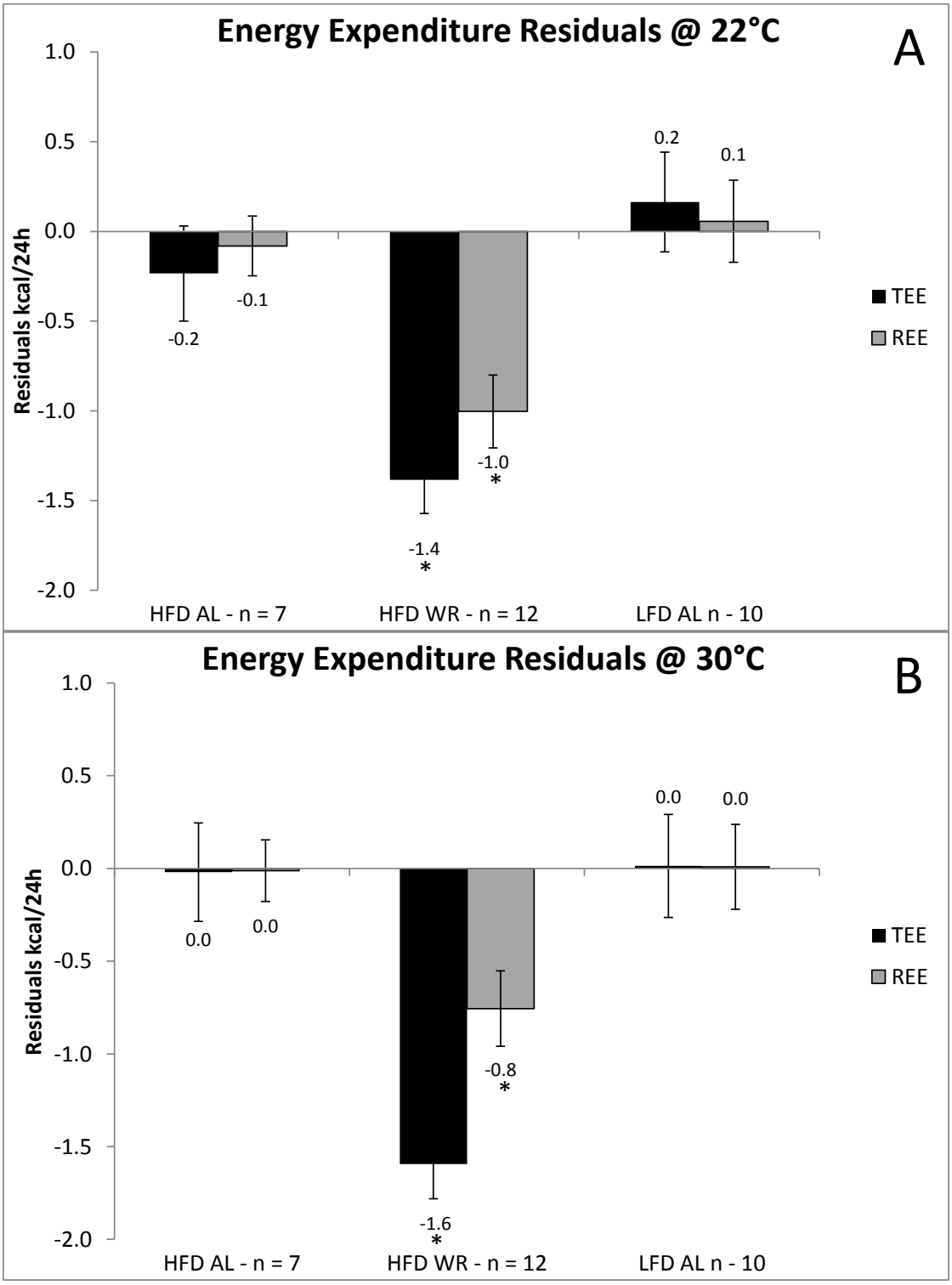

B
OB AL - n = 5
OB WR - n = 9
Body Weight (g)
90
80
70
60
50
40
30
20
22 Weeks
Calo 1
Calo 2
Calo 3
Calo 4
30 C
0
20
40
60
80
100
120
Days

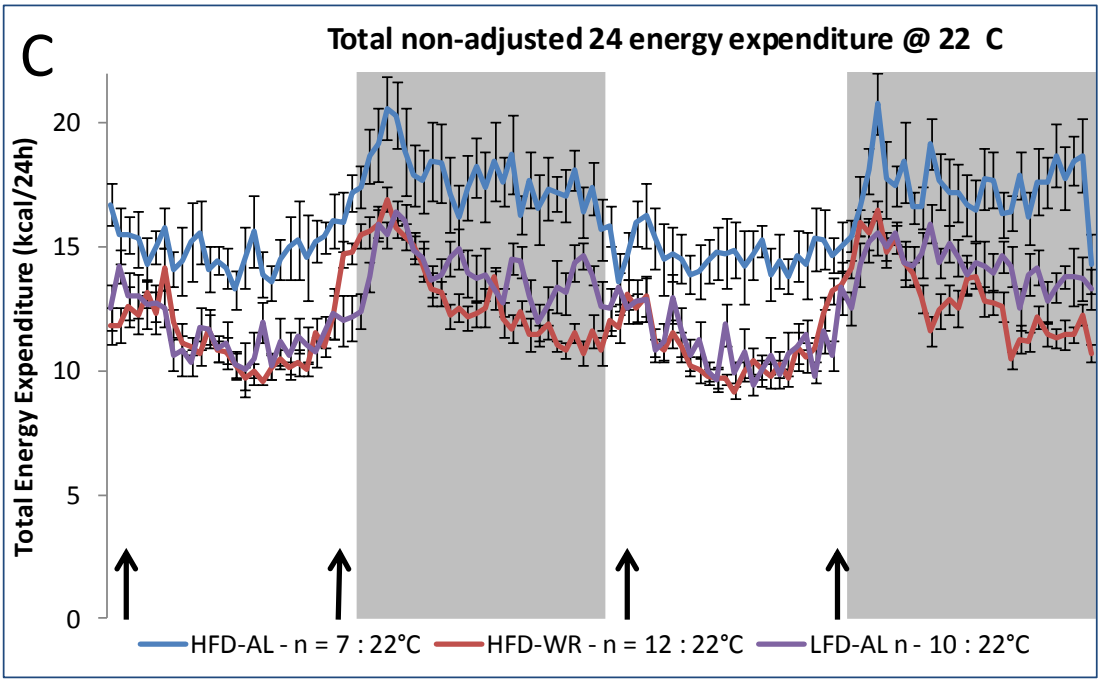
FIGURE 5.1 BODY WEIGHT AND TIMELINE

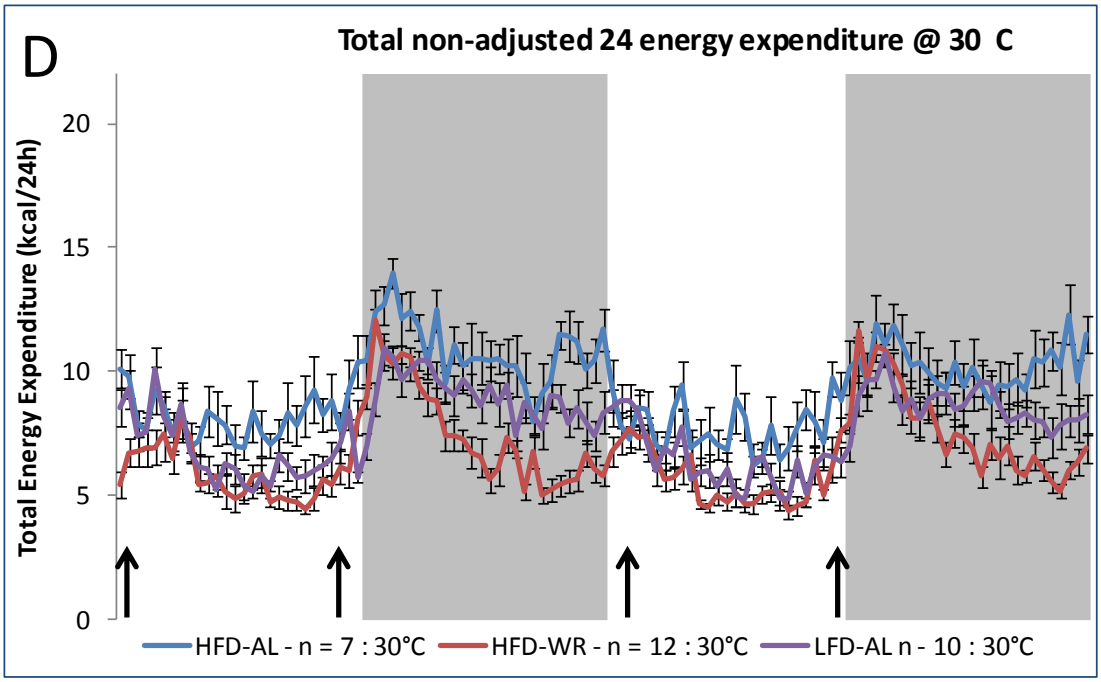
Mean (±S.E.) body weights of wild type **(experiment 1 - A)** or *Lep*^{ob} mice **(experiment 2 - B)**. Mice housed at 22°C at all times except where marked by thick black line during which ambient room temperature was maintained at 30°C. Indirect calorimetry sessions are indicated by thin black lines and 4 hour fasting bleeds at 1200 are indicated by circles (**experiment 1-A only**).

In **experiment 1-A**, mice were housed at 30°C and allowed *ad-libitum* access to: 1. high fat diet (60% kcal from fat: HFD-AL); 2. low fat diet (10% kcal from fat: LFD-AL); or 3. given a restricted amount (50% of normal food intake) of the high fat diet twice a day until they had lost 20% of their initial body weight (HFD-WR). HFD-WR were maintained at 80±1% initial body weight for 60 days on the high fat diet, during which period all mice were had indirect calorimetry performed at 30°C (Calo 2) and 22°C (Calo 3). After 80 days of food restriction, all mice were given *ad-libitum* high fat diet while housed at 22°C ambient (arrow).

In **experiment 2-B**, leptin deficient mice were given *ad-libitum* access to HFD from 6 until 22 weeks of age. A subset of these mice was calorie restricted by twice daily feeding (50% *ad-libitum* food intake) of the high fat diet until they had lost 20% of their initial body weight. After being maintained weight stable 5.for 30 days at 80±1% initial body weight by intercurrent adjustment of calories provided, the room temperature was increased from 22°C to 30°C. Following the switch in ambient temperature, HFD-WR mice were fed the same number of calories of HFD that they had received at 22°C for 16 days while being housed in the calorimeter (see calo 3 timepoint). On this regimen the animals gained weight. Upon reaching 90±0.8% initial body weight, caloric intake was reduced to match energy expenditure in order to maintain animals at 90±1% body weight. Mice subsequently underwent another round of indirect calorimetry (see calo 4 timepoint) while being maintained at this weight.


Energy Expenditure Residuals @ 22°C
A
Residuals kcal/24h
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-0.2
-0.1
-1.4
*
-1.0
*
0.2
0.1
HFD AL - n = 7
HFD WR - n = 12
LFD AL n - 10
TEE
REE
Energy Expenditure Residuals @ 30°C
B
Residuals kcal/24h
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0.0
0.0
-1.6
*
-0.8
*
0.0
0.0
HFD AL - n = 7
HFD WR - n = 12
LFD AL n - 10
TEE
REE


C
Total non-adjusted 24 energy expenditure @ 22 C
Total Energy Expenditure (kcal/24h)
20
15
10
5
0
HFD-AL - n = 7 : 22°C
HFD-WR - n = 12 : 22°C
LFD-AL n - 10 : 22°C


D
Total non-adjusted 24 energy expenditure @ 30 C
Total Energy Expenditure (kcal/24h)
20
15
10
5
0
HFD-AL - n = 7 : 30°C
HFD-WR - n = 12 : 30°C
LFD-AL n - 10 : 30°C

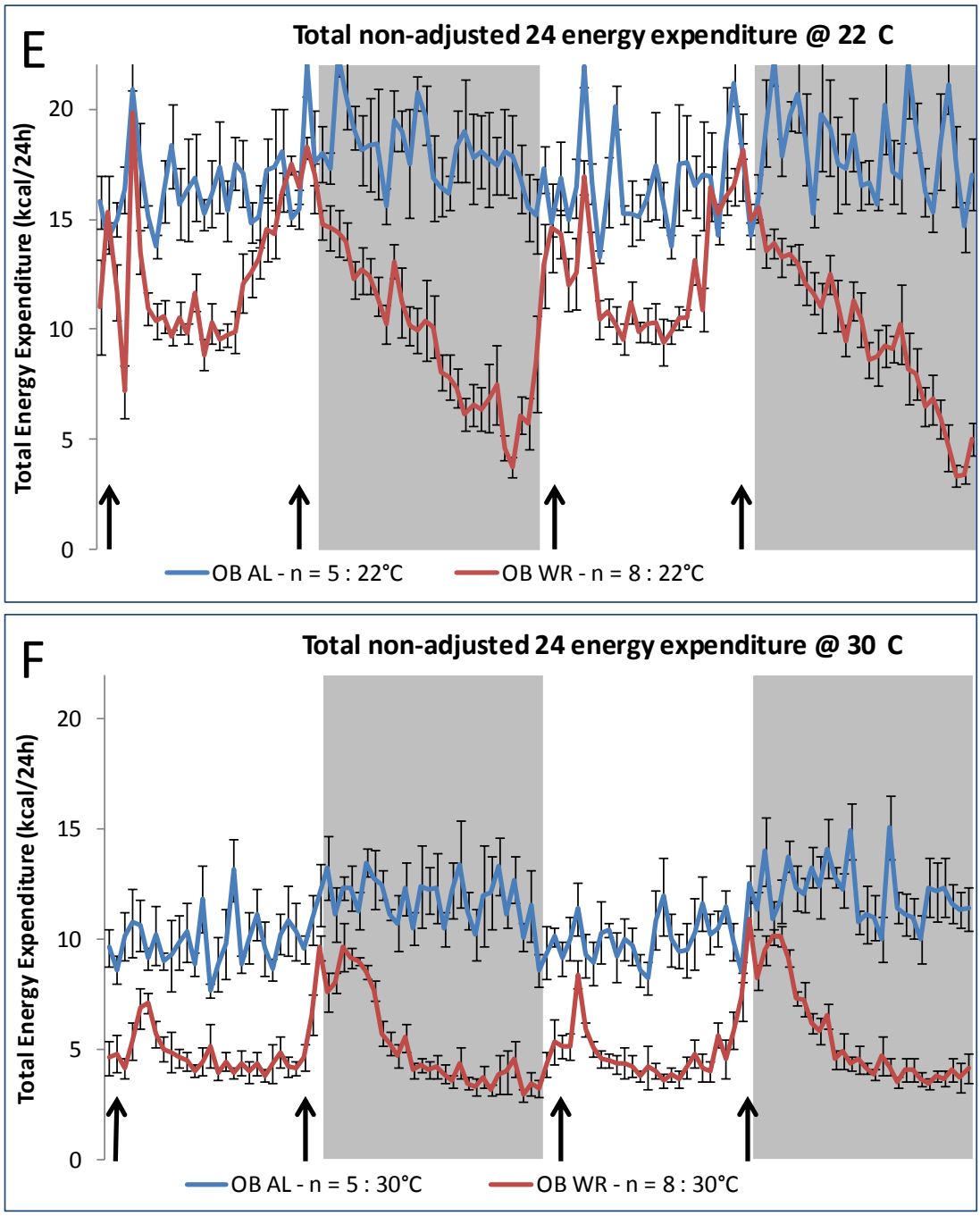


FIGURE 5.2 ENERGY EXPENDITURE PHENOTYPES IN LEP^{OB} AND WT MICE

Predicted values were obtained from multivariate regressions relating energy expenditure to FFM and FM of *ad lib* fed mice (HFD-AL + LFD-AL) at 30°C (TEE = 4.3 + 0.13 * FFM + 0.09 * FM; R^2 = 0.79, $p < 0.0001$, and REE = 3.6 - 0.01 * FFM + 0.11 * FM; R^2 = 0.78, $p < 0.0001$); and at 22°C (TEE = 1.2 + 0.45 * FFM + 0.15 * FM; R^2 = 0.87, $p < 0.0001$ and REE = 1.02 + 0.28 * FFM + 0.21 * FM at 22; R^2 = 0.93, $p < 0.0001$). Predictions of TEE and REE

based on measured FM and FFM were then made for each mouse at both ambient temperatures. The mean (±s.e.m.) observed-minus-predicted for each group 24-hour total (black bars) and 24-hour resting (grey bars) energy expenditure (kcal/24hr) for mice at 22°C **(A)** and 30°C **(B)** ambient room temperatures for experiment 1. **(C – F)** Non-adjusted (for FM and FFM) group mean (±s.e.m.) 24-hour total energy expenditure measured every 26 minutes over 48 hour period for WT mice (**experiment 1**) at 22°C **(C)** and 30°C **(D)** and *Lep*^{ob} mice (**experiment 2**) at 22°C **(E)** and at 30°C **(F)**. Black arrows represent feeding times for the HFD-WR mice in **C & D** OB-WR in **E & F**.

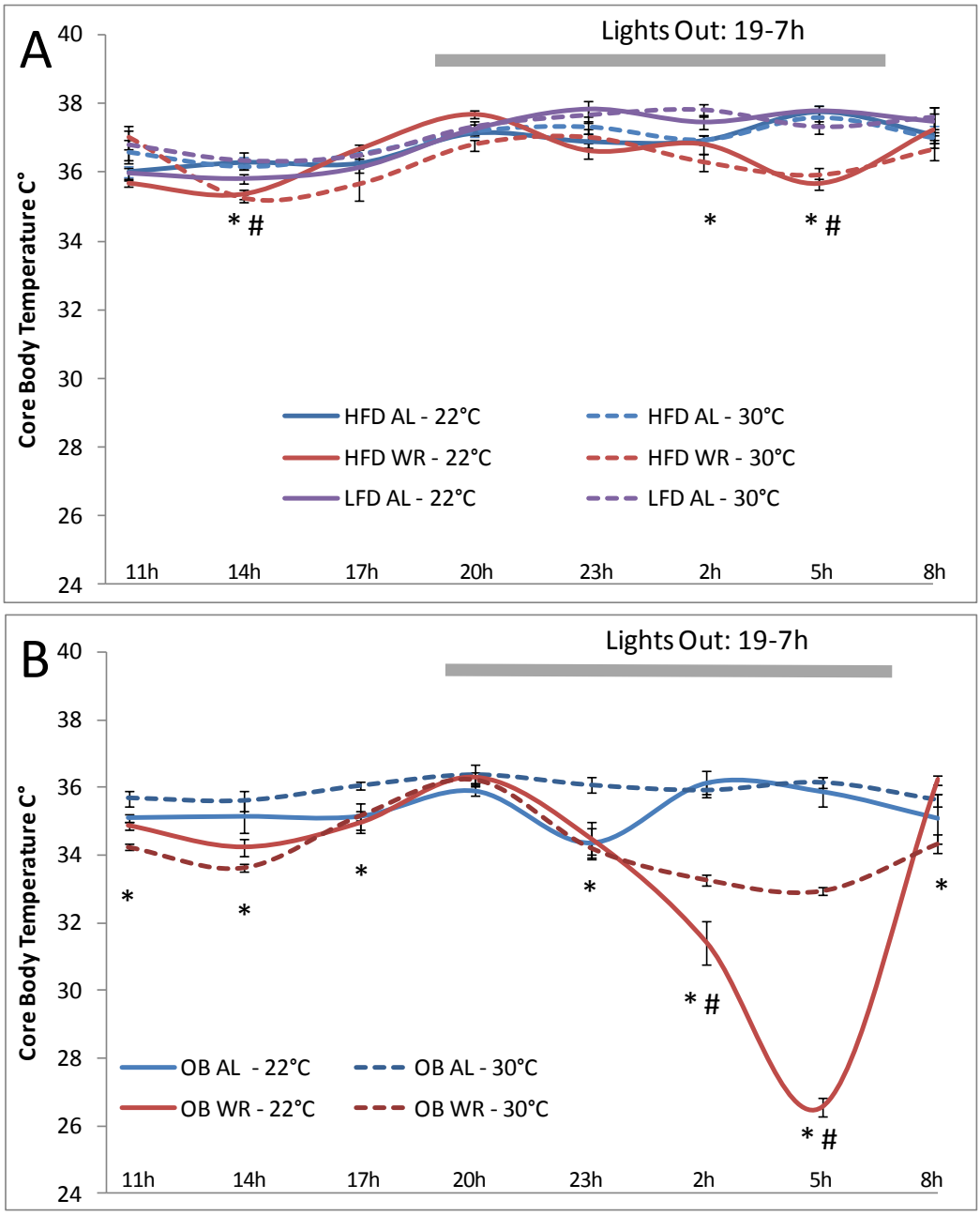
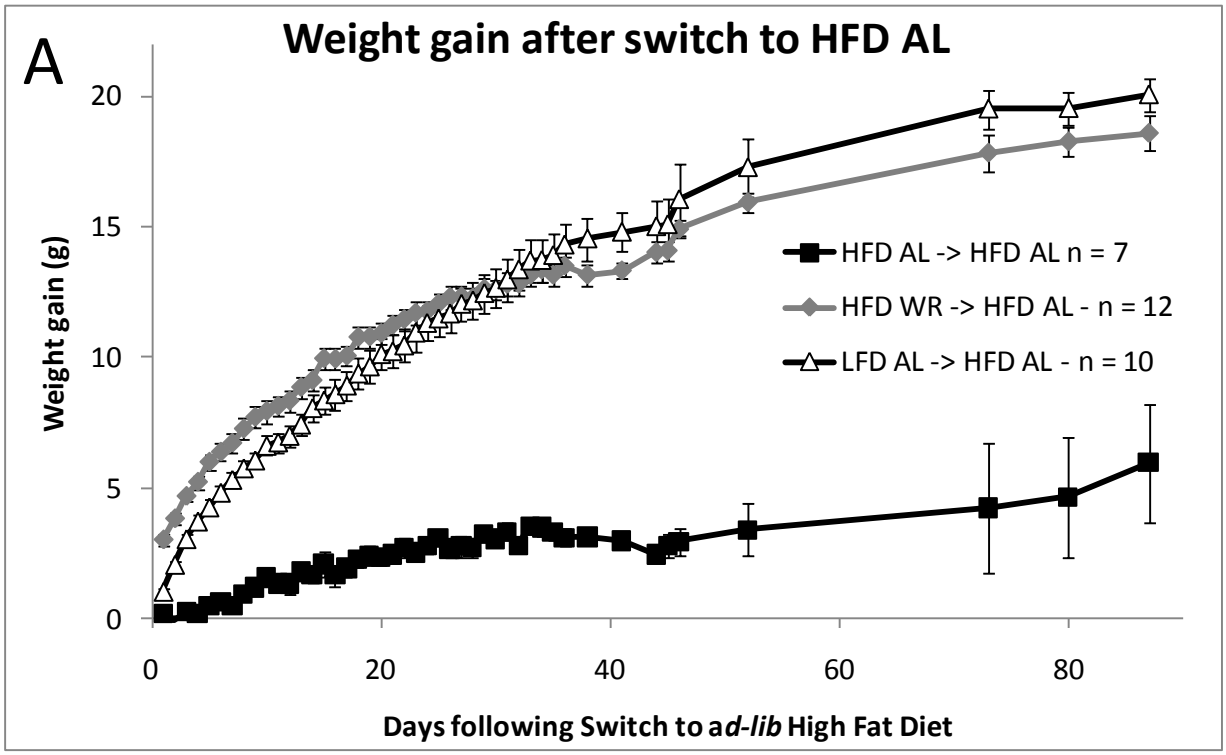
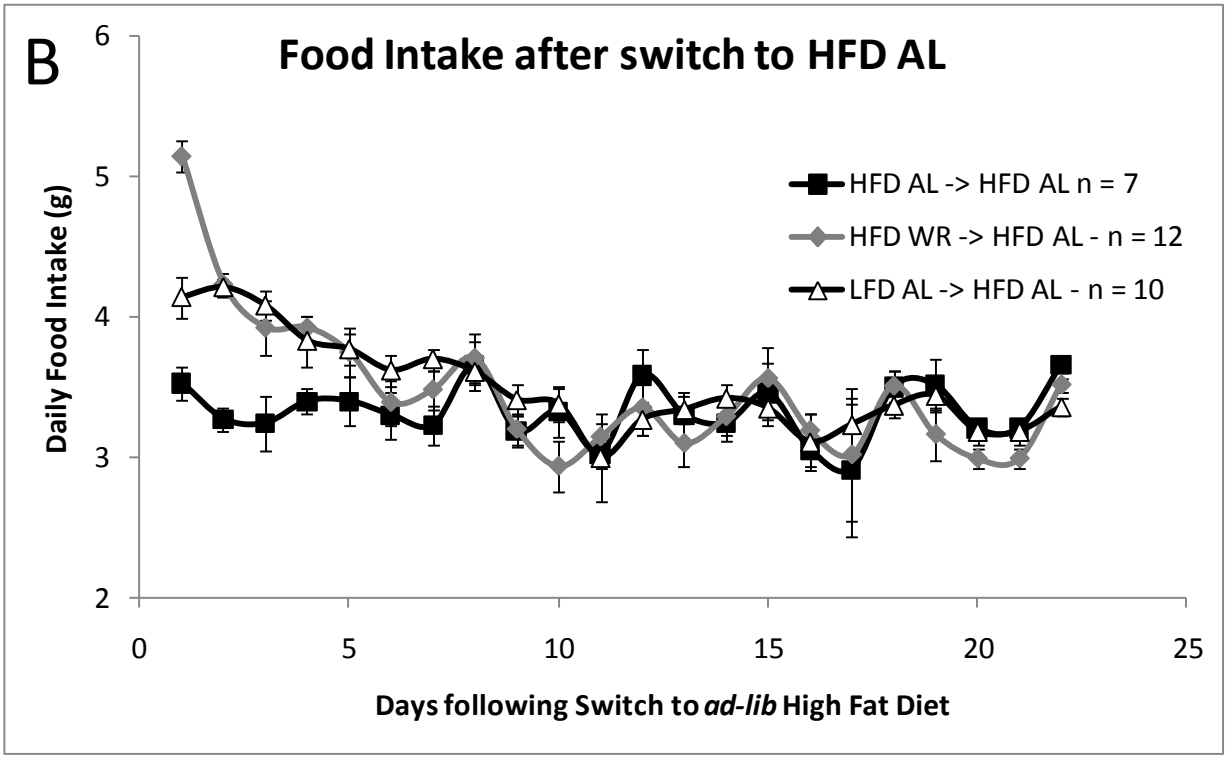


FIGURE 5.3: CORE BODY TEMPERATURE AT DIFFERENT AMBIENT TEMPERATURES

24 hour temperature profile. Mean core body temperatures (°C ±S.E.) were obtained in singly-housed mice (**experiment 1 – A**) or housed in pairs (**experiment 2 – B**) every 3 hours over two 24 hour periods. Solid lines represent core body temperatures when ambient temperature was 22°C; dashed lines represent core body temperatures when ambient temperature was 30°C. **(A)** C57BL/6J wild type mice (**experiment 1**): 1. *ad-libitum* access to high fat diet (HFD-AL); 2. weight-reduced by hypocaloric feeding of the high fat diet (HFD-WR); 3. given *ad-libitum* access to a low fat diet (LFD-AL). * and # denote significantly lower body temperatures in HFD-WR vs. both HFD-AL and LFD-AL groups at 30°C and 22°C, respectively. **(B)** C57BL/6J

leptin deficient mice (Lep^{ob} -/-, experiment 2) : 1. *ad-libitum* access to a high fat diet (OB-AL); 2. weight-reduced by hypocaloric feeding of the high fat diet (OB-WR). * and # denote significantly lower body temperatures in OB-WR vs. OB-AL groups at 30°C and 22°C, respectively.


A
Weight gain after switch to HFD AL
Weight gain (g)
0
5
10
15
20
0
20
40
60
80
Days following Switch to a*d-lib* High Fat Diet
HFD AL -> HFD AL n = 7
HFD WR -> HFD AL - n = 12
LFD AL -> HFD AL - n = 10


B
Food Intake after switch to HFD AL
Daily Food Intake (g)
2
3
4
5
6
0
5
10
15
20
25
Days following Switch to *ad-lib* High Fat Diet
HFD AL -> HFD AL n = 7
HFD WR -> HFD AL - n = 12
LFD AL -> HFD AL - n = 10

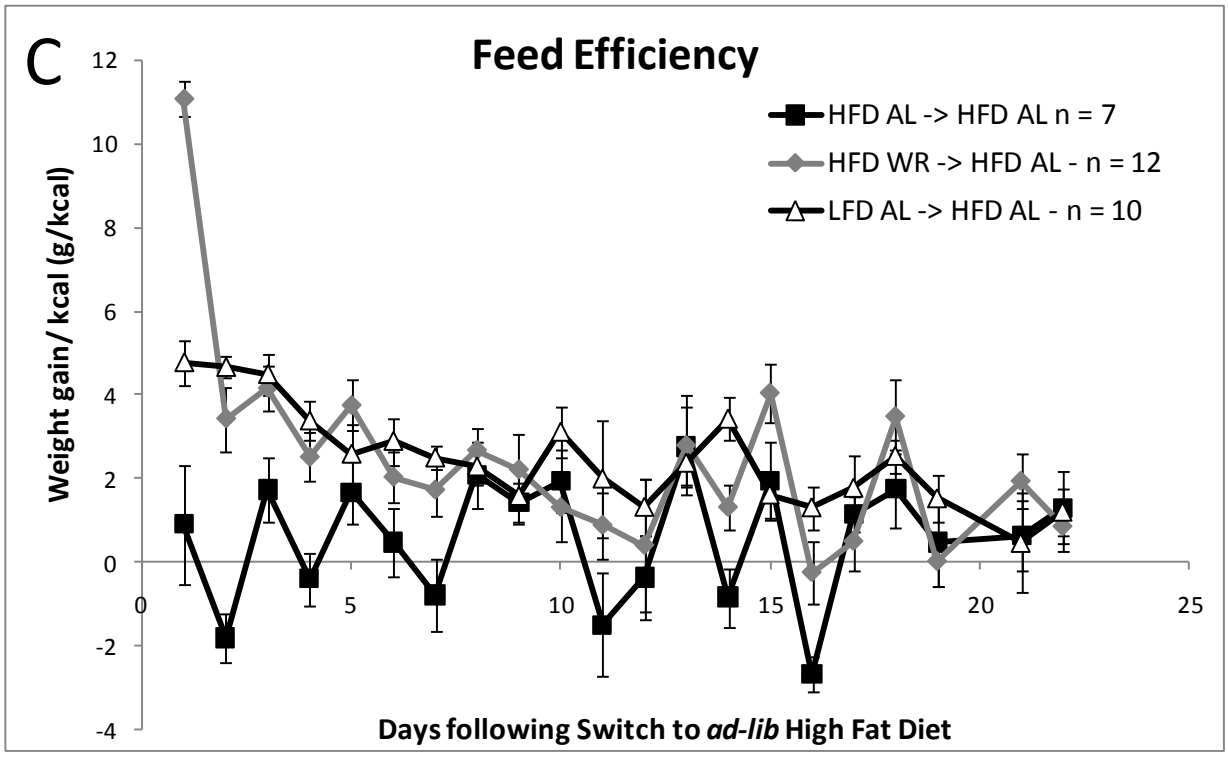


FIGURE 5.4: WEIGHT REGAIN FOLLOWING AD-LIBITUM ACCESS TO HIGH FAT DIET

After mice from experiment 1 were maintained on their respective feeding regimens for 79 days, both LFD-AL and HFD-WR WT mice were given *ad-libitum* access to HFD. Mouse weight **(A)** and food intake **(B)** were measured daily for all mice. Feed efficiency **(C)** is approximated by the ratio of weight gain (gram) to food ingested (calories) over a 24 hour period. HFD-WR and LFD-AL groups gained similar amounts of body weight (19.0±1.0 and 20.9±0.3 grams, respectively) with FM accounting for similar proportions of this increased mass (64±2% and 66-±2% respectively). Data presented as mean±s.e.m.

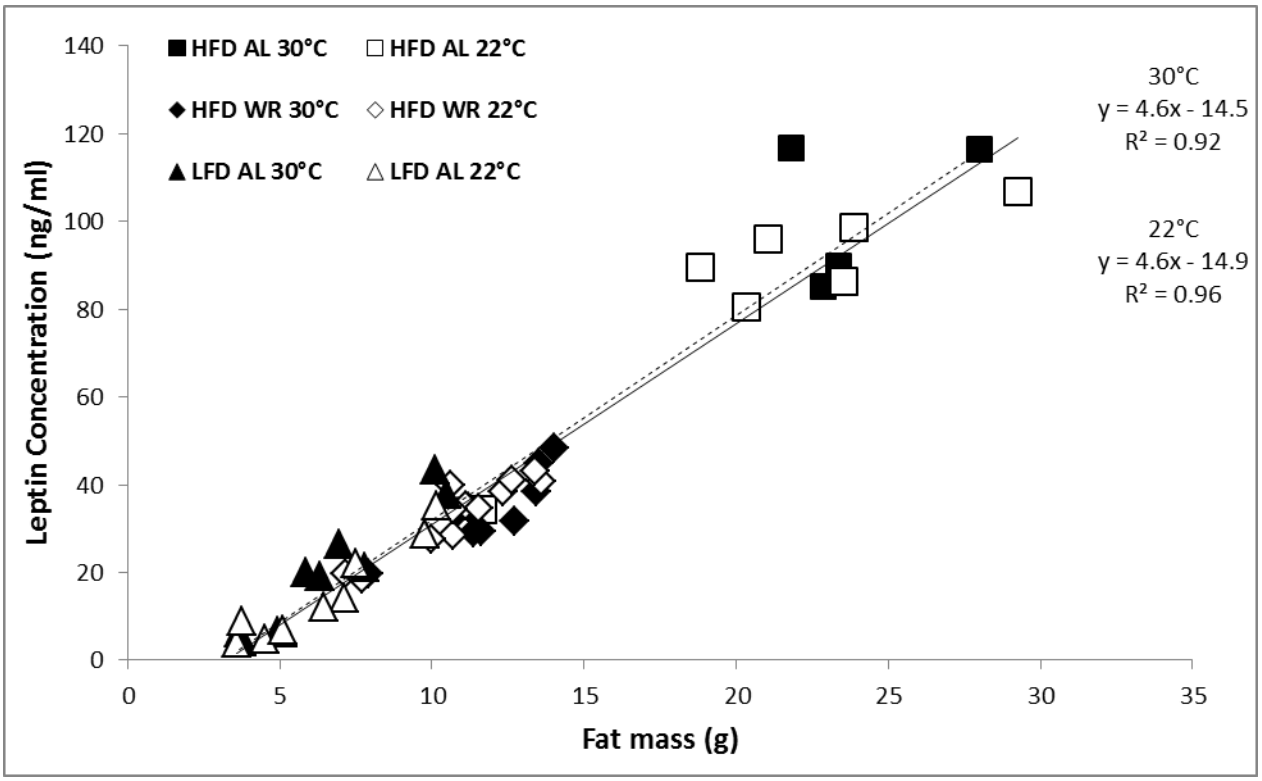


FIGURE 5.5: SERUM LEPTIN CONCENTRATION VERSUS FAT MASS AT DIFFERENT AMBIENT TEMPERATURES

Leptin (ng/ml) to fat mass (g). Linear regression for all mice; 22°C (solid line) and 30°C (dashed line). Squares represent HFD-AL, diamonds HFD-WR, and triangles LFD-AL at either 30°C (filled in shapes) or 22°C (open shapes).

	Body Weight & Composition			
	Body Weight	**Fat-Free Mass**	**Fat Mass**	**% Fat Mass**
Calo 2: 30°C - WR				
HFD-AL	53.4 ± 2.1^{A}	24.9 ± 0.3^{A}	21.2 ± 1.5^{A}	39 ± 1^{A}
HFD-WR	38.9 ± 2.1^{B}	23.2 ± 0.5^{B}	11.6 ± 0.5^{B}	30 ± 1^{B}
LFD-AL	34.2 ± 1.1^{C}	22.4 ± 0.4^{B}	6.4 ± 0.8^{C}	18 ± 2^{C}
Calo 3: 22°C - WR				
HFD-AL	54.4 ± 2.4^{A}	$26.5\pm0.5^{A€}$	21.2 ± 1.7^{A}	38 ± 2^{A}
HFD-WR	38.9 ± 1.1^{B}	23.4 ± 0.5^{B}	11.0 ± 0.6^{B}	28 ± 1^{B}
LFD-AL	33.9 ± 1.0^{C}	23.0 ± 0.4^{B}	6.3 ± 0.7^{C}	18 ± 2^{C}
Calo 4: 22°C - Post WR				
HFD-AL	56.3 ± 2.6^{A}	26.8 ± 0.6	22.2 ± 1.7^{A}	39 ± 1
HFD-WR	$51.8\pm1.2A^{B\$}$	$25.6\pm0.4^{\$}$	$19.5\pm0.6^{AB\$}$	$38\pm1^{\$}$
LFD-AL	$49.6\pm1.4^{B\$}$	$25.4\pm0.3^{\$}$	$18.0\pm0.8^{B\$}$	$36\pm1^{\$}$

TABLE 5.1: BODY WEIGHT AND COMPOSITION AT DIFFERENT AMBIENT TEMPERATURES

Body weight and body composition (FM and FFM) of the three mouse groups at 30ºC (Calo2) and 22ºC (Calo3) ambient: 1. during the weight perturbation period (i.e. HFD-WR maintained at 80% initial body weight); and 2. at 22ºC following weight regain of HFD-WR and LFD-AL subsequent to *ad-libitum* access to HFD (Calo 4). Phenotypes not connected by same letter within Calo measurement period are significantly different ($p<0.05$). € - Significantly different between Calo3 and Calo2 (ambient temperature comparison; $p<0.05$). $ - significantly different ($p<0.05$).between Calo4 and Calo3. HFD feeding for all group comparisons.

	Energy Expenditure					
	TEE (kcal/24h)	**TEE adjusted (kcal/24h)**	**REE (kcal/24h)**	**REEadjusted (kcal/24h)**	**NREE (kcal/24h)**	**Movement (1000x)**
Calo 2: 30°C - WR						
HFD-AL	9.3±0.3^{A}	8.6±0.4^{A}	5.6±0.3^{A}	4.7±0.3^{A,B}	3.7±0.1^{A}	158.8±28.6^{A}
HFD-WR	6.7±0.2^{C}	6.7±0.2^{B}	4.0±0.1^{B}	4.0±0.1^{B}	2.7±0.1^{B}	162.8±21.9^{A}
LFD-AL	7.7±0.1^{B}	8.1±0.3^{A}	4.1±0.1^{B}	4.6±0.2^{A}	3.6±0.1^{A}	251.7±24.0^{B}
Calo 3: 22°C - WR						
HFD-AL	16.3±0.7$^{A,€}$	13.2±0.5$^{A,B,€}$	12.4±0.5$^{A,€}$	9.8±0.4$^{A,B,€}$	3.9±0.1$^{€}$	106.7±16.4^{A}
HFD-WR	12.1±0.2$^{B,€}$	12.5±0.2$^{B,€}$	8.6±0.2$^{B,€}$	8.8±0.2$^{B,€}$	3.5±0.1$^{€}$	175.0±12.5^{B}
LFD-AL	12.8±0.4$^{B,€}$	14.5±0.4$^{A,€}$	8.7±0.3$^{B,€}$	10.2±0.3$^{A,€}$	4.1±0.1$^{€}$	210.1±13.7^{B}
Calo 4: 22°C - Post WR						
HFD-AL	15.8±0.7	14.7±0.4^{A}	11.7±0.7	10.7±0.3^{A}	4.1±0.2$^{\$}$	88.5±15.9
HFD-WR	15.8±0.4$^{\$}$	15.9±0.3$^{B,\$}$	11.9±0.4$^{\$}$	12.0±0.2$^{B,\$}$	3.9±0.3	131.0±12.7
LFD-AL	15.8±0.5$^{\$}$	16.5±0.3$^{B,\$}$	12.0±0.2$^{\$}$	12.6±0.2$^{B,\$}$	3.8±0.3	139.6±14.0

TABLE 5.2: ENERGY EXPENDITURE AND MOVEMENT AT DIFFERENT AMBIENT TEMPERATURES

Total energy expenditure (TEE: by indirect calorimetry), resting energy expenditure (REE: lowest one hour TEE period), non-resting energy expenditure (NREE: calculated as TEE – REE) and ambulatory movement (1000x) for all three mouse groups at 30ºC (Calo2) and at 22ºC (Calo3) during the weight perturbation period: 1. HFD-WR maintained at 80% initial body weight; and 2. at 22ºC following weight regain of HFD-WR and LFD-AL subsequent to *ad-libitum* access to HFD for all three groups (Calo 4). "TEE adjusted" and "REE adjusted" were calculated using ANCOVA with group as factor and FM & FFM as covariates. Values are group mean±s.e.m. Levels not connected by same letter within Calo measurement period are significantly different ($p<0.05$). € - Significantly different between Calo3 and Calo2 (ambient temperature comparison; $p<0.05$). $ - significantly different between Calo4 and Calo3 ($p<0.05$). HFD feeding for all group comparison.

	Blood Hormone and Metabolites				
	T3 (ng/dl)	**Glucose (mg/dl**	**Insulin (ug/l)**	**HOMA%S**	**HOMA IR**
Calo 2: 30°C - WR					
HFD-AL	3.3 ± 0.1^{A}	135 ± 4.1^{A}	0.26 ± 0.05^{A}	136.3 ± 20.3^{A}	0.92 ± 0.17^{A}
HFD-WR	3.1 ± 0.1^{AB}	118 ± 4.5^{AB}	0.16 ± 0.01^{B}	182.8 ± 9.5^{B}	0.56 ± 0.03^{B}
LFD-AL	2.8 ± 0.1^{B}	126 ± 6.4^{B}	0.14 ± 0.01^{B}	209.4 ± 7.1^{B}	0.48 ± 0.02^{B}
Calo 3: 22°C - WR					
HFD-AL	$5.2\pm0.4^{A€}$	138.9 ± 4.6^{A}	0.30 ± 0.03^{A}	106.2 ± 15.2^{A}	1.07 ± 0.12^{A}
HFD-WR	$3.9\pm0.1^{B€}$	118.2 ± 4.1^{B}	0.20 ± 0.01^{B}	154.8 ± 10.5^{B}	0.68 ± 0.05^{B}
LFD-AL	$3.3\pm0.1^{C€}$	124.1 ± 3.8^{B}	0.14 ± 0.01^{C}	205.1 ± 6.9^{C}	0.49 ± 0.02^{B}
Calo 4: 22°C - Post WR					
HFD-AL	4.5±0.3	145.3±10.4	0.29 ± 0.03^{A}	104.7 ± 12.3^{A}	1.05 ± 0.11^{A}
HFD-WR	4.0±0.1	$151.7\pm3.7^{\$}$	$0.40\pm0.08^{AB\$}$	$85.9\pm10.6^{AB\$}$	$1.46\pm0.27^{AB\$}$
LFD-AL	$4.0\pm0.3^{\$}$	$155.7\pm6.5^{\$}$	$0.56\pm0.08^{B\$}$	$61.5\pm13.0^{B\$}$	$2.04\pm0.30^{B\$}$

TABLE 5.3: SERUM HORMONES AND METABOLITES AT DIFFERENT AMBIENT TEMPERATURES

Serum hormones and metabolites for the three mouse groups at 30ºC (Calo2) and at 22ºC (Calo3) during: 1. weight perturbation period (i.e. HFD-WR maintained at 80% initial body weight); and 2. at 22ºC following weight gain of HFD-WR and LFD-AL subsequent to *ad-libitum* access to HFD for all three groups (Calo 4). Levels not connected by same letter within Calo measurement period are significantly different ($p<0.05$) by ANOVA with Tukey post-hoc analysis. € - Significantly different between Calo3 and Calo2 (ambient temperature comparison; $p<0.05$). $ - significantly different between Calo4 and Calo3 (HFD feeding for all groups comparison; $p<0.05$).

CHAPTER 6: EFFECTS OF CHRONIC LEPTIN INFUSION ON SUBSEQUENT BODY WEIGHT AND COMPOSITION IN MICE: CAN BODY WEIGHT SET POINT BE RESET?

INTRODUCTION

In weight-stable individuals, circulating leptin concentration is directly proportional to fat mass [160]. Loss of fat mass and calorie restriction cause decreases in circulating leptin concentrations [22,169,237], thus circulating leptin levels provide signals regarding the acute and long-term status of body fat stores. Following weight-loss, a decrease in circulating leptin provides one of the signals that induce a CNS-mediated decrease in energy expenditure and increase in hunger [161,169]. Restoration of leptin concentrations to pre-weight loss levels abrogates these metabolic and behavioral responses [22]. Diet-induced obese (DIO) mice that are weight-reduced by caloric restriction also respond by reducing energy expenditure. Compared to never-obese mice with similar body mass and composition, these 'post-obese' mice are hypometabolic [237]. Leptin administration to calorically restricted [105] or fasted mice [43] restores energy expenditure to levels similar to pre-weight loss and/or fast. In addition, 48-h fasted male mice show starvation-induced changes in gonadal, adrenal, and thyroid axes that are all leptin reversible [43]. Taken together, these findings indicate that circulating leptin concentration is a major afferent signal of overall energy availability, and that the hypometabolic phenotype of weight-reduced individuals is the result of a state of relative leptin insufficiency.

In contrast to the potent effect of leptin administration to humans or rodents with leptin insufficiency (weight-reduced, fasted, or congenitally leptin deficient), administration of a physiological dose of leptin to rodents or humans at usual or increased body weight has little to

no effect on total energy expenditure [101] or food intake. In fact, in the overweight and obese states, almost 10-fold elevations of plasma leptin concentrations are required before effects on energy expenditure and food intake [22,50,102] are observed. Such data suggest that leptin-sensing circuitry in the CNS is "designed" to be inherently more responsive to declines in ambient leptin than to increases. Evolutionary arguments for such "asymmetric" regulatory responses to changes in body fat have been proposed [13]. Determination of the "threshold" (minimum signal regarding fat mass) below which these responses are invoked is determined by genetic and developmental factors [13]. An important question is whether this threshold can be reset by environment. That is, whether sustained maintenance of a body weight higher (or lower) than that "encoded" by genetic and early developmental factors, could permanently alter the level of body fat "defended" by an individual. Such malleability would have major implications for efforts to prevent and treat obesity.

Elevations in the hypothalamus of fatty acids [238], cytokines (e.g. IL6) [239] and impairments of molecular stress responses in the endoplasmic reticulum [240] can impair acute and chronic leptin signaling, accounting for the persistence of high levels of body fat despite proportionate elevations of circulating leptin concentrations. Whether these various desensitization processes occur by a shared mechanism is unknown [241], and whether such desensitization is mechanistically related to the apparent "defense" of a higher body weight in mice chronically maintained at higher body weight by feeding of a HFD, is not clear. The achievement of an elevated body fat content is accompanied by many metabolic and endocrine changes that could contribute to this effect and are difficult to disarticulate experimentally. In the present study we sought to isolate the possible effects of high ambient leptin *per se* on this process. We examined the effects of 18 weeks of continuous exogenous leptin infusion on the

metabolic response of such animals. By infusing recombinant murine leptin at levels that mimicked those of age-matched diet-induced obese (DIO) mice, to lean mice consuming low-fat diet, we sought to create a mouse model of elevated circulating leptin concentrations without the metabolic "confounds" of diet-induced obesity (e.g. elevated triglycerides and glucose, insulin insensitivity, fatty liver, etc.). Our hypothesis was that a chronic elevation in leptin concentrations would result in a permanent elevation in the minimum level of defended body fat.

MATERIALS AND METHODS

Animals

48 C57BL/6J male 6 week-old mice were obtained from Jackson Laboratory (Bar Harbor, ME). Upon receipt, animals were housed 4 per cage in plastic pens with wood chip bedding in a pathogen-free barrier facility maintained at 22-24 ºC with a 12-h dark-light cycle (lights on at 0700 h). *Ad libitum* access to a low fat diet (LFD: Research Diets, Inc. D12450Bi, 10% kcal from fat) and water were provided during the entire experiment unless otherwise specified. Body weight and body composition were recorded every 14 days unless otherwise specified.

The protocol was approved by the Columbia University Institutional Animal Care and Use Committee.

Study Design Overview:

There were 3 phases to this study: 1. leptin infusion (**Figures 6.1 & 2**); 2. weight regain (**Figure 6.3**); and 3. food preference (**Figure 6.4**). All animals participated in all phases. After a 3 week period of acclimatization, cages were stratified based upon the total weight of the 4 mice occupying each cage. Cages were arranged in triplicates in ascending order of total body weight with cages 1 and 2 being assigned leptin (n = 36) and cage 3 of triad assigned PBS (n = 12). Mean body weights for the animals assigned to pbs (n=12) and lep (n=36) were 24±0.6g and 24±0.3g respectively. Mini-pumps were implanted in all mice. The LEP mice received recombinant murine leptin dissolved in PBS (7.9pH: Dr. A.F. Parlow; National hormone & peptide program); the PBS mice received vehicle and pumps were surgically replaced every two weeks to provide an 18 week infusion period (**leptin infusion phase**). Mice were placed in metabolic chambers 6 days after the implantation of the final mini-pump (25ug/day: Days 144-151: **Figure 6.1A** and **Figures 6.2A-C**). Mini-pumps were subsequently removed (day 155,

Figure 6.1A) and food intake and body weight were monitored for the following 25 days (**weight regain phase**: **Figures 6.3A-D**). Following 48 days of *ad-libitum* access to the LFD, a diet preference test was conducted (**diet preference phase**). Mice were individually housed and given simultaneous access to both a LFD (10% kcal from fat) and a medium fat diet (MFD: 30% kcal from fat: Research Diets, Inc. D09082404i) placed on the floor of the cage. Each food source was weighed daily for 10 days. Following 10 days of access to both LFD and MFD with no significant differences in diet preference between the LEP and PBS mice (**Figure 6.4**), mice were given ad-libitum access only to the MFD for the remainder of the experiment (a total of 60 days).

Leptin infusion phase:

Mini-pumps

Nine mini-pump implantations (at 2 week intervals) were performed in each animal over the 18 week infusion period. The first 6 mini-pumps used were Alzet model 1002 and the last 3 were model 2002 (Alzet; Cupertino, CA). The second model holds twice the volume (200 μl) of a model 1002, and was required to permit use of a lower the concentration of leptin in the pumps. Mini-pumps were placed in a dorsal subcutaneous pouch under inhaled isoflurane (1-1.5%) in oxygen anesthesia. Shaved skin was prepped with betadine and alcohol washes, and a 1.5-2 cm incision caudal to the interscapular region was made, avoiding the interscapular brown adipose tissue depot. The mini-pump was positioned with the orifice facing caudally. The metal flow moderator was replaced with PEEK medical microtubing (Durect Corporation, Cupertino, CA) to enable use of the time-domain-NMR for body composition analysis. To assess the effect of this retrofit on flow rate from the pump, we did not change out the metal flow modulator on

mini-pump change 7. This step accounts for the absence of body composition measures for that period (16ug/day; days 94 – 109: **Figures 6.1B & C**). Leptin dosing was based on pilot studies in which we correlated rates of infusion with intercurrent circulating serum concentrations of leptin. Using regression analyses of fat mass and serum leptin made earlier [237] our goal was to elevate circulating leptin concentrations in non-obese LFD-fed mice to those analogous of age-matched diet-induced obese mouse. The lowest dose administered was 1ug/day/mouse (41.6ng/hour) and the highest dose was 25ug/day/mouse (1021.7ng/hour); 3ug/day increments were added at each mini-pump switch time point (i.e. 4ug/day, 7ug/day, etc.). 4 hour fasting blood was obtained by retro-orbital bleeding 1 week after each mini-pump implantation.

Body weight and body composition

Body weight (BW) was measured (± 0.1 g) weekly using an Ohaus Scout Pro 200g scale (Nänikon Switzerland, between 07:45-08:15h). Body composition (fat mass: FM, fat-free mass: FFM, & extracellular fluid) were measured by time-domain-NMR (Minispec Analyst AD; Bruker Optics, Silberstreifen, Germany) every 2 weeks (1 week after previous mini-pump implantation and one day before retro-orbital bleeding).

Indirect calorimetry

Energy expenditure was measured with a LabMaster-CaloSys-Calorimetry System (TSE Systems, Bad Homburg, Germany) during the 25ug/day/mouse phase of study (days 144-151: **Figure 6.1A** and **Figures 6.2A-C**). O_2 and CO_2 measurements were taken every 26 minutes during a 72 hour period while mice were given *ad-lib* access to LFD and water. Because of possible initial stress related to transfer to the chambers, only the last 48 hours of measurements

were used to calculate total 24-hour energy expenditure (TEE; expressed in kcal/day) and respiratory quotient (RQ = VCO_2 / VO_2). Resting energy expenditure (REE in kcal/day) was defined as the lowest one hour period of energy expenditure, which coincided with the lowest 1 hour of total ambulatory activity during the 48-hour period and this value was extrapolated to 24 hours. Non-resting energy expenditure (NREE) was calculated as the difference between total energy expenditure (TEE) and REE. Physical activity was measured by an infrared beam system integrated with the LabMaster system. Total activity (beam breaks) in X, Y, and Z axis was stored every 26 minutes. The system is designed to differentiate between fine motor movement (defined as a single X or Y axis beam break), ambulatory movement (defined as the simultaneous breaking of two adjacent X or Y beams), and rearing, defined as the breaking of the Z axis infrared beam.

Weight regain phase

Following the removal of the last mini-pump (day 155; **Figure 6.1A**), BW and food intake (FI) were measured daily for 10 days each morning (0745h – 0815h) and every 2-3 days thereafter for the following 15 days when mice had *ad-lib* access to the LFD (**Figures 6.3A-D**). Food intake was measured per cage and divided by the number of mice in the cage in order to get an estimate of individual energy intake. Body composition was obtained prior to removing last mini-pump and then 5 and 41 days post excision of the last pump.

Diet preference phase

Once body weights had stabilized following excision of last mini-pump (approximately 5 weeks), mice were individually housed and allowed to acclimatize for 1 week. Both LFD and

MFD were placed on the bottom of each cage; body weight and food weight were determined daily (1000h – 1100h). Feed efficiency was estimated by dividing 24 hour weight change by the number of calories consumed during that period (g/kcal). Following 10 days of diet preference testing, mice were given *ad-lib* access only to the MFD. Body weight was measured every 1-2 weeks during this 8 week period.

Serum leptin and insulin

Blood was obtained by retro-orbital bleed following after a 4-h fast 1 week following every mini-pump switch during the 18 week leptin infusion phase as well as 5, 41, and 118 days following last mini-pump removal. Blood was allowed to clot for 2 h at room temperature, spun at 4°C for 20 min at 1,000 g, and serum was collected and frozen at −80°C until time of assay. Leptin was assayed using Quantikine ELISA kit (R&D Systems, Minneapolis, MN) and insulin using the Mercodia Ultrasensitive Mouse Insulin ELISA (Mercodia, Uppsala, Sweden).

Statistical Analyses

Data are expressed as means ± SE. Statistical analyses were performed using JMP (version 7; SAS, North Carolina). Where applicable, ANCOVAs were conducted using diet group (LEP or PBS) as factor with FM and FFM as covariates. Statistical significance was prospectively defined as $P\alpha < 0.05$.

RESULTS

Leptin infusion phase:

Body weight, body composition, and circulating leptin concentration during leptin infusion

Body weight and body composition upon implantation of the first mini-pump (day 24; **Figure 6.1A-C**) were indistinguishable between LEP- and PBS-treated mice (body weight: 24.0±0.4 vs. 24.0±0.6, fat-free mass: 17.8±0.4 vs. 17.7±0.4, and fat mass: 3.1±0.1 vs. 3.4±0.2 g, respectively). Body weight was significantly lower in the LEP group by the start of the 4ug/day dose of leptin (day 44; **Figure 6.1A**) with the majority of this difference accounted for by a decrease in FM (-0.9±0.1 g, representing a 30% decrease) in the LEP mice following implantation of the first mini-pump (1ug/day; **Figure 6.1B**). The decrease in FM following implantation of the 1st pump (1ug/day) then stabilized in the LEP group, and FM remained unchanged throughout the rest of the experiment. FM was not statistically different at the first dose (1ug/day; 2.2±0.1 g) compared to the final dose (25ug/day; 2.4±0.3 g) in LEP mice but rose significantly in PBS mice (2.9±0.2 vs. 4.6±0.3 g respectively; $p < 0.01$). Fat-free mass was slightly lower in the LEP group compared to PBS group starting at the 4ug/day dose, but reached statistical significance only at the 22ug/day time point (**Figure 6.1C**). A repeated measures ANOVA revealed strong treatment ($p < 0.001$), time ($p < 0.001$) and time*treatment effects ($p < 0.001$). Circulating leptin concentrations were significantly higher in the LEP compared to PBS group starting at 7ug/day (10.5±1.7 vs. 4.6±0.7 ng/ml respectively), and were > 7.5 fold times higher at the 25ug/day dose compared to the PBS group (47.3±6.0 vs. 6.3±3.0 ng/ml, respectively) (**Figure 6.1D**).

Energy expenditure, ambulatory activity, and respiratory quotient in LEP (25ug/day) and PBS mice

Non-adjusted (for body composition) mean total 24h energy expenditure (TEE) at 144-150 days was slightly but significantly lower in the LEP group compared to the PBS group (11.6±0.2 vs. 12.2±0.2 kcal/24h respectively; p = 0.04) (**Table 6.1** & **Figure 6.2A**), a reflection of the lower resting energy expenditure (REE) in the LEP group (8.4±0.1 vs. 9.1±0.2 kcal/24h; p = 0.01) (**Table 6.1**). Whether adjusted for FM and FFM (**Table 6.1**), or FFM only (not data shown), TEE was no longer significantly different between the groups (p = 0.09 and p = 0.1 respectively). The higher TEE observed in the LEP mice at the beginning of the first lights off phase (**Figure 6.2A**) correlates well with increased ambulatory activity (**Figure 6.2B**). When TEE and REE are adjusted for FM and FFM using multiple regression analysis [237], TEE were nearly identical between LEP and PBS mice (data not shown). Both TEE and ambulatory movement were shifted to the left in the LEP mice in the early lights off period (2000h – 0200h; **Figure 6.2B & C**). Mean 24 hour respiratory quotient (RQ) was similar in both groups (**Table1**) and only a few separations between the groups were seen over the entire 48 hours (**Figure 6.2C**).

Weight regain phase:

Body weight, food intake, and metabolic efficiency and circulating leptin concentrations;

Following discontinuation of the mini-pumps (Day 55, **Figure 6.1A**), body weight and food intake were measured every 24h for the first 10 days, and then every 2-4 days for the subsequent 15 days. Body weights of the LEP mice increased at ~0.2g/day until reaching a plateau on day 7. Mean 7 day weight gain was 1.3±0.2 grams (**Figure 6.3A**), representing a

5.2±0.8% increase in body weight (**Figure 6.3B**). PBS mice lost weight until day 4; mean weight loss in that period was 1.1±0.2 grams (**Figure 6.3A**), representing a 3.5±0.7% decrease in body weight (**Figure 6.3B**). Body weights returned to levels prior to mini-pump removal in the PBS group at about day 10. Despite their initial weight gain following removal of the leptin-containing pumps, body weights of the LEP mice remained slightly but significantly lower than those of the PBS group until the end of the experiment (118 days following mini-pump removal: LEP – 34.6±0.8 and PBS – 37.6±1.3 on last day of experiment) (**Figure 6.3A**). Food intake was significantly higher than PBS in LEP animals on days 2-6 post pump removal, but was similar to PBS mice following day 7 (**Figure 6.1C**). Feed efficiency, estimated by dividing the change in body weight (g) by the food intake (kcal) for 24h, was significantly higher in LEP mice on days 2-6. 4 hour fasting leptin serum concentrations were measured 5, 41, and 118 days following the cessation of leptin infusion (**Table 6.2**). LEP mice had significantly higher concentrations of leptin compared to PBS mice at both 5 (75% greater) and 41 (65% greater) days post cessation in spite of having lower fat mass (41% and 31% lower FM in LEP vs. PBS respectively; **Table 6.2**). When adjusted for fat mass, the differences were further accentuated. LEP mice had 3-5 fold higher adjusted circulating leptin concentrations at 5 and 41 days post-cessation of leptin administration ($p < 0.05$). 42 days following leptin infusion cessation, a diet preference test (see results in next section) was started in which mice were given free access to both MFD and LFD for 10 days before being switched to MFD only. On day 118 post cessation of leptin infusions, no significant difference in non-adjusted serum leptin concentrations was observed between LEP and PBS mice yet leptin concentrations were still significantly elevated when adjusted for fat mass (LEP = 44.3±4.2 vs. 24.8±6.8; $p < 0.05$). Leptin concentrations in either group had

significantly risen from the 41 day post-leptin infusion timepoint due to the increase in body fat resulting from ad libitum intake of the MFD.

Diet preference phase

Both LEP and PBS mice preferred the MFD, consuming more than 90% of their entire total daily caloric intake as MFD (**Figure 6.4**). The LEP mice ingested slightly fewer total calories as a result of relatively lower intake of the MFD during the first 5 days, a trend that was reversed on the last 2 days. When EI was normalized to estimates of FM and FFM, there was no longer a difference in energy intake data (data not shown). Following this 10 day period, all mice were given ad-libitum access to the MFD.

DISCUSSION

Leptin's role in signaling peripheral energy stores (i.e. adiposity) to the CNS has been well documented [103]. Whether decreased CNS sensitivity to leptin is a cause or a consequence of obesity is still highly debatable [241]. These possibilities are, of course, not mutually exclusive. Circulating molecules, such as free fatty acids, have been proposed as capable of inducing leptin resistance on their own thereby facilitating weight gain [238]. *Lep*^{ob} mice whose circulating leptin concentrations are "clamped" to those of lean animals by low dose administration of leptin via mini-pump remain leptin sensitive even after becoming obese by feeding of a high fat diet during 20 weeks [242]. Body weight and body-composition matched WT mice also fed a high fat diet had significantly higher leptin concentrations and were leptin resistant (i.e. decreased pSTAT3 activation in the hypothalamus following leptin administration) suggesting that leptin itself is required to induce leptin resistance [242]. The goal of the present study was to determine whether 18 weeks of hyperleptinemia (without the metabolic "confound" of obesity) was capable of resetting defended body in mice fed a low fat diet. We found that chronic elevations of circulating leptin concentrations, *per se*, did not lead to metabolic or behavioral "defense" of a higher body weight.

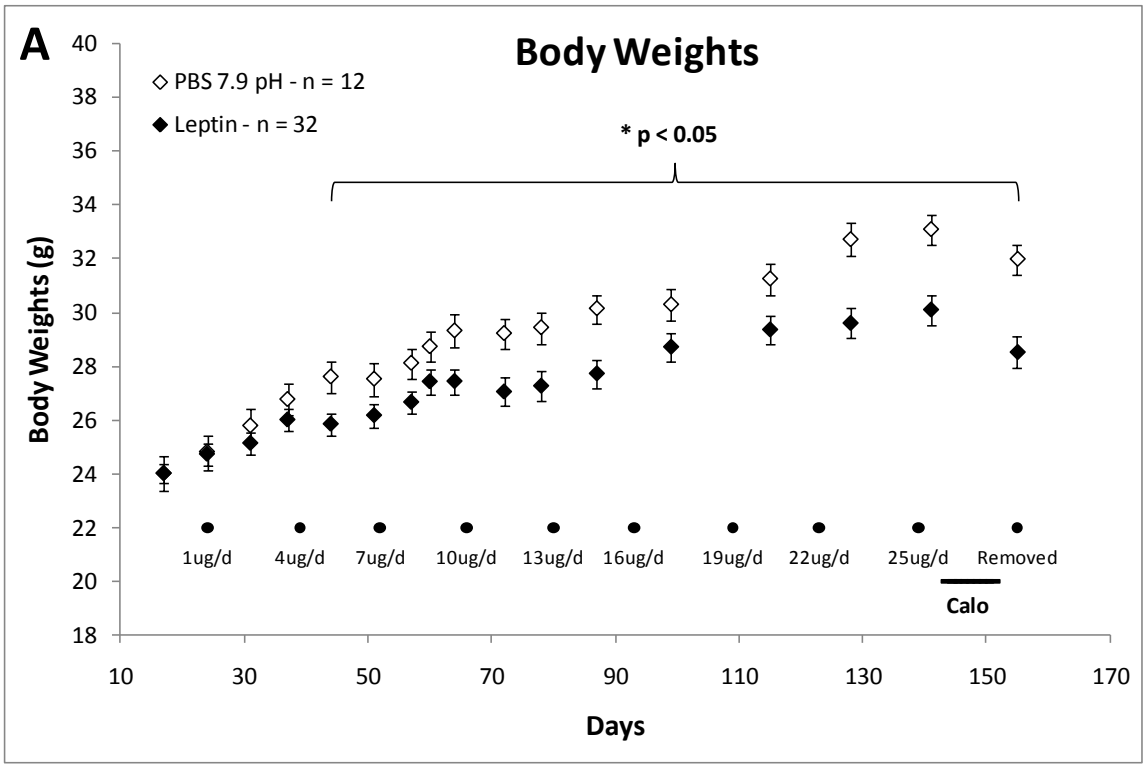
Leptin infusion resulted in lower fat mass accumulation starting at infusion rates of 4ug/day (serum leptin = 3.4±0.5 ng/ml) compared to PBS-infused control mice (serum leptin = 2.9±0.3). Harris *et. al* showed that *Lep*^{ob} mice administered peripheral doses of 2ug/day via osmotic minipump showed significant decreases in food intake and body weight, while a dose of 10ug/day was required to produce similar changes in wild type mice [243]. In a separate study, 200ng/hour infusion rates (i.e. 4.8ug/day) significantly decreased body weight of wild type mice by approximately 5% over a 14-day infusion period; there was a positive correlation between

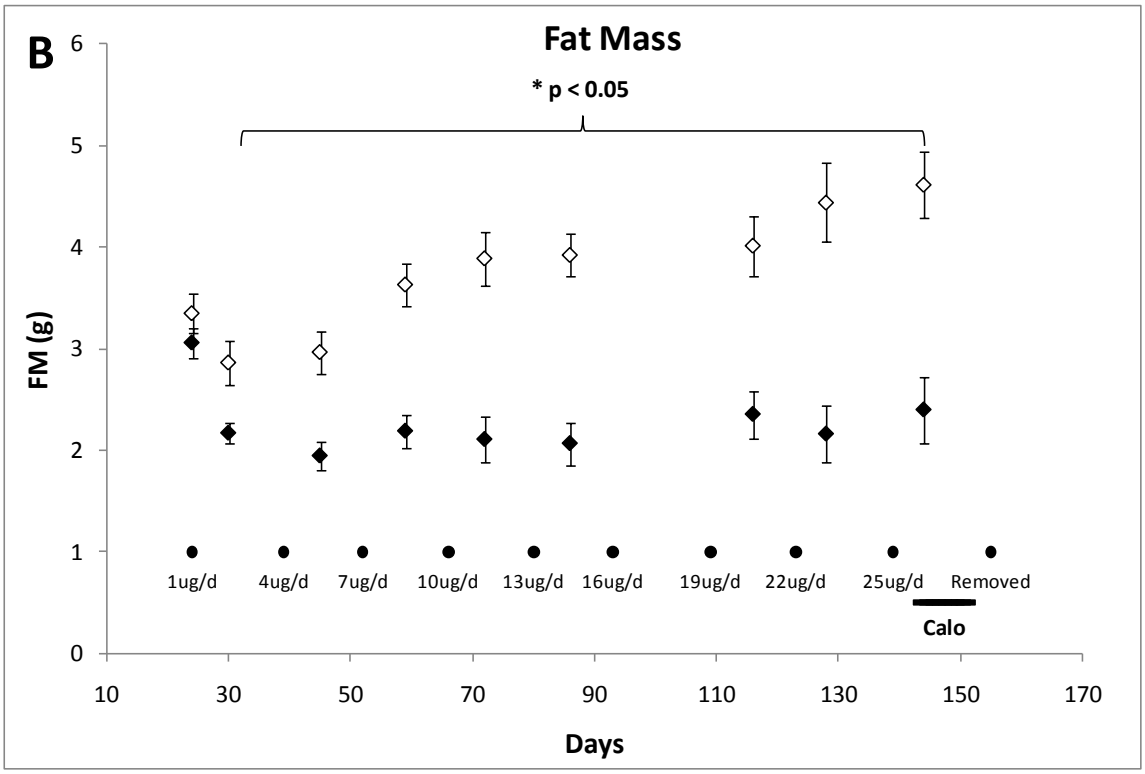
leptin infusion rates (200, 300, 400, and 500ng/hour) and maximal weight loss [73]. The highest dose administered in the present study (25ug/day) is higher than a reported dose of 10ug/day that was reported to greatly reduce somatic fat in mice [102]. 25ug/day in the present study did not decrease the RQ of LEP mice compared to PBS mice (0.88±0.01 vs. 0.89+0.01 respectively), suggesting that a gradual increase in circulating leptin results in a decreased sensitivity to leptin's effects on fat catabolism. When the leptin infusion was stopped, LEP-infused mice fed a low fat diet had a 5.2±0.8% weight increase during the first 10 days (vs. -1.0±0.8% in the PBS mice) and then stabilized at a body weight slightly (≈5%) but significantly lower than the PBS-infused mice suggesting that the "set point" for body fat had not been reset upwards, and may even have been moved lower. Weight regain following a period of exogenous leptin administration has been well documented and may result from a perceived leptin deficiency in the CNS following the discontinuation of leptin infusion yet since fat mass is reduced, other adipose tissue-mediated signals cannot be ruled out [73,243]. Acute leptin reduction should result in suppressed metabolic rate following removal of the pump and although not directly measured by indirect calorimetry in the present study, the high feed efficiency may be a combination of increased food intake and lower metabolic rate, not only increased food intake. A low metabolic rate coupled with increased food intake would operate in concert to quickly increase fat mass as observed in the present study (**Figure 6.2C** & **Figure 6.2D**). At the end of the leptin infusion phase, LEP mice had nearly 8-fold greater circulating leptin concentrations than PBS mice (47.3±6.1 vs. 6.2±1.1 ng/ml respectively) and lower absolute (2.4±0.3 vs. 4.6±0.3 g, respectively) and percent (7.7±1.0% vs. 12.7±1.4%, respectively) fat mass. The leptin concentrations observed in the LEP mice would be commensurate to those found in high fat diet fed mice at 16 weeks of age (10 weeks of high fat diet feeding). Once mini-pumps were removed, leptin concentrations in the LEP mice fell

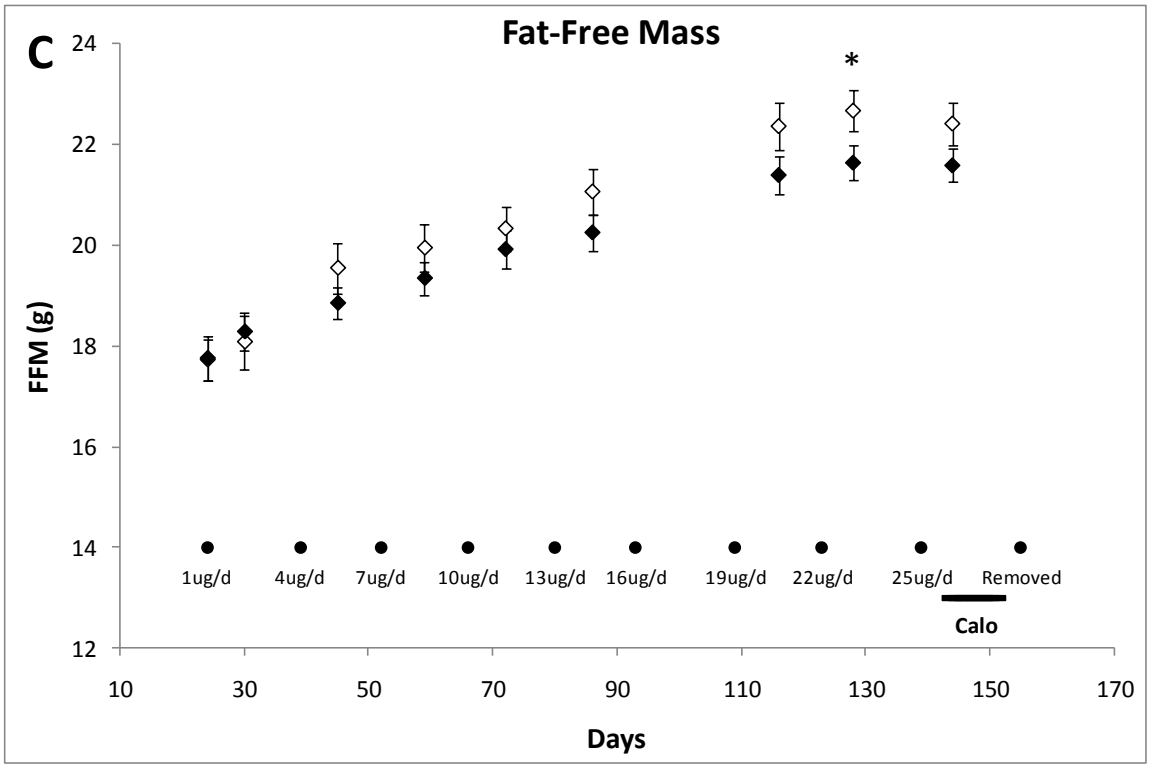
by >50% after 5 days, dropping from 47.3±6.1 ng/ml during the 25ug/day infusion period to 20.5±3.4 ng/ml. Yet this leptin concentration remained significantly higher than in the PBS treated mice, this in spite of LEP mice having significantly lower fat mass (≈40% lower; LEP = 2.4±0.3 vs. PBS = 4.4±0.4 g). LEP mice display increased circulating leptin concentrations adjusted for FM even 141 days following mini-pump removal. These apparently elevated leptin concentrations may arise for multiple reasons: 1) leptin is truly elevated but has diminished influence on metabolic parameters as hypothesized by the leptin threshold model; 2) problem in clearance or catabolism of leptin; 3) increase in the soluble leptin receptor (LepRe) that may decrease the bioavailability of leptin binding to LepRb; 4) the leptin infused mice may have been producing antibodies against leptin that could either decrease bioavailability and/or directly interfere with the ELISA kit used resulting in apparently elevated concentrations of leptin. 71% of human subjects that received 24 weeks of leptin injections developed antibodies[50]. Leptin has been shown to decrease food reward responses through actions mediated by the midbrain dopamine and opioidergic pathways[244,245]. 2 months following discontinuation of the leptin infusion, a diet preference test revealed no differences between LEP and PBS mice when offered both a 10% and 30% fat diets *ad-libitum* (**Figure 6.4**).

These results suggest that chronic elevations of circulating leptin concentrations *per se* do not result in major changes in defended body weight or diet preference. Peripheral leptin injection/infusion studies in mice have focused on the effects of leptin on body weight and composition, glucose homeostasis, and leptin resistance in the central nervous system. Infusion periods have ranged from single injections, to 28 day studies at infusion rates from 1ug/day to 40ug/day [73,102,243,246]. Our study was designed to examine directly the effects of incremental increases in circulating leptin concentrations over a period of 18 weeks on body weight

regulation. The design explicitly isolated the effects of elevated leptin *per se,* from the effects of increased somatic and dietary fat. We have previously suggested [237] that the hypometabolic phenotype observed in DIO mice that are weight-reduced to 20% below their "normal" body weight may result from perceived relative "hypoleptinemia". Chronic hyperleptinemia could affect both molecular and structural substrates for response to leptin in the central nervous system and elsewhere [237]. The fact that LEP mice in this study did not "defend" an elevated body weight following the discontinuation of the leptin infusion suggests that 1.) the period of leptin infusion what not sufficiently long; 2. diet/adiposity-mediated changes in the CNS are the responsible factors; 3. Elevated leptin concentrations and factors in #2 act synergistically to produce an elevation in the set point or threshold of defended body fat. When administered to either by CNS or peripheral infusion, leptin increases energy expenditure [247]. The 5% lower unadjusted (for body weight/composition) energy expenditure observed in the LEP mice receiving 25 ug/day leptin ($p = 0.04$), suggests that slow increases in ambient leptin by infusion may provoke desensitization to some of the metabolic and behavioral effects of acute leptin administration. These differences were no longer significant once TEE was corrected for FM and FFM.


A
Body Weights
◇ PBS 7.9 pH - n = 12
◆ Leptin - n = 32
* p < 0.05
Body Weights (g)
Days
1ug/d
4ug/d
7ug/d
10ug/d
13ug/d
16ug/d
19ug/d
22ug/d
25ug/d
Removed
Calo


B
Fat Mass
* p < 0.05
FM (g)
Days
1ug/d
4ug/d
7ug/d
10ug/d
13ug/d
16ug/d
19ug/d
22ug/d
25ug/d
Removed
Calo


C
Fat-Free Mass
*
FFM (g)
24
22
20
18
16
14
12
1ug/d
4ug/d
7ug/d
10ug/d
13ug/d
16ug/d
19ug/d
22ug/d
25ug/d
Removed
Calo
10
30
50
70
90
110
130
150
170
Days

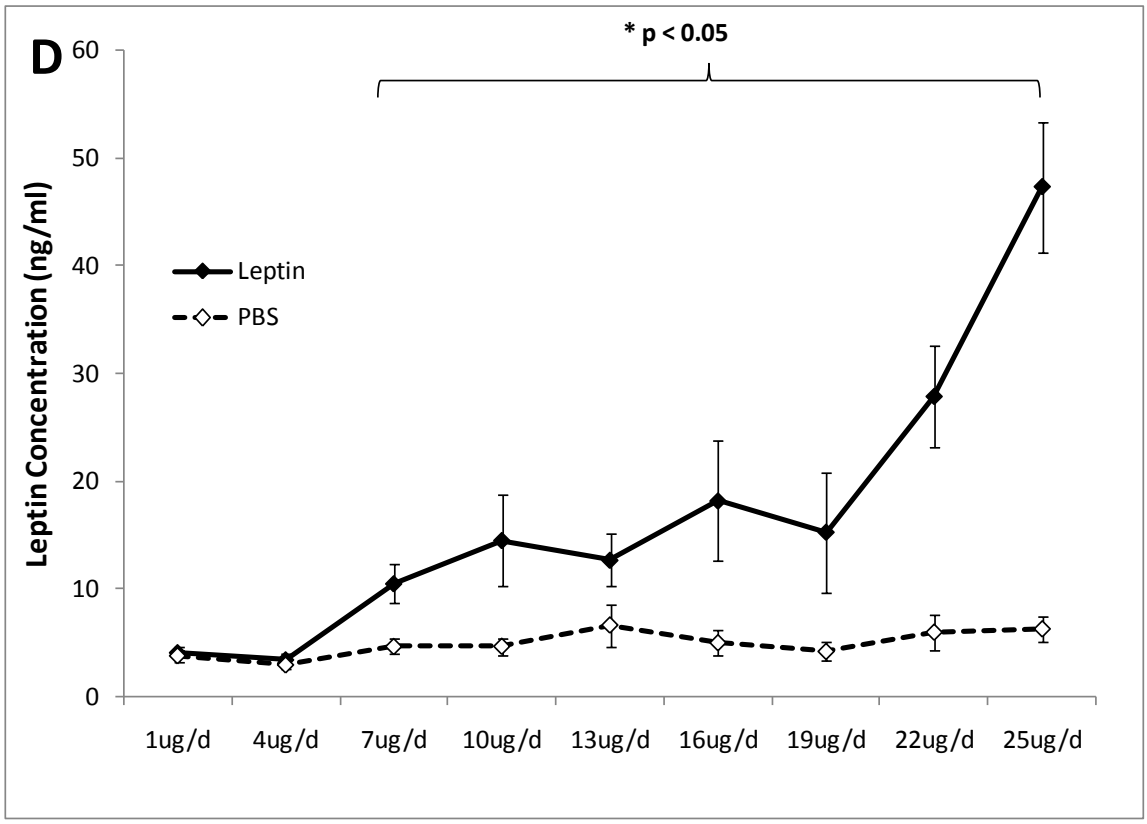
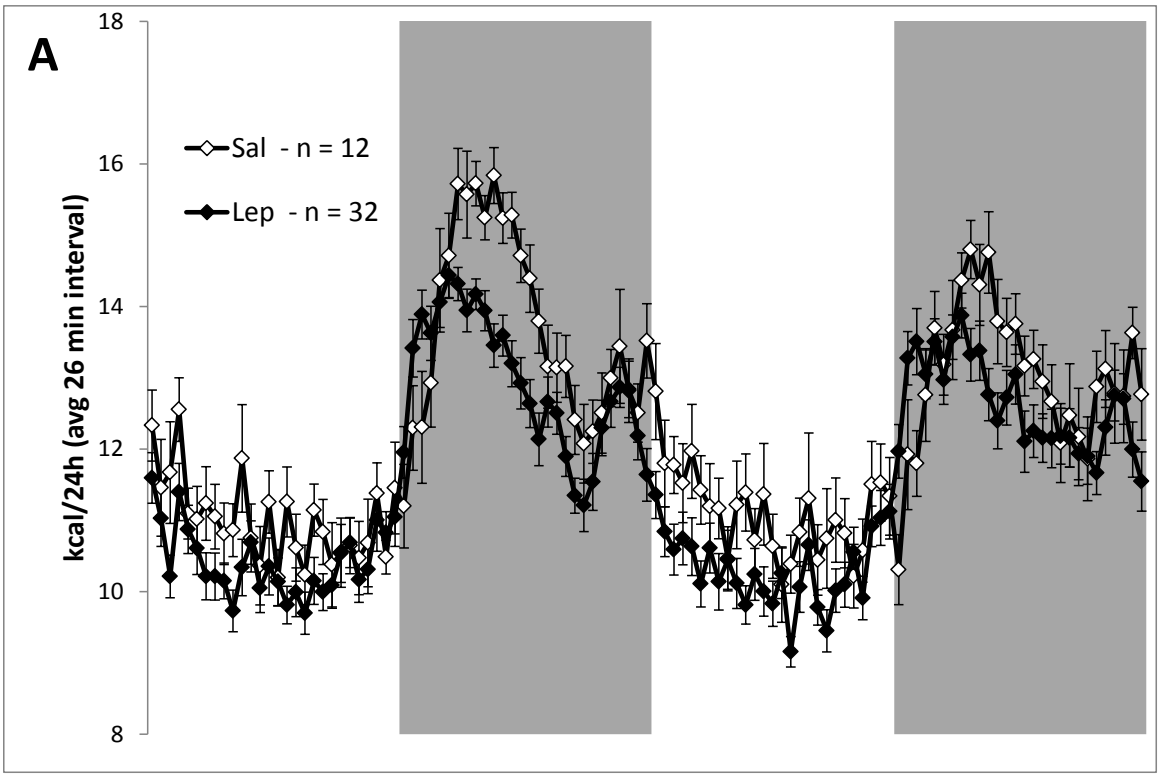
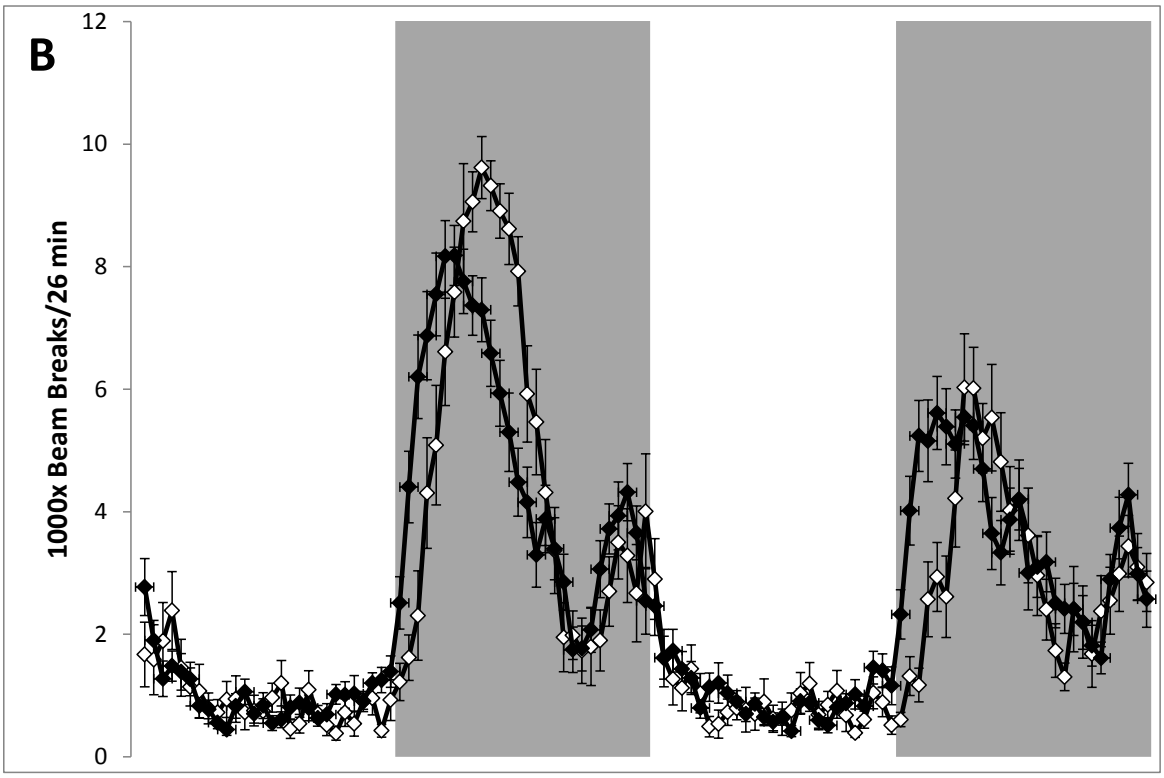

D
* p < 0.05
Leptin Concentration (ng/ml)
60
50
40
30
20
10
0
Leptin
PBS
1ug/d
4ug/d
7ug/d
10ug/d
13ug/d
16ug/d
19ug/d
22ug/d
25ug/d

FIGURE 6.1: BODY WEIGHT, BODY COMPOSITION, AND CIRCULATING LEPTIN CONCENTRATION DURING LEPTIN INFUSION

(A – C) Mean (±sem) body weight **(A)** fat mass **(B)** and fat-free mass **(C)** of +Leptin and +PBS infused mice. Black circles represent surgery days where mini-pumps were exchanged with successively increasing doses of leptin (doses given below each dot in ug/day). Black line on bottom right represents 72 hour indirect calorimetry measures for all mice. (**D):** Mean (±sem) circulating serum leptin concentrations (ng/ml) at different infusion rates (ug/day) of +Leptin and +PBS groups. 4 hour fasting blood was obtained 7 days following every implantation of new mini-pump.


A
Sal - n = 12
Lep - n = 32
kcal/24h (avg 26 min interval)
18
16
14
12
10
8


B
1000x Beam Breaks/26 min
12
10
8
6
4
2
0

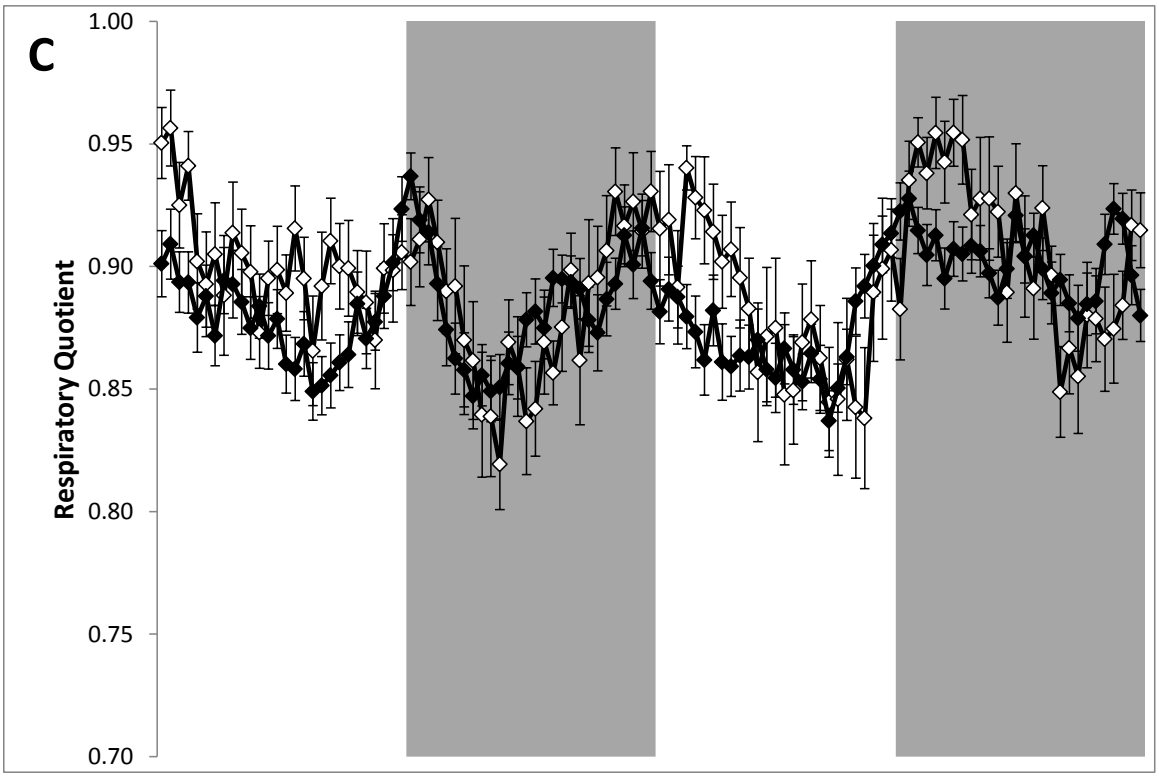
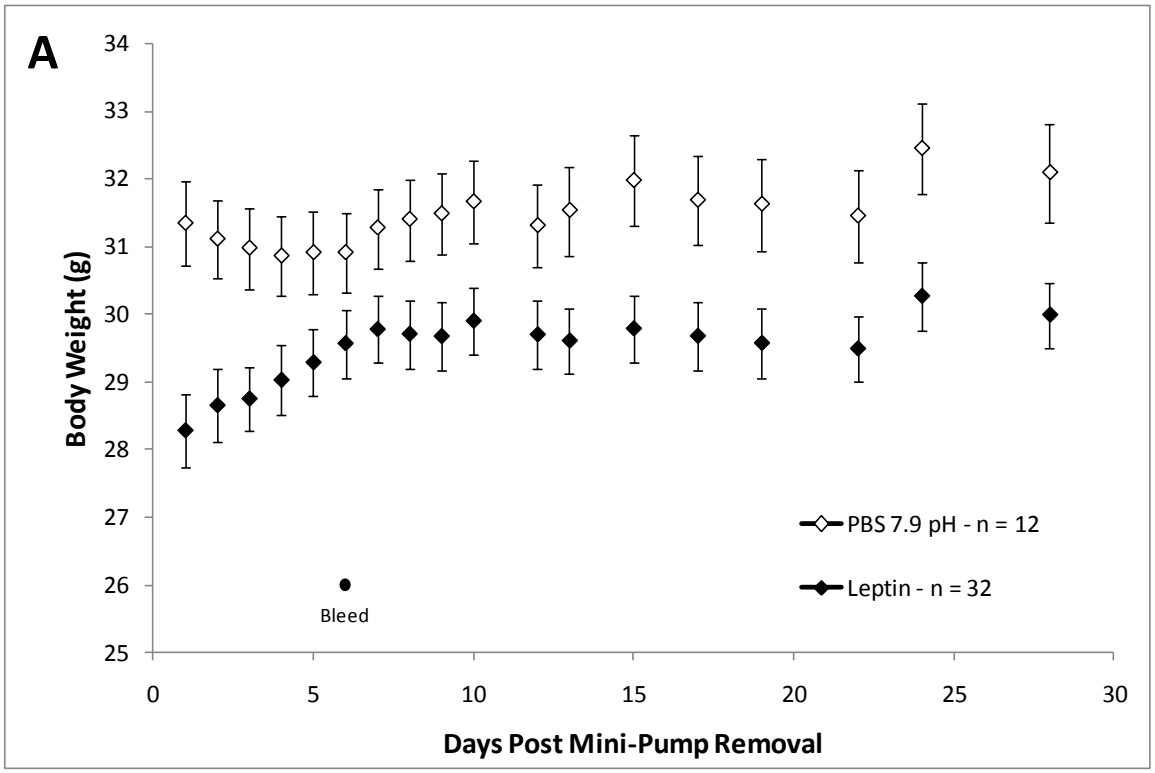
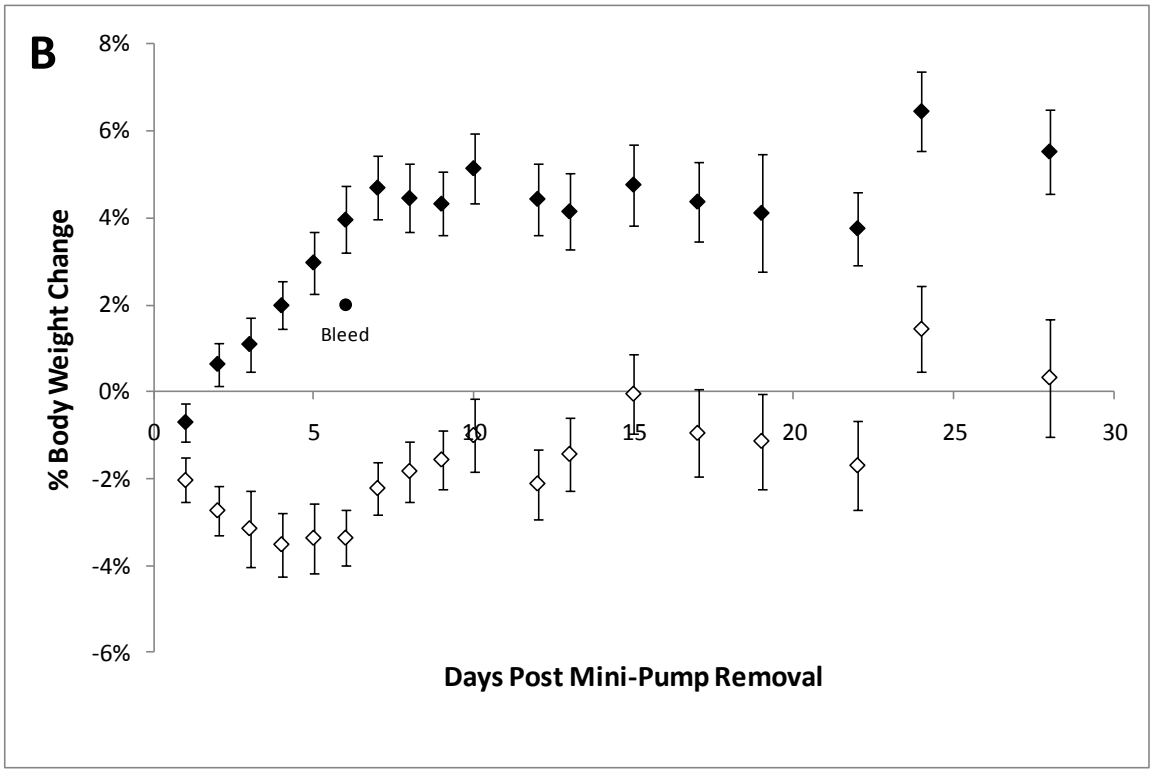
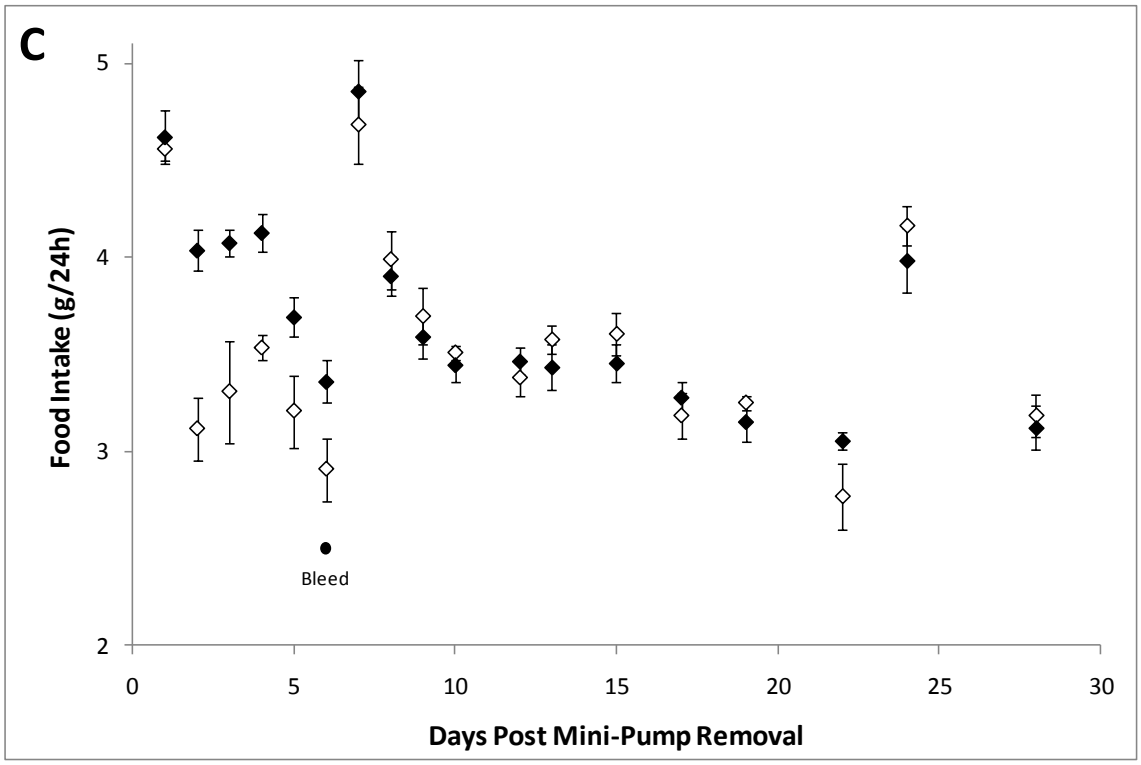


FIGURE 6.2: ENERGY EXPENDITURE, AMBULATORY ACTIVITY, AND RESPIRATORY QUOTIENT IN LEP (25UG/DAY) AND PBS MICE

Mean (±sem) non-adjusted (for body weight and composition) total energy expenditure **(A)**, cumulative movement **(B)**, and respiratory quotient (**C**) of +Leptin and +PBS infused mice. Grey shading represents lights off period. A total of the last 48 hours that mice were in the chambers are shown for all three figures.


A
Body Weight (g)
34
33
32
31
30
29
28
27
26
25
Bleed
PBS 7.9 pH - n = 12
Leptin - n = 32
0
5
10
15
20
25
30
Days Post Mini-Pump Removal


B
% Body Weight Change
8%
6%
4%
2%
0%
-2%
-4%
-6%
Bleed
0
5
10
15
20
25
30
Days Post Mini-Pump Removal


C
Food Intake (g/24h)
5
4
3
2
Bleed
0
5
10
15
20
25
30
Days Post Mini-Pump Removal

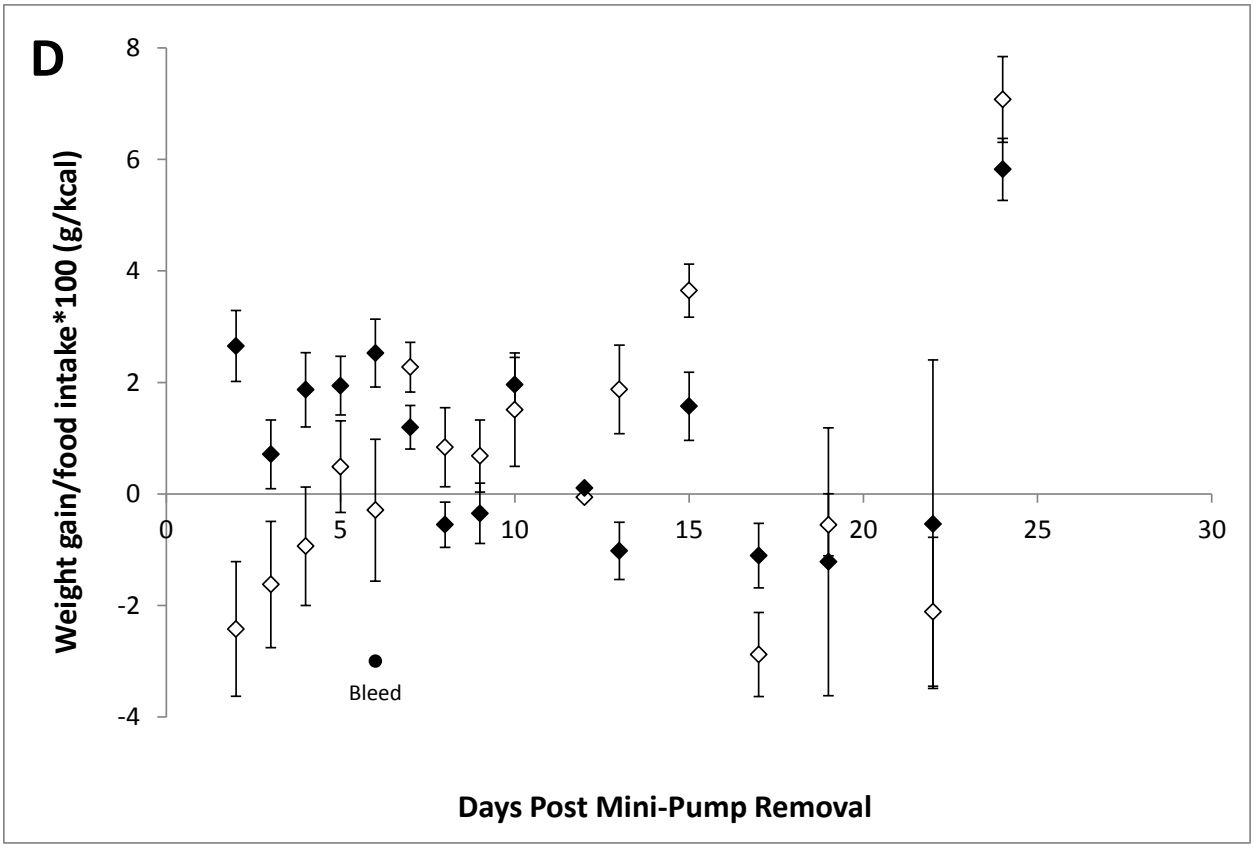

D
Weight gain/food intake*100 (g/kcal)
8
6
4
2
0
-2
-4
Bleed
0
5
10
15
20
25
30
Days Post Mini-Pump Removal

FIGURE 6.3: BODY WEIGHT, FOOD INTAKE, AND METABOLIC EFFICIENCY FOLLOWING LEPTIN INFUSION CESSATION

Mean (±sem) body weight **(A),** percent body weight change **(B),** 24 hour food intake of low fat (10% kcal from fat) control diet **(C)** and 24 hour feeding efficiency **(D)** of +Leptin and +PBS infused mice the 30 days following removal of the terminal mini-pump. Feeding efficiency was calculated by dividing 24 hour weight change (g) by 24 hour food intake (kcal).

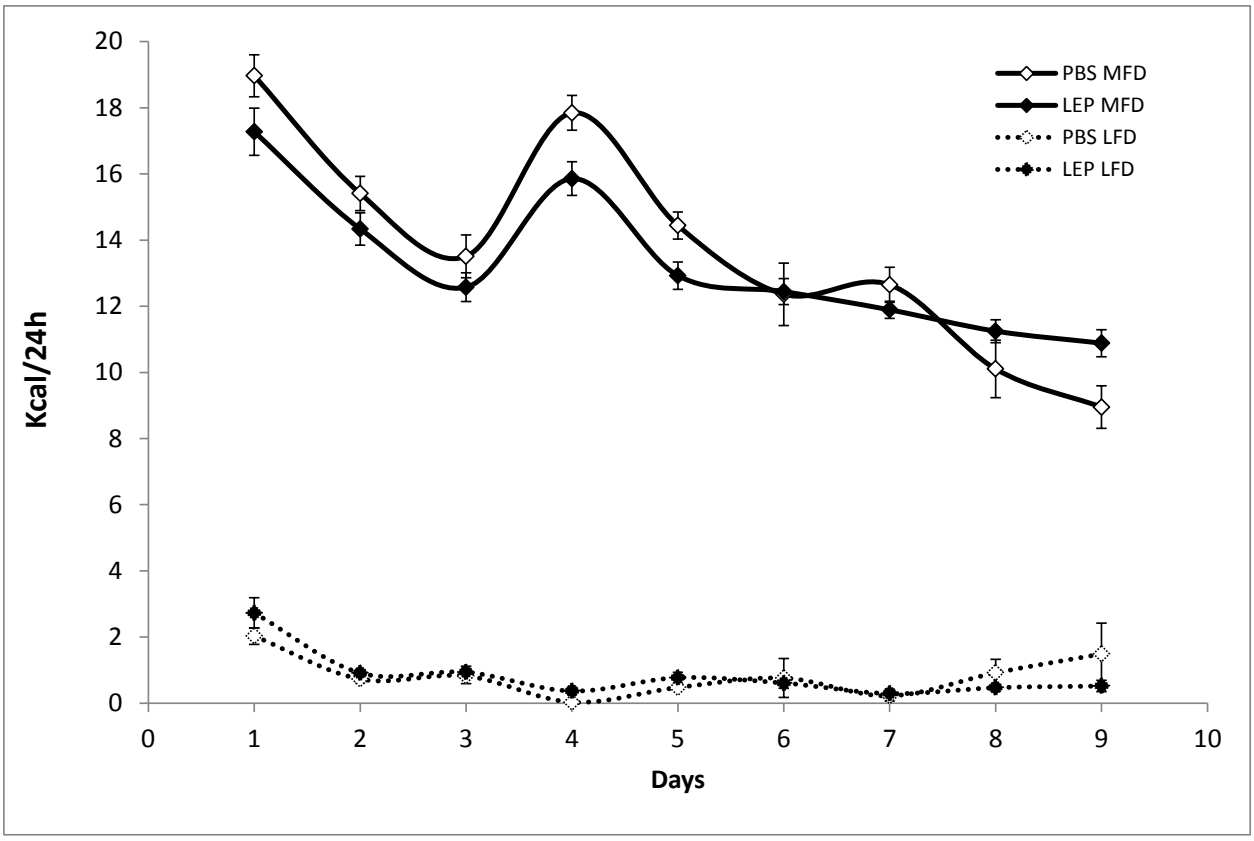


FIGURE 6.4: FOOD INTAKE DURING DIET PREFERENCE TEST

Mean (±sem) kcal/24h consumed of either LFD (10% kcal from fat: dashed lines) or MFD (30% kcal from fat: solid lines) for mice that had either received leptin (LEP mice; black diamond) or PBS infusion (PBS mice; open diamonds).

	Energy Expenditure			
	TEE (kcal/24h)	REE (kcal/24h)	NREE (kcal/24h)	RQ (24h)
Non-Adjusted				
PBS (n = 12)	12.2±0.2	9.1±0.2	3.2±0.1	0.89±0.01
LEP (n = 32)	11.6±0.2*	8.4±0.1*	3.2±0.1	0.88±0.01
Adjusted (FM & FFM)				
PBS (n = 12)	12.2±0.2	9.1±0.3	3.2±0.1	0.89±0.01
LEP (n = 32)	11.6±0.3	8.4±0.1#	3.2±0.1	0.88±0.01

TABLE 6.1: ENERGY EXPENDITURE PARAMETERS IN LEPTIN INFUSED MICE
Non-adjusted and adjusted (for FM & FFM) total energy expenditure (TEE), resting energy expenditure (REE), and non-resting energy expenditure of LEP and PBS mice during last mini-pump infusion (25ug/day of leptin). * significantly different by t-test ($P < 0.05$). # significantly different by ANOVA adjusted for FM.

Serum Leptin Concentration (ng/ml)

	Leptin (5 days)	Leptin (41 days)	Leptin (118 days)
Non-adjusted			
PBS (n = 12)	11.7±2.7	10.5±2.3	34.6±5.1
LEP (n = 32)	20.5±3.4*	17.3±3.7*	40.3±6.3
Adjusted mean (FM)			
PBS (n = 12)	4.7±5.1	5.7±5.4	24.8±6.8
LEP (n = 32)	23.1±4.7#	19.1±3.2#	44.3±4.2#

TABLE 6.2: SERUM LEPTIN CONCENTRATIONS FOLLOWING PUMP REMOVAL
Non-adjusted and adjusted (for FM) leptin concentration (ng/ml) at 3 time points (5, 41, and 118 days) following cessation of leptin infusions. * significantly different by t-test ($p<0.05$). # significantly different by ANCOVA using FM and FFM as covariates.

CHAPTER 7: DISCUSSION AND FUTURE DIRECTIONS

Genetic, epidemiological and physiological studies suggest that body weight is regulated [103]. The large increase in the number of obese individuals in many western and developing nations indicates that genes that favor energy accumulation and conservation may now have become detrimental in a new environment that includes easy access to energy dense foods and an increasingly sedentary lifestyle. Alleles of the relevant genes may have been selected for by cycling periods of feast and famine. This "thrifty" genotype would confer survival advantages to both a pregnant mother and her fetus. In the current "obesogenic" environment with abundant access to calorically dense food coupled with low energy requirements to obtain the food, these evolutionarily advantageous alleles may now promote excess body adiposity and its associated comorbidities. The "metabolic syndrome" [248], characterized by hypertension, dyslipidemia, high blood pressure, high fasting blood sugar concentrations and an elevated waist circumference, seems to reflect an interaction of the genetic predisposition to store fat with the current 'obesogenic' environment.

Clearly, there are differences in an individual's genetic propensity to obesity since overfeeding studies shows large differences in the amount of weight gained (reviewed in [249]). This was shown to be related to an individual's capacity to raise energy expenditure in response to overfeeding. In contrast, in response to the maintenance of 10% or 20% lower body weights, energy expenditure decreases more than expected compared to the decrease in metabolic mass[103]. The observation of the respective increases and decreases in energy expenditure observed in individuals with increased or decreased weight suggests a physiological feedback system which indicates the status of peripheral energy stores to the CNS ultimately affecting energy intake

and/or energy expenditure. Whether this feedback system for defense of body weight is "fixed" at a certain level for a given individual or whether it is "malleable" when faced with environmental challenges – such as dieting resulting in lower body weight or ingestion of high fat diet resulting in increased body weight – is not fully understood.

The aim of the work contained in this dissertation was to create mouse models in which to investigate multiple aspects of body weight regulation by exploring the effects of diets, ambient temperature, and leptin on physiological and molecular parameters involved in weight regulation.

METABOLIC ADAPTATION: DEFENSE OF ALTERED BODY WEIGHTS

The average US adult gains only 500-1000g per year in spite of consuming ≈ 900,000 – 1,000,000 kcal per year [42]. This small yearly weight gain ultimately translates to increases of 5 to 10 kilos over a decade if a persistent positive energy balance is maintained. The secular trend of increasing rates of obesity suggests that these small yet substantive yearly increases in body mass ultimately lead to an increase in the defended body weight; a rise in the "threshold". Clinical studies presented in the Introduction of this dissertation(chapter 1) gave evidence that both obese and non-obese humans who are maintained below "normal" body weight show evidence of metabolic adaptations (i.e. decreased EE and increased hunger) that are quantitatively and qualitatively similar [25,42,100,103]. This similarity in phenotypes suggests that the obese individuals are actively defending an elevated body weight.

The first weight perturbation study we conducted in mice was designed to determine whether environmental factors (differences in diet composition and food availability) could reset defended body weight in either an upward or downward direction.

CAN THE THRESHOLD FOR MINIMUM BODY FAT CONTENT BE RAISED?

Rothwell and Stock showed that short-term high fat-diet feeding in rats caused increased TEE that limited the amount of weight gained [250]. Long term access by rodents to a high fat diet increases adiposity to a degree that is strain-dependent [156,251,252]. Various weight-perturbation studies used outbred rats (Wistar, Sprague-Dawley) or inbred mouse strains that were either classified as obesity-prone or obesity-resistant depending on whether they were susceptible or resistant to weight gain when fed diets high in fat content [133-135,138,253-256]. The obesity-prone rats demonstrated numerous neuronal modifications, including decreased leptin sensitivity (decreased STAT3 phosphorylation and LepR quantity), abnormalities in serotonin and epinephrine turnover, decreased glucose sensing, and a decrease in ghrelin receptor in the ARC when maintained at a lower body weight by feeding of a low-fat diet [257,258] [259]. When presented with a high fat diet, the obesity-prone rats remained hyperphagic despite increases in leptin and insulin concentrations that should have curtailed further weight gain, whereas the obesity-resistant rats spontaneously reduce the number of grams eaten to compensate for the higher caloric density of the food [259]. Most of the neuronal changes found in the obesity-prone rats were only normalized once they had reached a higher level of adiposity, an elevated body weight that they then actively defended against further weight perturbations [133,134]. In the aggregate, these results suggest that a neuronal system capable of sensing changes in peripheral energy stores was being affected

directly by body weight (i.e. adiposity/leptin); the important question is whether these sensing mechanisms can be permanently altered resulting in defense of altered body weights. Many of these experiments were conducted in outbred rats that were specifically selected and then inbred to obtain the desired phenotype; that is to be obesity-resistant or obesity-prone. This genetic predisposition, carefully selected for by the investigators through an explicit breeding scheme, resulted in the creation of genetically distinct animals that are susceptible or resistant obesity upon high fat feeding. Whether the threshold for defended body weight could be altered through environmental means alone, not genetics, would need to be tested in genetically identical animals.

In the first study conducted as part of my thesis work, we demonstrated (chapter 2) that genetically identical C57BL/6J mice fed a high fat diet and subsequently weight reduced to 80% of maximal body weight, decreased their energy expenditure significantly more than predicted from the loss in metabolically active tissue (TEE ≈ -1.0 kcal/day or 8% below predicted based upon energy expenditure related to body mass and composition at maximum body weight). A decline in TEE disproportionately larger than the associated loss in body mass indicates that less energy was required to maintain a gram of tissue in a weight-reduced mouse than in that mouse at its *ad-libitum* higher body weight. This hypometabolic phenotype was accompanied by reduced circulating T3 concentrations, a decreased number of excitatory synapses onto leptin-sensitive POMC neuronal bodies, and increased hunger [203]. These phenotypes are almost identical – both in magnitude and direction – to those seen in weight-reduced animals that had never been obese. It is important to note that the decreased ratio in excitatory to total synapses onto the POMC soma, seen in both DIO-WR and CON-WR mice, was similar in magnitude to those observed in the *Lep*^{ob} mice [94]. This neuronal "signature" of the weight-reduced state – one

that is similar to that observed in mice deficient in leptin – further implicates relative hypoleptinemia, in the onset of physiological adaptations to maintenance of a reduced body weight. Characterizing neuronal "signatures" indicative of the weight-reduced and non-weight reduced states can be useful in drug testing paradigms. Compounds capable of reversing such neuronal abnormalities thereby abrogating metabolic adaptation would be useful in preventing weight reduced humans form regaining lost weight. No significant differences in the relationships in TEE or REE and body weight/composition were observed between CON-AL mice fed a chow diet and DIO-AL maintaining an elevated body weight. This finding indicates that DIO-AL mice had normal, not elevated, energy expenditure parameters relative to body mass further supporting the notion that physiological parameters at this new higher body weight had been normalized compared to the increased EE observed in short-term weight-gain/overfeeding studies [16,260].

Together, these observations suggest that diet-induced obese mice are actively defending a new elevated body weight compared to genetically identical non-obese mice and that the threshold for defense of body weight can be raised by maintaining an elevated body weight for a sufficiently long period of high fat feeding. The biological basis for this apparent upward shift in the threshold, whether related to some aspect of the diet, the obese state (e.g. hyperleptinemia, hyperinsulinemia, increased FFA) *per se,* or to an interaction of the two was further explored in subsequent experiments (see Leptin's Role in Defense of Body Weight).

CAN THE THRESHOLD FOR MINIMUM BODY FAT CONTENT BE LOWERED?

Studies in human subjects demonstrated that successful maintenance of a lower body weight, even for more than six years, was still accompanied by disproportionately (relative to body mass and composition) decreased EE [25,26]. Whether metabolic adaptation can be abolished (i.e. threshold lowered) following sufficiently long periods of maintenance at a lower body weight is unknown, although seemingly unlikely since even six years of weight reduction caused no attenuation of the hypometabolic phenotype[25]. The DIO-WR and CON-WR mice described in chapter 2 were maintained at 80% of maximal body weight for ≈ 3 months before undergoing indirect calorimetry and more than 5 months before being sacrificed. After 3 months of decreased body weight, the WR animals still had significantly suppressed TEE compared to their AL-fed counterparts. Given the average life span of mice (≈2 years) vs. humans (≈80 years), this time period is comparable to maintaining humans at a reduced body weight for 10 years. In a subsequent study (chapter 5), weight-reduced mice (HFD-WR) were maintained at a lower body weight for 78 days before being presented with *ad-libitum* access to the high fat diet. This sudden access to *ad-libitum* high fat diet resulted in rapid weight regain and within 90 days, body weights were no longer significantly different from those of HFD-AL mice. The rapid increase in body weight seen in the HFD-WR mice in the first week following switch to AL HFD feeding is due to the combined effects of lower TEE and increased hyperphagia. Taken together, these data suggest that even extended periods of weight-reduction do not result in abatement of the hypometabolic phenotype. In light of the evolutionary pressures on survival and reproduction discussed above, this result is not surprising. Previous studies in rats support this idea. Even 16

weeks of weight-reduction is followed by rapid weight regain once food is provided *ad-libitum* [130,133,134,255,256].

It is interesting to note that the non-obese mice that had not been previously exposed to the HFD (LFD-AL mice) readily gained similar amounts of body weight as the HFD-WR mice once switched to the HFD. The issues related to the hedonics of the high fat diet and the possible neural effects that this diet may have on the CNS and/or leptin sensing are discussed below (see Leptin's Role in Defense of Body Weight).

The data presented in chapters 2 and 5 support the concept that the threshold can be increased but not lowered. These findings are plausible in light of the 75-85% recidivism to obesity in otherwise successfully weight-reduced humans, the increasing prevalence of obesity, and the evolutionary arguments related to defense of body weight presented in the Introduction.

Does Torpor Contribute to the Hypometabolic Phenotype?

Fasted mice at sub-thermoneutral ambient temperatures (<30°C) decrease metabolic rate resulting in a sharp decline in core body temperature, a response that is likely engaged to protect themselves from life-threatening starvation during periods of caloric insufficiency[113]. The mice described in chapter 2 were all studied at conventional sub-thermoneutral mouse-facility temperatures (22°C), which imposes a constant thermal stress in these rodents. We therefore undertook a second weight-perturbation study (reported in chapter 5) to explore the physiological and metabolic changes in weight-reduced mice when studied at both sub-thermoneutrality (22°C) and thermoneutrality (30°C). This study was designed: 1. to assess whether torpor played a role in the metabolic adaptation of weight-reduced animals; 2. to assess whether thermal stress at

22°C affected the ability to detect subtle differences in energy expenditure in weight-reduced animals. These studies address the relevance of qualitative and quantitative responses of WR mice at 22°C ambient to metabolic adaptation observed in weight reduced humans. The weight-reduced formerly obese mice (HFD-WR) showed similar absolute declines in TEE adjusted for body composition at both 22°C (-1.4kcal/day) and 30°C (-1.6kcal/day) when analyzed using multiple regression analysis. This analysis is conducted by creating a regression equation using the "normal" eumetabolic mice only, in this case the *ad-libitum* fed mice (HFD-AL and LFD-AL), relating TEE to metabolic mass (FM and FFM) against which all other mice are compared (chapter 2 contains more details on this mathematical approach). The second analytical approach used to investigate these data was ANOVA. The analysis of energy expenditure data using FM and FFM as covariates revealed significantly lower TEE in the HFD-WR mice at 30°C but not at 22°C. These results reveal that the analyses of EE are sensitive to the mathematical approach used and that 22°C ambient may blunt or obscure the full effect of metabolic adaptation due to high thermogenic requirements at these sub-thermoneutral temperatures. 24-hour body temperature measurements revealed that none of the mice became torpid (defined as a core body temperature of <31°C) at either ambient, confirming that torpor does not play a role in the metabolic adaptation seen at 22°C. A small but significant difference in core body temperature (≈-1.5°C) was recorded in the HFD-WR mice at 0500h compared to the AL fed mice (both HFD-AL and LFD-AL). Whether the small decrease in body temperature observed in the HFD-WR mice is primary or secondary to the lowering of metabolic rate is not known. Some data favor the latter possibility; decreases in metabolic rate precede the drop in body temperature in animals in incipient torpor [113]. Due to the high surface-to-volume ratio of many small rodents, a lowering of metabolic rate (i.e. hypometabolic phenotype) would lead to decreased core body

temperatures through rapid surface area heat loss. The findings that the absolute metabolic adaptation (at least when using the multiple regression analysis) is equivalent at both ambient temperatures and that the mice do not enter torpor, suggest that the increased energy efficiency observed in the weight reduced state is the result of other adaptive mechanisms not related to torpor.

Weight-reduced leptin deficient animals (*Lep*^{ob}) were also studied at both 22°C and 30°C ambient temperatures (results reported in chapter 5). The metabolic consequences of weight-reduction in these leptin deficient animals are discussed in detail in the following section (see Leptin's Role in Defense of Body Weight). The weight-reduced OB mice (OB-WR; chapter 5) became torpid between the hours 0200h and 0500h (average body temperature 26.5±0.3°C) but only when studied at 22°C. At 30°C, a significant decrease in body temperature was also observed between 0200h and 0500h in the OB-WR, with mean core body temperatures reaching 32.9±0.1°C, yet none of the mice became torpid. Leptin replacement in calorically restricted mice [105] and *Lep*^{ob} [115] mice blunts both the number and the severity of bouts of torpor. In our studies, torpor occurred only in weight-reduced congenitally leptin deficient mice (OB-WR) housed at 22°C.

Regression analysis revealed comparable leptin to FM relationships in all WT mice including the HFD-WR mice, suggesting that: 1) these mice were weight stable and not calorically restricted relative to metabolic rate; and 2) the leptin axis had not been disrupted by weight reduction *per se*. Consistent with leptin's capacity to increase energy expenditure, leptin administration to *Lep*^{ob} mice quickly (within 2 hours) increases norepinephrine turnover in brown adipose tissue [261], and in WT rodents central infusions and large peripheral injections

increase energy expenditure and UCP1 content, the major thermogenic molecule found in BAT[262 263]. Other studies have found no upregulation of UCP1 content or EE in rats and mice that were infused peripherally with physiological doses of leptin[264,265]. These studies suggest that when rodents are "euleptinemic", they are "eumetabolic" and only supraphysiological injections are capable of further elevating metabolic rate.

When leptin levels decline (i.e. due to acute food deprivation), the concentrations are no longer sufficient to maintain a thermogenic drive resulting in the initiation of torpor. *Ucp1-/-* and *Lep^{ob}-/-* double knock-out mice do not survive due to hypothermia below 12°C ambient unless leptin is administered highlighting leptin's capacity to increase thermogenic responses [226]. The frequency of torpor initiation in C57BL/6J seems to be weight/adiposity dependent since male mice that are over 25 grams (that have higher percent body fat and leptin concentrations) rarely enter torpor upon a 24 hour fast whereas torpor is much more prevalent in mice less than 25 grams (personal communication Dr. Steve Swoap: Williams College). More specifically, it seems to be directly linked to absolute leptin concentrations and not some other adipose/weight-dependent factor since *Lep^{ob}* mice, that have excessive body fat but no functional leptin, readily enter torpor even under fed conditions[113]. In light of this result and the fact that leptin inhibits torpor, we hypothesize that the absence of torpor in the WT HFD-WR mice is due to the presence of sufficient circulating leptin.

One possibility to further investigate leptin's role in the onset of torpor would be to use antibodies or the soluble leptin receptor to decrease the amount of bioavailable leptin in circulation in a time-specific and dose-dependent manner. Dose-dependent responses of torpor onset could be explored while mice are housed at different ambient temperatures. One danger

with such an approach is the animal may start producing antibodies against such molecules. The human leptin trials conducted in the late 90s concluded that 71% of the participants that underwent 24 weeks of leptin treatment developed antibodies against the hormone [50]. Using the *Lep*^{ob} heterzygote mice (*Lep*^{ob} /+), that produce ≈30% less leptin per unit fat mass[266] than +/+ mice, could similarly be tested at different ambient temperatures and differing lengths of fast. *Lep*^{ob} /+ mice were shown to survive a prolonged fast significantly longer than normal homozygous mice (+/+) but significantly shorter than *Lep*^{ob}-/-[47] suggesting a gene dose effect that may influence frequency and depth of torpor in these mice.

LEPTIN'S ROLE IN DEFENSE OF BODY WEIGHT

Leptin's capacity to reverse many of the physiological adaptations observed under fasting/weight-reduced conditions in both humans and rodents has been well documented [13,42,103]. These models, in which physiological doses of leptin have metabolic effects, have sometimes been described as functionally "hypoleptinemic" because of the similarity of phenotype with leptin deficient humans and rodents. The capacity of leptin to reverse many of the physiological modifications in the weight-reduced state, suggests that leptin is being actively sensed by the organism (in CNS and possibly elsewhere) and reacting to decreased absolute concentrations of the hormone. In chapter 2, we reported that weight reduced DIO mice (DIO-WR) had similar physiological phenotypes (low EE, suppressed T3, increased food-seeking behavior) to weight-reduced never obese mice (CON-WR), suggesting that they were now defending an elevated body weight (i.e. raised body weight [fat] threshold). We hypothesize that leptin replacement in these DIO-WR mice would indeed reverse these phenotypes as occurs in weight-reduced human subjects [22,42,161] and fasting mice [43] that are given replacement doses of leptin. If leptin is capable

of reversing the phenotypes observed in the WR mice as has been shown in humans, this would be further support for the inference that the diet-induced obese mice (both DIO-AL and DIO-WR) had adapted to and required elevated circulating leptin concentrations in order to be "eumetabolic".

If we posit that the leptin threshold model proposed in the introduction (**Figure 1.3**) and elsewhere [13,42] is involved in an organism's capacity to resist weight [fat] loss and that the DIO mice have an elevated threshold as a consequence of maintaining an elevated body weight, some aspect of the obese state and/or the high fat diet must be involved in alterations in CNS-based sensing of leptin. Attenuation in leptin signaling induced by obesity, sometimes referred to as "cellular leptin resistance", could increase the amount of weight gain induced by genetic and environmental factors [241]. A decrease in sensitivity to circulating leptin concentrations would result in further increases in adipose tissue until enough leptin is produced to generate sufficient signal intensity in the hypothalamus. In such a "feed forward" model, in which obesity and/or components of the high fat diet decrease CNS leptin sensitivity resulting in further weight gain, it is hard to distinguish mechanisms that cause initial weight gain from those that result from it. Various causes of cellular leptin resistance have been proposed. Suppressor of cytokine signaling (SOCS)3 is upregulated by leptin receptor activation and functions as an inhibitor of leptin signaling by binding to Tyr_{985} [62,267]. SOCS3 KO models are more leptin sensitive resulting in decreased food intake and protection from obesity when presented with a high-fat diet [268,269]. Protein tyrosine phosphatase (PTP)1B dephosphorylates Jak2, reducing leptin signaling both in cultured cells and in vivo [270,271]. PTP1B neuronal KO models, similar to the SOCS3 KO models, have increased leptin signaling and decreased adiposity[272,273]. Endoplasmic reticulum stress and low level inflammation have also been implicated in decreased leptin sensitivity[240,274]. Decreased

CNS-mediated leptin signaling is therefore a plausible neural explanation for apparent defense of elevated body weight observed in the DIO-WR (chapter 2) and HFD-WR (chapter 6) mice.

Whether hyperleptinemia *per se* in the obese animals was responsible for the apparent rise in the regulatory threshold was investigated in two separate but related studies. The first study used *Lep*^{ob} mice (chapter 5) to determine whether weight loss in leptin deficient animals was associated with metabolic adaptation. The second study characterized WT non-obese mice infused with leptin so that circulating concentrations of leptin were consonant with those of age-matched diet-induced obese mice, in essence isolating hyperleptinemia without the confounds of obesity-related physiological changes (chapter 6).

In the *Lep*^{ob} study we hypothesized that no metabolic adaptation would be seen following weight reduction since the CNS would not receive the afferent leptin signal indicating a decrease in body weight. However, we found that leptin deficient mice did become hypometabolic when weight reduced at both 22°C and 30°C ambients. The OB-WR mice became torpid and hypometabolic at 22°C ambient. At 30°C, their body composition-adjusted TEE was 25% below that of the OB-AL controls. Hence, in this admittedly special case of congenitally leptin deficient animals, a change in circulating leptin concentration was not required to invoke a hypometabolic response to weight loss.

The leptin infusion experiment (chapter 6) tested whether 18 weeks of hyperleptinemia – in the absence of obesity - could raise the threshold. Upon cessation of leptin infusion, we hypothesized that these mice would increase body weight (adiposity) and plateau at an elevated body weight compared to PBS infused mice, due to permanent modifications in leptin sensitive pathways of the CNS. These CNS based modifications would result in decreased sensitivity to

leptin, which upon leptin cessation would cause a perceived "hypoleptinemia" resulting in physiological adaptations associated with the weight-reduced state: decreased EE and increased EI. These changes would ultimately lead to increased adiposity. These changes would have been equivalent to the WR mice described in chapters 2 and 5. The sudden drop in leptin would have been perceived by the brain as a sharp decline in body adiposity, the physiological equivalent of rapid weight loss. *Lep*^{ob} mice whose circulating leptin concentrations are "clamped" to those of lean animals by low dose administration of leptin via mini-pump remain leptin sensitive even after becoming obese by feeding of a high fat diet during 20 weeks [242]. Body weight and body-composition matched WT mice also fed a high fat diet had significantly higher leptin concentrations and were leptin resistant (i.e. decreased pSTAT3 activation in the hypothalamus following leptin administration) suggesting that leptin itself is required to induce leptin resistance [242]. Contrary to our hypothesis, we found that the highest infusion rate (25ug/day) was not associated with elevated EE. This finding suggested that these mice had accommodated to the elevated concentrations of leptin, reflecting an increase in the concentration of leptin at which an animal is "eumetabolic". Upon cessation of the leptin infusion, the mice did not defend elevated body weights and even plateaued at slightly lower body weights compared to PBS-infused mice. This finding suggested that hyperleptinemia alone is not capable of raising the threshold for defense of body fat, implicating other factors related to the obese state and/or the diet in the changes in body weight set point. Elevations in the hypothalamus of free fatty acids [238], cytokines (e.g. IL6) [239] and modified endoplasmic reticulum stress biology [240] have all been shown to impair acute and chronic leptin signaling. This impairment may account for the persistence of high levels of body fat despite elevations of circulating leptin concentrations. Whether these various desensitization processes occur by shared mechanisms is unknown[241],

and whether such desensitization is mechanistically related to the apparent "defense" of a higher body weight in mice chronically maintained at higher body weight by feeding of a HFD, is not clear.

A few possible major confounds to the inferences reached above should be mentioned. Human leptin trials showed that more than 70% of participants that received exogenous leptin injections for 24 weeks were positive for anti-leptin antibodies[50]. If such antibodies were produced in the current experiment, the increased circulating leptin concentrations may have been rendered biologically unavailable for CNS signaling. A second possible confound is that the antibodies themselves may have artificially increased the apparent leptin concentrations when measured by ELISA by direct interaction with the antibodies used in the assay itself. Gene expression levels of leptin in WAT could give us further insight into this possible confound and is currently being undertaken. Finally a third possible confound is that the endogenous short form leptin receptor (LepRe) could have been upregulated again causing a decrease in the amount of bioactive leptin that can reach the brain. We unfortunately do not have enough serum to test whether LepRe has been upregulated in these mice but this type of assay should be conducted in future experiments.

LEPTIN & MICROBIOTA

The microbiota sequencing project was conducted to test whether the metabolic adaptation observed in weight-reduced mice might be related to changes in bacterial populations that could confer metabolic advantages to the host. The correlations between circulating leptin concentrations and specific bacterial species were of particular interest. Chief cells in the

stomach can produce leptin [275] and leptin upregulates mucin production in the intestine[187,188] thereby altering local bacterial niches. Some of the bacteria interact directly with mucin; *Akkermansia* subsists on mucin [185]and *Mucispirillum* colonizes the mucus layer[186]. Elevated leptin concentrations have been linked to the up-regulation of pro-inflammatory cytokine gene expression (e.g. *Il6, CXCL1*) in mouse colon and leptin signaling in the colon has been linked to colon cancer[276-279]. Observational studies have shown an inverse association between dietary fiber intake and body weight[280,281]. It has recently been suggested that this relationship is the result of the anaerobic breakdown of fiber by intestinal bacteria into short-chain fatty acids (SCFA). There are SCFA specific receptors in the gut[282,283] that, upon activation, increase the release of the "ileal break" gut hormone peptide YY (PYY) [284]. Interestingly, *in vitro* and *in vivo* application of SCFAs to mouse adipocytes has been shown to regulate leptin expression while feeding of propionate (a SCFA) to mice led to elevated circulatory leptin concentrations[285]. To directly test whether leptin concentrations have a direct effect on bacterial abundance in the gut, we collected fecal samples from the leptin infused mice (chapter 6) at low, medium and high infusion rates as well as following the removal of the mini-pumps. These samples are currently being analyzed. Demonstration that circulating leptin concentrations directly modulate bacterial abundance of certain species in the gut would open a new line of investigation.

FUTURE DIRECTIONS

The apparent defense of elevated body weight in mice following high fat diet feeding suggests a change in the body weight set point (chapters 2 & 5). This resetting does not seem to be the result of hyperleptinemia alone since mice receiving 18 weeks of leptin infusion did not defend an elevated body weight upon cessation of the infusion (chapter 6). Free fatty acids, ER

stress, and low level inflammation have all been implicated in the CNS's decreased sensitivity to circulating leptin concentrations possibly underlying defense of elevated body weight. The weight-reduced mice studied in this thesis (chapters 2 and 5) were generated by feeding hypocaloric amounts of the high fat diet (60% kcal from fat). Whether the dietary fat content is capable of diminishing leptin sensitivity in the CNS resulting in turn in the defense of a higher body weight [238] was tested by conducting a large scale diet switch experiment. Diet-induced obese mice (fed HFD for 26 weeks) then switched to a low-fat control diet (10% kcal from fat) lost all excess body weight plateauing at weights similar to those of never obese mice. Indirect calorimetry did reveal small but significant decreases in adjusted energy expenditure (-0.45±0.15 kcal/day) suggesting a "defense" of a higher body weight. Whether the full magnitude of this adaptation is masked by the increased thermogenic demand at 22°C is unknown. A subset of the mice from this study were injected with bolus doses of leptin (3mg/kg) in order to determine leptin signaling in different areas of the brain. The analysis of these brain regions and the discovery of leptin sensitive areas should dictate the brain regions in which altered leptin signaling plays a direct role in body weight homeostasis. In light of the data presented in chapter 5 on the role of ambient temperature on energy homeostasis phenotypes in mice, we are also currently collaborating with scientists at The Pennington Biomedical Research Center to conduct a dietary switch study at both 22°C and 30°C ambient to determine whether removal of the thermogenic stress induced at sub-thermoneutral temperatures (22°C) would "unmask" attempts by formerly obese mice to defend higher body weights when provided diets lower in fat content.

CONCLUSIONS

The obesity epidemic in the U.S presents great medical and economic challenges. The high prevalence rates of obesity, T2D, and the metabolic syndrome dictate the need for wide-ranging research into the causes and potential preventative treatment options for obesity. Understanding the defense of body weight and metabolic adaptation when faced with weight perturbation is one of the key components to understanding obesity as the interaction between genes and environment. The experiments presented in this thesis aimed to demonstrate how body weight regulation can be influenced through environmental changes such as high fat diet, availability to calories, and ambient room temperature. Further understanding of the communication between energy stores and the CNS will be crucial in developing effective prevention strategies and therapies for obesity.

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