THE EFFECT OF CHRONIC OVERFEEDING ON PHYSICAL ACTIVITY IN MICE

A Dissertation

by

HEATHER LEIGH VELLERS

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Chair of Committee,	J. Timothy Lightfoot
Committee Members,	James D. Fluckey
	Christopher Woodman
	Wei-Jung A. Chen
Head of Department,	Richard B. Kreider

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ABSTRACT

Physical inactivity, together with poor nutrition, make up the second leading cause of death in the United States, and also contribute to the development of a host of diseased states. While strong evidence suggests only modest increases in daily activity is required to decrease all-cause mortality and premature death, activity levels in the U.S. remain low. Indirect results suggest that chronic overfeeding decreases physical activity, though the mechanisms linking overfeeding and decreased activity remain uninvestigated. The most potent biological regulators of daily activity known thus far are the primary sex hormones, and there are reports that chronic overfeeding alters sex hormone levels. Thus, these separate data sets lead to the hypothesis that the primary sex hormones directly link chronic overfeeding and reduced activity. To investigate this hypothesis, inbred mice (C57Bl/6J) were bred, and the resulting offspring were weaned at three weeks of age and randomly assigned to either a control (CFD) or high fat/high sugar (HFHS) diet. Daily wheel running activity (distance, duration, and speed) of the mice was measured along with weekly measurements of nutrition intake and body composition. Upon sacrifice, blood samples were extracted via a cardiac puncture for determination of testosterone and 17β-estradiol levels in male and female mice, respectively.

The mice on the HFHS diet showed a significantly increased daily caloric intake and percent body fat composition. Additionally, the HFHS diet significantly reduced acute wheel running distance for both male and female mice (≈61%). The HFHS-

induced reduction in distance was the result of different mechanisms in the male and female mice; the reduction in activity in males due to reduced activity duration, and in females, a decreased speed of activity. A two-week period of wheel access was not sufficient to alter HFHS-induced reductions to activity or increases in body fat. Further, there were no significant effects of chronic overfeeding on serum levels of testosterone or 17β-estradiol, suggesting that the overfeeding-induced decrease in activity occurred independently of sex hormone levels. In conclusion, chronic overfeeding significantly decreases daily activity in mice and shows sexual dimorphism in responsible indices.

DEDICATION

I would like to dedicate this dissertation to my wonderful parents, Gary and Lisa Vellers, and also to my siblings, Ashley Shaw and William Vellers. I will always be grateful for the love, support, encouragement, and belief that you all have had in me throughout my life, and since coming to Texas A&M. To my father, I have learned so much from you. Your tireless hard work, dedication to our family, and selflessness, is something that I have always admired, and sincerely appreciate about you. To my mother, thank you for being the loving, kind, and caring person that you are, and instilling in me those qualities. To my sister and brother, thank you for the unwavering friendship I have with each of you no matter the distance we are apart.

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1. INTRODUCTION

Physical inactivity, in combination with poor nutrition, is the second leading cause of actual death in the United States, and also the primary contributor to a host of chronic diseases including obesity, heart disease, type II diabetes, and some forms of cancer (93). As a result, it has been estimated that physical inactivity has led to over $400 billion in health care costs yearly in the U.S. (24). While an increase in physical activity by even a modest amount (~1000 total calorie expenditure per week), has been shown to significantly decrease all-cause mortality (20-30% reduction) (70) and lowers premature death (14), activity levels still remain low and continue to decline. Objective measurements of daily activity (126) have estimated that only 3.5% of U.S. residents aged 20 years and older meet the physical activity participation guidelines recommended by the American College of Sports Medicine (ACSM) and the American Heart Association (AHA) (53, 99). Blair and colleagues (3, 25, 134) have further emphasized the importance of regular physical activity through demonstrating that physically active individuals, even with an existing relative risk factor (e.g. hypercholesterolemia, high blood pressure, obesity, and etc.), have a significantly lower premature death rate due to cardiovascular disease when compared to sedentary individuals with no known relative risk factors. Thus, increasing activity for otherwise healthy - yet sedentary individuals - in addition to diseased populations, would lead to substantial gains for both the health of the individual and aid towards eliminating much of the health care cost burdens to the

U.S. It is therefore critical to have a greater understanding of the factors influencing and regulating physical activity for the individual.

The regulation of physical activity is understood to be largely controlled by genetic/biological mechanisms (25- 92% influence)(75) and to date, the primary sex hormones in both males (testosterone) and females (17-β estradiol) are suggested as the most potent regulators of physical activity (14, 16, 132). Substantial literature dating back to 1925 (132), in addition to work from our laboratory (14, 16), identified the primary sex hormones in males (testosterone) and females (17β-estradiol) as potent biological regulators of daily physical activity (14, 16). The removal (via orchiectomy or ovariectomy) of testosterone or 17β-estradiol resulted in ≈ 90% decreases in activity, while endogenous replacement of these hormones resulted in varying levels (35-110%) of recovered baseline activity (16). Furthermore, these findings showed that the recovered activity distance and duration were primarily influenced by testosterone administration, whereas the recovery of speed was primarily dependent on the replacement of 17β-estradiol. Thus, this data, along with other investigations (76, 132) suggest an androgenic and/or estrogenic pathway is involved in regulating physical activity.

To date and to our knowledge, studies have yet to investigate whether a single factor known to disrupt and/or reduce the levels of the primary sex steroid hormones would directly lead to decreases in physical activity. One such factor, which has recently been confirmed by Bouchard and colleagues to directly decrease both testosterone and 17β-estradiol, is chronic overfeeding (13, 103). In this work, it was

demonstrated that overfed male subjects (1000 kcal/day, six days/week) resulted not only in decreased sex steroid hormones, but also, that the androgens (e.g. testosterone) were amongst the most sensitive to the changes affected by overfeeding (13). While this particular investigation did not consider the effect of overfeeding and its' associated responses with physical activity, there are additional studies that have provided indirect and anecdotal evidence suggesting that overfeeding does lead to decreases in physical activity. For example, Levine and colleagues (71, 114) showed that overfeeding in both lean and obese human subjects, by 1,000 kcal/day, decreased their free-living walking. While the aforementioned studies support the hypothesis that overfeeding may decrease physical activity through overfeeding-induced decreases in the primary sex steroid hormones, no study has directly tested this hypothesis.

Determining whether a causal link between chronic overfeeding and physical inactivity exists would be a first step towards initiating a line of potentially impactful mechanistic studies directly bearing upon the biological mechanisms controlling daily activity, as well as obesity and other chronic health diseases. Thus, the purpose of this study was to determine the effect of chronic overfeeding on measures of physical activity and the level of the primary sex hormones.

2. LITERATURE REVIEW

2.1 Introduction

Chronic overfeeding has been shown to alter the status (function and concentration) of the primary sex hormones in both males (testosterone) (13, 112) and females (17β- estradiol) (82) and has been indirectly linked to alterations in daily activity (71, 114). Given that the primary sex hormones have a potent role on physical activity regulation (14, 16, 17, 110, 132), and that overfeeding alters these hormones leads to speculation regarding a role of the primary sex hormones as potential mechanisms linking the two behaviors. To date, a direct influence of chronic overfeeding on physical activity levels through sex hormone involvement has yet to be tested. Therefore, the focus of this review is to discuss the literature that has provided direct and indirect links between diet and activity as well as the literature regarding the effect of chronic overfeeding on the primary sex hormones. Together, these topics are integrated to bring forth the evidence linking overfeeding reductions on activity through altered levels and function of the primary sex hormones.

2.2 Chronic Overfeeding and Physical Activity Regulation

Indirect evidence in humans (71, 114) and in non-human primates such as the baboon (55), suggest overfeeding may be an independent factor associated with

decreases in physical activity. For example, in humans, Levine et al. (71) showed that overfeeding both lean and obese human subjects decreased their free-living walking. In this study, both male and female subjects (ages 39 ±8 years) were overfed by 1,000 calories per day for nine weeks, and objective activity measures were taken and compared 10 days prior- and 10 days post-overfeeding period. Regardless of whether the subjects were healthy lean, or obese, their activity, measured as distance covered, reduced by similar amounts when overfed (~ 1.5 miles/day). Additionally, another study by Schmidt et al. (114), observed that chronic overfeeding (overfed by 1.4 times an individuals' daily basal energy needs) significantly decreased free-living walking activity in obesity-prone (~40%), but not obesity-resistant individuals. Furthermore, a recent longitudinal epidemiological study conducted in England consisting of 25,639 men and women aged 39- 79 years over a ten-year period, showed that higher rates of weight gained during this period correlated with future levels of decreased physical activity (48). From this study, however, conclusive associations could not be made for the isolated and/or combined effects of weight gain and dietary intake as predictors for reduced future activity. Anecdotally, Higgins et al. (55) observed that baboons (*Papio hamadryas Sp*.) , when given excess calories through a fructose drinking solution in replacement of normal water, displayed observational decreases in daily activities (climbing, playing, etc.).

Similar to humans and non-human primates, there are also studies in mice that support the linkage between chronic overfeeding and physical activity. For instance, one study by Funkat et al., showed that C57BL/6J mice fed a specialized very high fat

(60.4% fat) diet for a six week period before instituting running wheel access, demonstrated that the diet resulted in reduced wheel running activity compared to mice fed a normal chow diet (4% fat) (46). Each of these studies however, utilized excessively high fat diets (60% fat versus 45% fat) that are 25- 35% higher fat content than what the typical adult would consume (4, 10). Most recently, a study by Rendeiro et al. (105), showed that high fructose consumption (18% fructose), which closely resembles the average fructose consumption among U.S. adults (15- 23%) (85, 123), in mice resulted in acute decreases in wheel running activity.

While most literature suggests that overfeeding reduces daily activity, there are also other studies that have suggested that overfeeding actually increases daily activity. Brown et al. (18) demonstrated that C57BL/6N female mice, when fed a high fat diet (45% kcal fat) and exposed to a running wheel at the same time, exhibited activity that was increased to match the level needed to compensate for the increased caloric consumption in order to maintain normal body weight (18). A further line of evidence by Meek and Garland also showed increases in activity when feeding high-activity selectively bred mice a standard rodent westernized diet (42% fat). However, since these mice were selectively bred for high voluntary wheel running activity and due to their high active nature, these authors speculated that this response in these mice was a result of their ability to utilize dietary lipids for sustained exercise and increased performance (87, 88).

To the best of our knowledge, the aforementioned studies in mice are the only existing studies that have suggested a potential link between nutritional overload and

physical activity. Thus, there are some studies (88) that showed that overfeeding increased activity and some studies (71, 114) that showed that overfeeding decreased activity. Overall, it appears that chronic overfeeding alters activity, but the direction of that alteration is unclear and cannot be elucidated based on present literature. While there are other confounding variables such as age, the time of running wheel application and/or the type of diet utilized that prevent the understanding of whether a direct link exists between chronic overfeeding and physical activity, there is support for an effect of diet on activity level.

2.3 Caloric Restriction and the Physical Activity Response

While there is no direct evidence of effects of overfeeding on activity *per se*, there is copious literature regarding the effect of moderate caloric restriction on activity. Literature starting from the early 1900's to the present have provided strong evidence linking reduced caloric intake with increased physical movement (23, 27, 30, 31, 40, 52, 100, 104, 106, 107, 109, 111), and this phenomenon has been described in both human (22, 54, 59, 121) and rodent models (30, 61, 101, 129).

In humans, the effect of caloric restriction on increased activity is difficult to experimentally test because of ethical concerns. However, case literature dated back to 1868 (50) for anorexia nervosa (AN), has reported observations of heightened levels of activity (e.g. "restlessness" and "increased drive" to move), which typically, is only observed after following a period of self-starvation (22). In such case reports, clinical

observations consist of AN patient-specific findings such as restlessness (2), fondness for particularly long-walks (47), exhibiting an internal urge to be highly active (131), and "overactive" (92). These observations of heightened activity following a period of caloric restriction, was discussed in a comprehensive review by R. Casper (22), where the author proposed the hypothesis that the caloric restriction of AN leads to increased physical activity in humans as opposed to the prevailing belief that increased physical activity led to AN.

In a similar fashion, rodent models of AN (aka: the Activity Based Anorexia or ABA model) also display hyperactive responses (e.g. increased wheel running behavior) following a caloric restricted regimen (30). The ABA model is an established rodent model of activity anorexia that simulates the activity response of humans with AN. The protocol for the ABA model, first established by Routtenberg and Kuznesof (107, 109), places rats/mice on an investigator-imposed food restricted regimen (restricted to one hour of food access per day), and provides free access to a running wheel (107, 109). In this model, running wheel activity significantly increases due to time-restricted feeding (107). Similar observations regarding the increase in activity with a decrease in caloric consumption has occurred numerous times in the literature (5, 22, 54, 61). Thus, taken together, the human and animal studies provide evidence of a direct effect of caloric reduction on increasing physical activity levels.

The mechanisms controlling this hypocaloric increase in activity have not been as clear as the phenomenon itself, though there is a long history of investigations attempting to uncover the underlying mechanisms. In 1922, Richter was the first to

show that rats in a semi-starved induced state (up to 25% caloric restriction), showed significant increases in their activity (i.e. wheel running), which later led to the development of the ABA rodent model (101). Richter's work was later expanded upon by other investigators including Routtenberg and Kuznesof (108), and then Epling and Peirce (30), where they further examined this link. Routtenberg and Kuznesof (109) demonstrated that food deprivation in rats led to not only parallel increases in wheel running activity and sharp declines in body weight, but also that these responses to caloric restriction led to a downward spiral ultimately leading to their death. Some investigators (9, 28, 120) have suggested that this viscous cycle of a paradoxical increase in wheel running activity under a semi-starved-induced state is a result of an activated "foraging mechanism" (e.g. foraging gene) that is activated in an attempt to prompt increased movement in search for food.

While the possibility of an activated foraging mechanism linking caloric restriction and increased activity still persists, some evidence suggests otherwise. Epling and Pierce (100) were the first to illustrate that a continual hyperactive response following the period of food restriction (in the activity based anorexia model in rats) was independent of a foraging mechanism. They showed that semi-starved rats conditioned to voluntarily access more food with the push of a button, ignored the additional food source and continued to increase their wheel running activity regardless of having food access (30). In all, the findings of Epling and Pierce's study demonstrated that the increased activity effects induced by semi-starved states overrode the effects of food deprivation. Additionally, the authors also noted from their findings

that there is likely a point of an energy deprivation where the drive to search for food is not the primary cause for the continual high volume of wheel running. Likewise, in humans with AN, where heightened activity responses are observed to follow a period of self-starvation, the hyperactive behaviors continue regardless of having food readily accessible (22) . Thus, these findings highlight a direct regulatory effect of caloric restriction on increased activity that is ultimately without dependence on a foraging mechanism because the hyperactivity continues in spite of food availability.

Mechanistically, various neurochemicals central to the brain, have been associated with the hypocaloric hyperactivity response, including leptin (5, 35, 54, 129), serotonin (56), and dopamine (64). The adipose tissue-derived hormone leptin, is one of the most widely known and investigated mechanisms associated with dietary restriction and increased activity (5, 37, 38, 54, 59, 73, 129). The secretion of leptin is correlated with fat mass, where leptin secretion is increased with increases in fat mass, and decreased with decreases in fat mass (1). The involvement of leptin in diet and activity regulation has been tested through leptin administration in patients diagnosed with AN and in ABA rodent models, where increased leptin reduced the hyperactive responses in these subjects (37, 73). A study by Verhagen et al. (129) demonstrated that the low levels of leptin associated with increased wheel running was mediated through the ventral tegmental area of the brain. The authors further speculated that the suppressed leptin levels induced an increased firing rate of the dopaminergic neurons, which highly express leptin receptors (39), and may provide one mechanism through which leptin participates in activity regulation in response to caloric restriction. Interestingly, this

finding is also in line with the well accepted theory that activity is regulated within the mesolimbic system (located in the midbrain, which connects the ventral tegmental area of the brain to the nucleus accumbens), where dopaminergic signaling regulates locomotion (39, 44, 45) . Although the study by Verhagen et al.(129), as well as other studies (62, 119) have solely focused on increased activity associated with caloric restriction, these studies add support to the hypothesis that the dopaminergic mesolimbic system has a central role in activity regulation.

While plasma leptin has been suggested as a key player between caloric restriction and hyperactivity in both humans with AN (5, 54, 59) and in ABA rats (35), these studies are correlational and have not been directly tested. Some evidence suggest the hypocaloric-induced increases in physical activity are independent of leptin levels (56, 95). For example, one study by Morton et al. (95) demonstrated a hypocaloric-induced increased activity response independent of leptin, through fasting (fasted for 24-hours) wild-type and *ob/ob* (leptin deficient) mice and found that fasting in these mice led to significant increases in both ambulatory and wheel running activity, independent of leptin. Another study by Hillebrand et al. (56) also supported that the hypocaloric hyperactivity interaction was not dependent on leptin. The authors of this study evaluated the effect of the atypical antipsychotic drug, Olanzapine (Zyprexa), on wheel running in the ABA rat model, and also physical activity in a cohort of AN patients who were classified as 'hyperactive' upon hospital admittance. Olanzapine is considered a theinobenzodiazepine compound, which has a high affinity for 5-HT $_{2A/AC}$ receptors, histamine (H1) receptors, adrenergic (a1 receptors), and a moderate affinity for

dopamine (D1-D4) receptors (20, 21, 94, 116). Olanzapine was found to inhibit the heightened wheel running in ABA rats, and hyperactive responses in patients with AN; however, Olanzapine inhibited the hyperactive responses without altering plasma leptin levels. Thus, the ability of Olanzapine to inhibit hyperactivity without a change in leptin levels, suggests other mechanisms are involved leading Hillebrand et al. (56) to hypothesize that the hyperactivity resulting from hypocaloric conditions could be through serotonin activation (5-HT $_{2A/AC}$ receptors), histamine receptors, and/or dopamine given the high-moderate affinity of Olanzapine on these systems. Klenotich et al. (64) expanded on the finding that Olanzapine blunted ABA, through selectively blocking the 5-HT $_{2A/AC}$ receptors and dopamine receptors (DR) - D2R and D3R, and found that selective antagonism of D2R and D3R reduced ABA, whereas antagonism of 5-HT $_{2A/AC}$ did not. Therefore, this study suggested that the D2 and D3 receptors are the mechanisms through which Olanzapine reduces ABA, and thus suggest significant involvement of D2 and D3 to increase activity in the hypocaloric state.

The pathways underpinning how neurochemicals, like dopamine, mediate the effect of caloric restriction with increased physical activity remain unclear; however, evidence suggests that the sirtuins, specifically neuronal sirtuin 1 (*Sirt1*), are required for this response. The sirtuins are intriguing central mechanisms, given that they are believed to sense the metabolic status of an organism, and subsequently, direct various downstream metabolic responses as well as increasing physical activity of an organism (113). Chen et al. (23) found through comparing physical activity in wild-type and *Sirt1* knockout mice following nine-months of caloric restriction (60% restriction of ad

libitum values), that activity significantly increased in the wild-type, but not the *Sirt 1* knockout mice suggesting that *Sirt1* is required for caloric restriction to induce increased physical activity. A further study on *Sirt1* involvement between caloric restriction and increased activity, was conducted by Cohen et al. (27), where it was demonstrated that caloric restriction reduces *Sirt1* in the hypothalamic neurons encompassing somatotropic signaling to the lower axis, and consequently results in decreased locomotor behavior.

Thus, the evidence presented by the aforementioned studies provides a direct link between caloric restriction and increased activity. As such, given that caloric restriction directly increases physical activity and that there are supported mechanisms mediating this effect (5, 38, 64), it is unclear why the converse would not also apply: that excessive caloric intake would lead to an inhibition of physical activity.

2.4 Average Daily Caloric Consumption among U.S. Adults: Eating More; Eating Less; or the Same?

When considering the relationship between chronic overfeeding and daily activity, for translational application it is important to set this question within a societal context: do humans – especially in the United States - overeat? According to a report by Ford et al. (42), which analyzed self-reported nutritional data from the National Health and Nutrition Examination Survey system (NHANES), the average daily caloric intake among US adults, aged 20- 74 years, has decreased by ≈ 74 calories per day (kcals/day) in years 2009-2010 when compared to 2003-2004. In children (ages 2-12

years) and adolescents (12- 18 years), Mendez et al. (89) demonstrated through analyzing four cycles of the NHANES nutritional database (2003-2004, 2005-2006, 2007-2008, and 2009-2010 cycles), that the average daily caloric intake in adolescents increased in the most recent years by 59 and 97 kcals/day in boys and girls, respectively. The children in this same investigation appeared to have leveled off with only minimal changes in caloric intake in the most recent years (-11 to 40 kcals/day). A recent study, concurrent with the aforementioned studies by Ladabaum et al. (66) supported the finding that according to self-reported dietary intake, the average daily caloric intake has not changed significantly; however, this investigation also showed substantial increases in obesity. Ladabaum et al. found the prevalence of obesity from 1988 to 2010 increased 24.9% to 35.4% in women, and 19.9% to 34.6% in men (66). Thus, the increased prevalence of obesity in most recent years is in contrast to data suggesting caloric intake is slightly decreasing or remaining the same. Ladabaum et al. (66) and others (126), show that physical activity is low and rapidly declining; however, it is uncertain whether physical inactivity is the sole reason for the increased rates of obesity. Therefore, the validity of these dietary intake reports have been somewhat controversial, because while representing large scale estimates, they are derived from subjective, self-reported data, and thus, may significantly underestimate true dietary intake. In particular, Archer and colleagues (4) have suggested that there are serious flaws associated with the NHANES data base system.

Archer, et al. (4) examined nutritional reports contained in the NHANES data sets from years 1971 to 2010 to determine if the reported nutritional intake data was

valid. The investigators compared individually reported energy intake to estimated basal metabolic rates for each individual. These estimates were based on the ratio of reported energy intake (rEI) to basal metabolic rate (rEI/BMR) and the difference between rEI and estimated total energy expenditure from the Institute of Medicine's (IOM) predictive equations. Over the 39 years of NHANES data considered, Archer, et al. (4) determined that on average, men and women aged 20- 71 years underreported their daily caloric intake by -365 calories and -281 calories per day, respectively. Interestingly, the severity of underreporting was greatest in obese men (-716 calories/day) and women (-856 calories/day; (4). Given this large underreporting of caloric intake it is uncertain whether a correcting of the NHANES values would reveal excess caloric intake; however, this issue was not discussed by Archer, et al. (4). Archer and colleagues' primary conclusion was that self-reported nutritional information cannot be trusted as a valid representation of what likely represents the average U.S. adult. Unfortunately, large-scale methods to adequately assess true caloric consumption in a large population as well as whether there have been historical changes in daily intake remain largely unknown. Thus, caution is warranted when utilizing the self-reported nutritional NHANES data to support and/or further research stemming from its' contents.

Even while the work by Archer, et al. (4) appeared to discredit the validity of the self-reported data contained in the NHANES data set, their data analysis and conclusions are still limited because they are based upon estimates (self-reported nutritional intake), with other estimates (formula to estimate basal metabolic rate) to further extrapolate estimates (estimate of the amount of under- or over-reporting nutritional intake). Thus,

the combination of the various estimates cannot and do not provide a solid foundation upon which to base an answer to whether humans eat more than they did in the past.

The rise of overweight and obese individuals in the US over the past 30 years (43, 97) seems to infer that human caloric intake has increased, and that diet-induced body compositional changes has led to decreased physical activity. For example, in a longitudinal study by Metcalf et al.(91), a cohort of 202 children was studied to determine the association between body fatness and physical inactivity. The participants were assessed from 7 to 10 years of age, and physical activity was measured daily five days per week using accelerometers and body composition was measured annually using dual-x-ray absorptiometry. The results showed that physical inactivity was a result of, rather than the cause of fatness, i.e. inactivity was dependent upon on obesity (91). While nutritional intake was not measured by Metcalf et al. (91), this study suggested that physical inactivity was occurring after the onset of increased fatness supporting our hypothesis that diet could be altering physical activity regulation.

Anecdotally, it may seem obvious that excess caloric consumption is prevalent among U.S. residents; however, as Archer and colleagues have shown, the actual caloric intake – whether in excess, unchanged, or decreased – cannot be determined by the current data available (4). Further, using obesity as a marker of overeating, or emphasizing that inactivity is the cause of obesity, are all difficult arguments given the scant data investigating these questions.

2.5 Associations between Body Composition and Physical Activity

When considering whether chronic overfeeding has an independent- or co-dependent effect on physical activity, it is important to isolate the contribution of conditions resulting from chronic overfeeding toward inactivity. Alterations to body composition, including increases in total body weight, increases in fat mass, and/or decreases in lean muscle mass, are well-established and accepted consequences of chronic overfeeding (13). Some literature reports that increased adiposity (58, 91), and/or increased total body weight (48) are associated with decreased levels of physical activity, whereas other studies have suggested that body composition has no role in activity regulation (7, 77, 84). Given that chronic overfeeding has been indirectly associated with decreased physical activity and that altered body composition is an established effect of chronic overfeeding, it is important to clarify whether the diet and physical activity interaction is an independent effect of excess dietary intake or arises from the increase in percent body fat composition resulting from overfeeding.

In support of the hypothesis that altered body composition has a role on the regulation of physical activity, a longitudinal study conducted in England with a cohort of 25,639 subjects (men and women aged 39-79 years), demonstrated that the rate of weight gained over an 18-month period was a significant predictor of future reductions in physical activity in both moderate (0.5-2 kg) and heavy (>2 kg) body weight gainers (48). The conclusions of this study (48) must be tempered because of the use of a subjective physical activity survey to assess activity levels. However, there are other

studies that have measured physical activity using objective measures that have shown similar findings. For example, Levine et al. (71) demonstrated that weight gain in adults following a nine week period of overfeeding (1000 kcals/day above weight maintenance needs) was correlated to decreased walking activity, and that walking activity continually declined in association with increases in weight in both lean and obese subjects. However, isolating the effects of caloric intake on body weight versus physical activity in this study was not accomplished. Lastly, a longitudinal analysis by Metcalf et al. (90) using children aged 7-10 years old (202 total with ~53% considered overweight), showed that physical inactivity in this cohort was predicted by increased adiposity, but that physical inactivity was not a significant predictor of increased adiposity (90). Taken together, these investigations suggest that increased body weight and fat mass could independently be associated with decreased daily activity.

Conversely, there are also studies which support chronic overfeeding as an independent regulator of physical activity, regardless of increased body weight, fat mass, and/or decreased lean muscle mass (76). For example, Schmidt et al. (114) in studying the effect of overfeeding on spontaneous physical activity in obesity prone versus obesity resistant individuals (men and women aged 25- 35 years), observed that the obesity prone (>30 kg/m^2) and obesity resistant (16.9- 25.5 kg/m^2) individuals receiving the eucaloric (weight maintenance diet) did not differ in any of the activity measures observed. However, following a three-day period of overfeeding, Schmidt et al. found that only the obesity prone individuals showed decreases in walking and walking intensity suggesting that body composition played a role in overfeeding-induced

reductions to physical activity. (76). In children, inconsistencies are reported within the literature with regards to the actual contribution of body composition on regulation of their activity levels, and have instead focused on the effect of parental influences (29, 57). A systematic review by Edwardson et al. (29) demonstrated a significant influential effect of parents on child activity, where the parents influenced the type, and intensity of activity of the child, and additionally that parental modeling, transport, and encouragement also played significant roles in child activity involvement. Additionally, another review of correlates for physical activity in preschool children (57) showed body mass index was not a significant correlate of activity participation, but similar to the Edwardson, et al. review (29), children tended to be more active when they had parents that were active. As such, the reliability of the studies investigating the relationship between body composition and daily activity in children should be considered with caution, particularly if parental influences were not controlled for. In adults, a clearer role of body composition is present. Overall, whether chronic overfeeding has an independent- or codependent-effect on activity remains unclear, though there are a larger number of studies that favor an independent role of caloric intake on physical activity.

2.6 The Effect of Sex Steroid Hormones on Physical Activity

Substantial literature dating back to 1925 (132), in addition to work from our laboratory (14, 16) have identified the primary sex hormones in males (testosterone) and

females (17β-estradiol) as potent biological regulators of daily physical activity (14, 15). From our lab, Bowen et al. (15) identified specific influences of testosterone and 17β-estradiol on physical activity in mice using removal (via orchiectomy or ovariectomy) of testosterone or 17β-estradiol. Removal of the sex hormones resulted in ≈ 90% decreases in activity in both males and females, while endogenous replacement of these hormones resulted in varying levels (35-110%) of recovered baseline activity. Furthermore, these findings also showed that the recovered activity of distance and duration were primarily influenced by testosterone administration, whereas the recovery of speed was primarily dependent on the replacement of 17β-estradiol.

Earlier work by Roy and Wade (110) suggested that estradiol had the most potent effect on patterns of physical activity through the aromatase complex. In brief, the enzyme aromatase, when activated, functions to induce conversion of testosterone into estradiol. In Roy and Wade's work, they reported that a direct effect of an estrogenic mechanism was required for regulation of physical activity responses through the aromatase complex. They further examined the effect of the non-aromatizable (dihydrotestosterone proprionate) and aromatizable (testosterone proprionate) androgen administration in male rats that underwent orchidectomies (110). Roy and Wade's results showed that the aromatizable form of testosterone had the greatest effect on physical activity, while the non-aromatizable form of testosterone had no effect on activity (110). In all, Roy and Wade interpreted these results to suggest that the activity response was dependent on testosterone conversion to estradiol via the aromatase complex. Interestingly, more recent work by Bowen et al. (14), has opposed the idea

that the aromatase complex is necessary for the regulation of physical activity through the sex hormones. In this work, it was demonstrated through administration of reversible and irreversible aromatase pharmacological interventions – in orhidectomized male mice – that wheel running behaviors remained unaffected when using aromatase blockers suggesting that the aromatase complex was not required for physical activity regulation (14).

Regardless of the aromatase literature regarding regulation of activity in males, there is evidence that suggest estrogen may also be involved (49). In a study conducted by Gorzek et al. (49), female ovariectomized mice displayed significant decreases in voluntary physical activity by up to 80%, while physiological replacement of estrogen with either 17β-estradiol or tamoxifen stimulated increases in activity in these mice (but only to ≈54% of baseline). Thus, these collective sources of data (49, 76, 132) suggest both an androgenic and estrogenic mechanisms are directly involved with the regulation of physical activity.

2.7 Overfeeding-Induced Alterations to the Primary Sex Steroid Hormones

While studies have associated an effect of overfeeding on reduced levels of activity (72, 114), it is unclear what the potential mechanisms are that could mediate this response. We hypothesize that a potential mechanism that could be involved would be the primary sex steroid hormones in males (testosterone) and in females (estrogen: 17β-estradiol/E2).

In males, consistent evidence shows that chronic overfeeding leads to direct reductions in the level of testosterone and its' precursor metabolites (13, 103, 112). Work by Pritchard et al. (103) analyzed the effect of long-term overfeeding in identical twin pairs where the subjects were overfed 1000 kcal per day for 6 days per week over a 6-week period, and found that while genotype had a significant effect on the response in testosterone, that in all subjects the reductions in testosterone to overfeeding were significantly correlated with higher gains in abdominal fat, i.e. abdominal fat gainers showed a larger decrease in testosterone with overfeeding . A more recent overfeeding study conducted by Bouchard and colleagues (13), using the same protocol as Pitchard et al. (103), observed a variety of responses to overfeeding such as increased leptin levels, decreased total testosterone and testosterone metabolites, decreased maximal aerobic capacity, decreased muscle oxidative potential, and decreased lean body mass. To a lesser degree, other less consistent factors were also shown to correlate with chronic overfeeding effects including larger abdominal fat cells, lower proprandial energy expenditure, increased estrogenicity, decreased Dehydroepiandrosterone (DHEA), and decreased cortisol (13). Lastly, a study by Sato et al. (112) evaluated the effect of chronic overfeeding in healthy men, with and without a family history of type 2 diabetes, for a period of 28 days by 5200 KJ/day, on both plasma testosterone and metabolism of testosterone local to the skeletal muscle. In this study, Sato et al. found that while there was not a significant overall effect of overfeeding on plasma levels of testosterone, there was a significant effect of group, where the men with a family history of type 2 diabetes had a greater reduction in testosterone compared to men without prior family history of

type 2 diabetes. Furthermore, Sato et al. also demonstrated significant disruptions to testosterone local to skeletal muscle (not in plasma), where the expression of 3β-hydroxysteroid dehydrogenase (HSD) and 17β-HSD enzymes which are involved in the formation of testosterone were significantly reduced by overfeeding. Therefore, while local concentrations of testosterone in the skeletal muscle of these men were not assayed in this study, Sato, et al.'s findings that these steroidogenic enzymes were decreased following overfeeding suggest there may have been a local effect of chronic overfeeding on the skeletal muscle that was not indicated by circulating concentrations of testosterone.

The literature that has examined the effects of overfeeding, and/or its' development into obesity (13, 125), supports that while circulating testosterone levels is significantly reduced, the levels of estrogens (i.e. 17β-estradiol and estrone) increases in males. Interestingly, a study by Schulte et al. (117) found that testosterone values were significantly increased following a 12 week period of caloric restriction (800kcal/day) in morbidly obese men with a mean BMI of 47.2 kg/m^2. The authors found the increased production of testosterone with caloric restriction in obese men occurred through two distinct mechanisms: 1) Improved testicular function (i.e. increased testosterone production); and 2) Reduced conversion of testosterone into 17β-estradiol in adipose tissue by aromatase activity. It has been additionally shown that with gains in body fat due to overfeeding (51, 115) there were significant increases in the conversion of testosterone to estrogen through increased aromatase activity. Thus, aromatase activity was dependent on body fat content; i.e. a higher body fat content was directly correlated

with higher aromatase activity, and would result in an increased conversion rate of testosterone into 17β-estradiol (8, 102).

The mechanism through which overfeeding directly alters androgenic and/or estrogenic function remains unclear, though it has been shown that the decreased levels of testosterone with overfeeding were correlated with higher gains in fat mass (13, 103). Additional work by Blouin et al. (12) showed the reductions in testosterone were specifically correlated with higher gains in abdominal visceral (omental) fat mass. These findings suggest, in men, that the reductions in testosterone due to overfeeding may be due to changes occurring in adipose tissue (e.g. through increased aromatase activity). What is unclear with low levels of testosterone in these conditions is whether the overfeeding-induced reductions are a result of the caloric excess, or if the reductions are a result of increased adiposity local to omental fat gains. In overweight and obese men with greater gains in visceral abdominal fat accumulation, this enzyme expression often correlates with increased visceral fat accumulation localized to the abdominal area, and thus, presumably is a factor responsible for increased estrogen production in obese men (12). The sex hormone-binding globulin (SHBG), which is a glycoprotein that binds androgens and estrogens, has also been shown to decrease proportionately with increases in visceral adiposity, and with obesity, can decrease the half-life of testosterone, which could inhibit delivery of testosterone to target tissues in men (103, 125). The decrease in SHBG as a consequence of chronic overfeeding and obesity is thought to occur as a result of hyperinsulinemia, where insulin inhibits SHBG production from the liver (130). Thus, decreases in SHBG can decrease circulating

testosterone and limit testosterone availability to target tissues. Pritchard et al. (103) overfed a set a 12 identical male twin pairs (21±2 years of age), by 1,000 calories for 6 days per week for 8 weeks, and found that overfeeding decreased SHBG by 15.9 ±1.6%, and that these reductions were significantly correlated to gains in visceral adiposity. Most recently, however, a study by Bouchard et al. (13) in overfed men showed that SHBG was not significantly altered. Taken together, there are a variety of suggested mechanisms through which overfeeding reduces testosterone; however, there is not agreement on the mechanism that is active.

In women, there are no studies to date, that have investigated the specific effects of overfeeding, though there are studies in women with diet-induced conditions such as obesity and polycystic ovarian syndrome (POS), which provide evidence of sex hormone irregularities. While men generally display reductions of serum testosterone with overfeeding (118), women have significant increases in the level of testosterone with overfeeding (32) This sex-dependent paradox was recently addressed in a review by Escobar-Morreale et al. (32), where they explained that while the effect of overfeeding on sex hormone responses was in opposition in men and women, that the altered sex hormone status effects on body composition is the same. Escobar-Morreale et al. pointed out that the higher levels of testosterone in men needed for support of lean muscle mass and other male specific traits are diminished in a metabolically compromised state, such as overfeeding. When comparing the relative levels of testosterone in men and women under a metabolically compromised state, the high levels of testosterone in women still do not meet the levels of overfed men. Thus, even while

circulating levels of testosterone is increased in women with overfeeding, the levels are not high enough to meet the metabolic demands for lean muscle mass gains, and therefore, heightened levels of testosterone instead supports increases in fat mass (32, 33). Additionally, in women with hypersecretion of testosterone, 17β-estradiol is decreased and this further exacerbates poor whole-body metabolism given that 17β-estradiol has metabolic protective effects in pre-menopausal women (122). While serum levels of 17β-estradiol are typically lower in obese women, Blouin et al. (12) demonstrated altered androgenic metabolism local to adipose tissue, where significant androgen deactivation activity was observed in women classified with visceral abdominal obesity. In this study, Blouin et al. (12) evaluated the expression and activity of two enzymes in the aldo-keto reductase (AKR) superfamily, specifically the AKR1C subfamily – AKR1C3 (17β-HSD-5) and AKR1C2 (3α-HSD-3) – in the abdominal tissue of women, and found that the latter enzyme (3α-HSD-3), which is an androgen deactivator, was more predominant in abdominal adipose (mRNA expression), and that the conversion rate of this enzyme was significantly increased with abdominal obesity. Thus, this study by Blouin et al., suggest that in women, there is a potential effect of chronic overfeeding on sex hormone metabolism in specific tissues, such as adipose tissue.

Estrous cycling irregularities in obese females have been observed in both rodent (74) and human models (81, 98). Overweight and obese women also display abnormalities of the hypothalamic-pituitary-gonadal axis, which is a key regulator of ovarian production of estrogen (60). Additionally, it is well established that 17β-

estradiol is associated with metabolic conditions such as obesity and type 2 diabetes, where in females, 17β-estradiol is reduced. For example, a study by Litwak et al. (82) in female mice, demonstrated that 17β-estradiol administration during 12 weeks of a high fat diet (45% fat) was able to prevent reductions in 17β-estradiol, estrous cycling dysfunctions, and overall significant increases in total body weight, body fat, and visceral fat accumulation association with high fat diet. Additionally, the findings in this study also showed that 17β-estradiol administration in high fat diet mice reduced their overall food intake, increased whole body energy expenditure, and increased locomotor activity, showing recovery from the obesogenic effects of high fat diet. An additional important finding from this study was that 17β-estradiol was identified to be reduced as a direct result of high fat diet, rather than a subsequent effect of increased fat mass associated with the diet. Moreover, the 17β- estradiol reductions led to the increases in fat mass and percent fat. Thus, in females, 17β- estradiol decreases as a direct result of diet, whereas in males, testosterone is reduced as a direct result of diet (13, 103).

Given that the levels of the primary sex hormones in males (testosterone) and females (17β- estradiol) are directly associated with dietary intake, and that physical activity has been associated with sex hormone level (16, 110, 132) leaves the question of whether diet alters activity through the sex hormones. To date, this question has not been directly answered.

2.8 Summary and Future Directions

To date, studies have yet to test the hypothesis that overfeeding-induced alterations to the sex hormones elicit a regulatory response on daily activity. Given the low and continually declining levels of physical activity in the U.S. (126) and the increased rates of overweight and obese individuals (66) there is a need to examine the interaction between diet and activity. As discussed, ambiguity exists regarding whether diet has a co- or independent effect with body composition, on activity levels. Future studies can be organized such that each variable can be isolated to determine the actual effect of each on activity. Conducting such experiments will be interesting because of the potential mechanistic link between excess caloric intake and decreased activity and because of the potential for such investigations to stimulate further lines of research geared towards dietary interventions and/or possible pharmacological manipulations to prevent physical inactivity. The central hypothesis to this study was that chronic overfeeding will significantly lead to reductions in physical activity in mice; and secondarily, that the reductions to activity will be related to overfeeding-induced reductions to the primary sex hormones in male (testosterone) and female (17β-estradiol) mice. Thus, the purpose of the experiments in this dissertation was to examine the effect of chronic overfeeding on the regulation of physical activity, and sex hormone levels, in mice.

2.9 Purpose of Study

The purpose of the below experiments was to examine the effect of chronic overfeeding on the regulation of physical activity (wheel running) in mice. Given the evidence showing that testosterone and 17β-estradiol can directly affect physical activity and that the levels of these sex hormones are significantly reduced following chronic overfeeding (13), suggest the possibility of direct regulation of activity by overfeeding. Thus, in conjunction with our extensively validated model of physical activity, (65, 68, 69, 78-80) we have incorporated a model of overfeeding in mice to investigate the effect of chronic overfeeding on activity and a potential responsible biological pathway(s).

3. METHODS

3.1 Overview

Animals: This protocol conformed to the standards of humane animal care and was approved by the Texas A&M University Institutional Animal Care and Use Committee (AUP 2013-0274). C57BL/6J inbred breeder mice (Jackson Laboratory, Bar Harbor, ME) were used in both experiments (study aims 1 and 2) because of their consistent use in the scientific literature and genetic homogeneity of the strain.

Purpose: The primary purpose of the following experiments was to determine if chronic overfeeding decreased physical activity, and secondly, whether the primary sex hormones in males (testosterone) and females (17β-estradiol) were associated with potential overfeeding-induced alterations in daily activity. Thus, we conducted two sets of experiments:

Experiment 1) Determine the direct effect of chronic overfeeding on the sex hormones levels and overfeeding's acute effect on voluntary wheel running activity in mice;

Experiment 2) Determine the effect of chronic overfeeding on physical activity patterns, and whether two weeks of wheel running access – while still being overfed - reversed the effects of the HFHS diet.

3.2 Experiment 1 Methods

Four breeder pairs of C57BL/6J mice (8♀, 4♂) were purchased from Jackson Laboratory (Bar Harbor, ME). For breeding purposes, two female mice were housed in a single cage with one male breeder. At three weeks of age, offspring (pups) were weaned, and individually housed using random assignment to one of two diets: the control fed diet (CFD) or the high fat high sugar (HFHS) diet (Table 1 and see below for diet composition). Random assignment was used to assign these mice to one of the four groups defined in Table 2, with the goal of each group having an equal distribution of male and female mice per group (≈ six per group).

Figure 1 provides an overview of the timeline for this experiment. Groups one and two (Table 2) served to determine the direct effect of the HFHS fed diet on the level of primary sex hormones in males (testosterone) and females (17β-estradiol), and were sacrificed at 12.5- 13 weeks of age without access to running wheels. Groups three and four were given wheel running access for three days at 12 weeks of age to measure acute running wheel activity (see below). The first two days of running wheel access in these mice was designated as an acclimation period, while day 3 of running wheel activity was measured and used in the analysis. All groups of mice were sacrificed at approximately the same age (12.5- 13 weeks of age; Figure 1), with females sacrificed based on when they were confirmed to be in the Proestrus stage of the estrous cycle. Further, in order to eliminate circadian cycle influences on sex hormone levels, all male mice were

sacrificed between 9:00- 11:00 am, and female mice were sacrificed between 12:00-2:00 pm (see below section measurement of estrous cycle phases).

At sacrifice, the mice were anesthetized using 3-4% isoflurane and then blood samples were collected via cardiac puncture. The blood samples were centrifuged for 20 minutes at 4°C centigrade at 10,000 rpm to separate red blood cells and serum. The serum samples were snap-frozen and stored at -80 degrees for later assessment of serum testosterone and 17β-estradiol using competitive enzyme-linked immunosorbent assay (ELISA) kits from Alpco and Abcam, Inc. respectively (see below).

Measurement of Wheel-Running Activity: Wheel running activity was determined by collection of daily distance, duration, and speed of wheel running using our standard lab protocol (79, 128). Running wheel activity has been show to peak and plateau when mice reach 9-12 weeks of age (124), and this age range has been consistently studied by studies from our lab (78, 80, 127); thus, we chose to also measure running wheel activity at 12-weeks of age in order to compare repeatability and reproducibly for our control animals. Running wheels were provided at 12 weeks of age, and access was given to select groups of mice for three days. The first two days of running wheel access was used for mice to acclimate to the presence of a running wheel, where the third day was used to analyze the acute physical activity response to diet. Briefly, plastic running wheels with a 410mm circumference and a solid running surface were mounted to the cage tops of standard rat cages. The magnet from a cycling computer (BC8.12, Sigma Sport, Batavia, IL) was glued to the wheel and the cycling computer was attached to the outside of the cage and calibrated to the size of the wheel. Running distance (km/day)

and duration (mins/day) data were collected on a 24-hour cycle in the morning, and the average daily running speed (m/min) was calculated from the daily distance and duration measures. The sensor alignment and freeness of the wheel were checked daily and adjusted as needed.

Dietary Protocols: The composition of the diets is provided in Table 1. At three weeks of age the mice were randomly assigned to one of the six groups provided in Table 2. Utilizing the standardized high fat diet from Research Diets, Inc., in conjunction with a 20% fructose solution to replace normal water, induced chronic overfeeding. The combination of the high fat feed and the 20% fructose solution represented the typical high fat/high sugar diet (HFHS) of U.S adults (85, 123). For the control diet, we used a normal 4% fat chow diet (Harlan Labs, Houston TX) in conjunction with normal water, which is the standard rodent diet provided by our animal care facility. Food (grams) and fluid intake (ml) were weighed and recorded on a weekly basis to estimate the average daily/weekly caloric intake.

Measurements of Body Composition: Body composition was measured on a weekly basis beginning at four weeks of age and continued until sacrifice (12.5- 13 weeks of age). Weight and body composition were determined using an Echo MRI mouse body composition device (EchoMRI, Houston, TX). Body composition measurements – weight, body fat percentage, and lean tissue weights - were determined by placing the mouse in the appropriate analysis tube of the MRI machine. These measurement tubes have a plunger that is used to inhibit the movement of the animal without discomfort. Each measurement took approximately 60 seconds and the animal

was then put back in its cage.

Measurement of Estrous Cycle Phases: Because sex hormone levels in female mice cycle every three to four days, the estrous cycle phases were determined using vaginal lavages (86) beginning at 12 weeks of age so that hormone levels were taken at the same time of the cycle in each animal (i.e. Proestrus phase). All female mice were terminated between the hours of 12:00 pm- 2:00 pm, given a recent study by McLean et al. (86) suggest 17β-estradiol reaches its' highest peak during between these hours of Proestrus. Prior to performing the procedure, distilled water was autoclaved and cooled to physiological temperature (i.e. 37°C). The mouse was taken out of its' cage and placed on the top of the cage grid and while continuing to allow the mouse to grip the cage grid top with the front paws, the base of the tail was grasped to lift the hind limbs. One hundred µl of the double distilled water was drawn into a sterile 200 µl glass pipette and the end of the sterile pipette tip was placed at the opening of the vaginal canal. Approximately 25-50 µl of the double distilled water was displaced into the opening of the vaginal canal and pipetted 4-5 times to ensure a sufficient number of cells were taken, and the sample was smeared on a glass slide, then allowed to completely dry at room temperature before analysis. Once the estrous smears dried, the slides were then stained using the three-step Hema 3 Fixative and Solutions containing fixative, hematoxylin, eosin, and four deionized waters (for destaining). The slides were then viewed at 10X under a microscope. The estrous cycle phases were determined by cell typology using the guidelines provided by McLean et al. (86).

Sex Steroidal Hormone Assays: On the day of sacrifice, the mice were anesthetized via inhalation of 3-4% isoflurane with subsequent cervical dislocation. After euthanasia, blood samples were collected via a cardiac puncture using a 20-gauge needle, and the blood samples were immediately centrifuged for 30 minutes at 4°C centigrade to separate red blood cells and serum. The serum samples were stored in our -80 degree freezer for future assessment of testosterone (Alpco Serum Testosterone, Salem, NH), and 17β- estradiol (Abcam, Cambridge, MA) using competitive ELISA kits. In brief, ELISA on the testosterone (ng/ml) samples were completed by measuring triplicate samples for each male per manufacturer's instructions. The ELISA for 17β-estradiol (pg/ml) was also performed in triplicates for each female sample per the manufacturer's instructions.

Measurement of Uterine Horn Weights: In the event of unreliable values from the 17β-Estradiol ELISA analysis, we also collected uterine horn weights for the female mice. Prior studies have utilized horn mass as an indirect indicator of biologically active estrogens (133, 135). On the day of sacrifice, the left and right uteri of the female mice were dissected, and immediately weighed on an electronic scale (Mettler Toledo). The uterine horns were measured after removal of adhering fat and mesentery tissue. Given the female mice in this current study differed significantly in total body weight because of dietary treatment, uterine weights were standardized by calculating the ratio of the uterine wet weight (mg) and total body weight of each mouse (mg).

Statistical Analyses: Physical activity measures (distance, duration, and speed) and body composition measures (percent body fat, fat mass, lean mass, and total body

weight), were analyzed by a one-way ANOVA. The nutritional intake data were analyzed by a two-way ANOVA with time being the repeated measure with an alpha level set *a priori* at 0.05. In the event of significant main effects, a Tukey's *post-hoc* test was employed. Serum concentrations of testosterone (males) and 17β-estradiol (females) were compared using one-way ANOVAs to analyze for differences in the HFHS and CFD fed male and female mice, respectively. Physical activity data (distance, duration, and speed), collected on the third day of wheel running access, was compared between groups using a one-way ANOVA in male (HFHS versus CFD fed) and female (HFHS versus CFD fed) mice. Separate one-way ANOVAs, for male and female mice, were employed given it is well-established that activity measures in male and female mice significantly differ in their normal wheel running activity, where females typically run ≈20% farther on average than males (79). Thus, an effect of sex was not determined in our analyses given the difference in their normal running wheel activity. All statistical analyses were completed using JMP statistical software (SAS Inc., Cary, NC), while all graphs were developed using GraphPad Software (La Jolla, CA).

3.3 Experiment 2 Methods

The same breeder pairs used in Experiment 1 were also utilized in Experiment 2. At three weeks of age, offspring (pups) were weaned, and individually housed with a random assignment to one of two groups based on the diet types defined in Experiment 1 (i.e. CFD or the HFHS diet Table 1; Groups 5 and 6), with the goal of each group having

an equal distribution of male and female mice per group (four- six per group; Table 3). All mice remained on their randomly assigned diet from the wean date until their specified time of sacrifice (Figure 2). At 12 weeks of age, running wheel access was provided for two weeks for all mice until 14 weeks of age. A daily record of running wheel data (distance, speed, and duration ran) was recorded during this time period using methods previously defined in Experiment 1. Similarly, weekly measurements of food and fluid consumption, weight, and body composition were made as they were in Experiment 1. At 14 weeks of age, the mice were sacrificed using methods described in Experiment 1. The procedure for estrous cycle phase was similar to Experiment 1; however, this procedure was conducted starting at 14 weeks of age, instead of 12 weeks of age as in Experiment 1. Additionally, the procedure for analyzing serum concentrations of the sex steroid hormones was similar to Experiment 1; however, the levels were analyzed at 14 weeks of age, instead of 12 weeks of age as in Experiment 1.

Statistical Analyses: All statistical analyses were conducted using similar approaches as in Experiment 1. However, daily activity values (distance, duration, and speed) across the two-week period were analyzed using a two-way ANOVA with diet as a factor and time as the repeated measure, with the alpha-value set *a priori* at 0.05. A one-way ANOVA was further employed to compare the mean differences between acute running wheel data in experiment 1 and day three date in experiment 2, by calculating mean differences between CFD and HFHS acute running wheel activity (distance, speed, and duration) for both male and female mice. In the comparison analysis, we calculated the percent change in distance, duration, and speed of activity between the HFHS and

CFD mice (percent change= (μCFD-$\bar{x}$HFHS)/ (μCFD*100)), and compared the mean percent changes between experiment 1 and 2 for each activity measure for male and female mice.

4. RESULTS

4.1 Experiment 1 Results

Offspring Demographics: Forty-seven pups (25♀, 22♂) were analyzed for this experiment with an average litter size of seven pups (±1) in the control fed (CFD) mice and eight pups (±1) in the high fat/high sugar (HFHS) fed mice (Table 1).

Caloric Intake: From three weeks of age (weaning age) to 12 weeks of age, the HFHS fed male mice consumed significantly more kilocalories per day (kcals/day) compared to their control counterparts (CFD vs. HFHS: 10.51 ±0.55 vs. 14.95 ±0.94 kcals/day; $p<0.0001$; Figure 3). Similarly, female HFHS-fed mice consumed significantly more kcals/day per day compared to the CFD female mice (12.51 ±0.56 vs. 10.04 ±0.73 kcals/day; $p<0.0001$; Figure 4). During this period, the HFHS fed male and female mice on average consumed 29.5% and 19.6% more kcals/day than the CFD mice, respectively.

Body Composition: At 12 weeks of age (week of, but prior to running wheel access) body composition measurements were made and the total body weight (grams), fat mass (grams), lean body mass (grams), and percent body fat were compared between the HFHS and CFD diet groups. The HFHS fed males displayed significantly higher values in total body weight (CFD vs. HFHS: 24.5 ±2.1 vs. 32.1 ±4.1 gms; $p<0.0001$), body fat percentage (13.6 ±4.6 vs. 40.7 ±11.9%; $p<0.0001$), fat mass (2.8 ±21.0 vs. 8.9

±3.0 gms; p<0.0001), and lean mass (20.4 ±1.6 vs. 21.8 ±1.0 gms; p=0.0223) when compared to CFD male mice (Figure 5).

Likewise, the HFHS fed female mice displayed significant alterations to body composition as indicated by higher increases in total body weight (CFD vs. HFHS, 19.8 ±0.9 vs. 23.0 ±2.5 gms; p=0.0003), body fat percentage (12.6 ±3.1 vs. 25.4 ±9.3%; p=0.0002) fat mass (2.5 ±0.5 vs. 4.4 ±1.8 gms; p=0.0002), and lean mass (16.3 ±0.9 vs. 17.3 ±0.9 gms; p=0.0181; Figure 6).

Wheel-Running Data: In the HFHS fed male mice, acute wheel running distance (Figure 7) was significantly lower compared to the CFD mice (CFD vs. HFHS: 7.42 ±5.1 vs. 2.89 ±1.9 km/day; p=0.0.0497), but neither the duration (p=0.0552) nor speed (p=0.7392) of activity were significantly different compared to controls. The duration in the male mice, while not significant, trended toward significantly reduced in the HFHS animals with a moderate statistical power (power=0.5048) and low least significant number estimate (n=10) for significance. The HFHS fed female mice also ran significantly less distance than the CFD mice (9.14 ±2.9 vs. 3.58 ±3.6 km/day; p=0.0229) with neither duration (p=0.2257) nor speed of activity (p=0.0964; power=0.3817; LSN=14) being significantly affected by the HFHS (Figure 8). When considering the daily distance ran, the HFHS fed male and female mice ran approximately 61.2% and 60.8% less than the CFD mice, respectively.

Sex Steroid Assays: In males, serum testosterone (ng/ml) in the CFD (1.4 ±2.81 ng/ml) and HFHS (3.35 ±4.04 ng/ml) fed mice levels were not significantly different when measured following nine weeks of diet treatments (p=0.2662; Figure 9). The

average coefficient of variance between triplicates from each sample was 7.2% (±3.8). All mice had values falling within range of the assay kit's assay range (0.1- 25 ng/ml, with a sensitivity of 0.066 ng/ml). No data points were eliminated based on our standard criteria for elimination (three or more standard deviations above or below the mean).

In female mice, serum 17β-estradiol concentration values in the CFD (20.8 ±10.5 pg/ml) and HFHS (17.4 ±6.6 pg/ml) fed mice were not significantly different (p=0.5983; Figure 10a). The average coefficient of variance between triplicates from each sample was 3.17% (±1.5). The 17β-estradiol assay is able to detect 20-2000 pg/ml (sensitivity of 8.68 pg/ml) and the values for all of our analyzed samples fell within this range.

Uterine Horne weights: As a secondary confirmatory measure to the 17β-Estradiol values, we also calculated the uterine horn to total body weight in the female mice. We found no significant difference (p=0.5390; Figure 10b) between the CFD (5.1 mg ±1.78) and HFHS (4.33 mg ±1.78) fed mice, supporting our estrogen values.

4.2 Experiment 2 Results

Offspring Demographics: Twenty-four offspring (14♀, 10♂) were analyzed for this experiment with an average litter size of eight pups (±2) in the CFD mice and eight pups (±2) in the HFHS-fed mice (Table 3).

Caloric Intake: From weaning (3 weeks of age), until the time of sacrifice (14 weeks; Figure 2), the HFHS-fed male mice consumed significantly more kilocalories per day (kcal/day) when compared to their control counterparts (CFD vs. HFHS: 11.43 ±1.4

vs. 15.45 ±2.6 kcals/day; p<0.0001; Figure 11). Similarly, the female HFHS-fed mice also consumed significantly more kcal/day per day compared to the CFD female mice during this time period (10.25 ±0.6 vs. 15.01 ±4.9 kcals/day; p<0.0001; Figure 12). Throughout this time, the HFHS fed male and female mice on average consumed 24.7% and 27.9% (respectively) more than their control counterparts.

Body Composition: The HFHS-fed males had significantly higher total body weight (CFD vs. HFHS: 25.24 ±0.6 vs. 35.0 ±3.1gms; p<0.0001), body fat percentage (10.65 ±2.3 vs. 50.40 ±7.6 % p<0.0001), and fat mass (2.29 ±0.5 vs. 11.09 ±2.1 gms; p<0.0001), but not lean mass (21.54 ±0.5 vs. 21.93 ±1.2 gms; p=0.0551; Figure 13). The HFHS fed female mice also displayed significant alterations to body composition as indicated by higher increases in total body weight (CFD vs. HFHS: 21.49 ±2.7 vs. 26.93 ±2.7 gms; p=0.0003), body fat percentage (14.72 ±4.1 vs. 36.32 ±11.4%; p=0.0005) fat mass (2.56 ±0.7 vs. 6.73 ±2.2 gms; p=0.0005), and lean mass (17.39 ±0.6 vs. 18.44 ±0.5 gms; p=0.0043; Figure 14).

Wheel-Running Data: The two-week period of running wheel access did not alter the HFHS-induced decreases in physical activity. The HFHS fed male mice compared to the CFD mice revealed an overall significant decrease in distance (CFD vs. HFHS; 8.2 ±2.1 vs. 2.3 ±0.4 km/day; p=0.0118), but not the speed (p=0.4534) or duration (p=0.0556) of activity (Figure 15). In the female HFHS fed mice, there was also a significant reduction in the distance (CFD vs. HFHS; 10.27 ±1.4 vs. 7.20 ±1.6 km/day; p=0.0218) with no change in duration (p=0.4915), but with a significant decrease in speed (42.46 ±4.6 vs. 32.42 ±5.6 m/day; p=0.0147; Figure 16).

As a confirmatory measure to Experiment 1, we also analyzed day three of running wheel activity of the mice in this experiment and compared these results to the previous results from Experiment 1. Similar to experiment 1, we found significant acute reductions in distance ran for HFHS fed male ($p=0.008$) and female ($p=0.0014$) mice; however, in this experiment, the reduction to duration in males ($p=0.0095$), and reduction to speed of activity in females ($p=0.006$), became significant (Figures 17 and 18). For the male mice, the percent change in acute running activity between experiments 1 and 2 was not significantly different for the distance ($p=0.096$) or duration ($p=0.0689$) ran, however, there was a significant difference in the speed of activity ($p=0.021$; (Figure 19) indicating that speed was altered more in the experiment 2 male mice. In the female mice, the percent change in acute running activity between experiments 1 and 2 was not significantly different for the distance ($p=0.2265$), duration ($p=0.2674$), or the speed of activity ($p=0.1544$; Figure 20). Thus, the fact that the percent change in distance and duration between the CFD and HFHS mice were not significantly different between experiments 1 and 2 supports reproducibility of HFHS diet-induced acute reductions to daily distance ran.

Effect of Wheel-access on Body Composition: In male mice, a two-way ANOVA indicated an overall significant difference in body composition ($p<0.0001$), with post-hoc testing indicating this difference was due to diet type ($p<0.0001$), and not due to running wheel access ($p=0.2980$; Figure 21). Similarly, in female mice, a two-way ANOVA indicated an overall significant difference ($p<0.0001$) with this effect also only

being due to diet type ($p<0.0001$) and not due to body compositional changes with running wheel access ($p=0.0728$; Figure 22).

Sex Steroid Assays: In male mice, there was a not significant difference in serum testosterone concentration values between the CFD (3.79 ±6.0 ng/ml) and HFHS (5.33 ±5.6 ng/ml) mice at the completion of two weeks of having wheel running access ($p=0.6868$; Figure 23) with all values falling within range of the assay kit's assay range (0.1- 25 ng/ml, with a sensitivity of 0.066 ng/ml). The average coefficient of variance between triplicates from each sample was 3.3% (±1.5%). No data points were eliminated based on our standard criteria for elimination. While we attempted to analyze 17β-Estradiol serum concentrations in the female mice for this aim (three times total), the results we obtained for each of the ELISAs were undetectable (~50% undetectable), presumably due to very low estradiol concentrations in these mice according to the manufacturer of the analysis kit. Given that further analyses were not possible due to lack of sample, uterine horn weights were used as an indirect measure for estrogenic status in the female mice for this aim. By comparing the uterine horn to total body weight in the female mice in experiment 2, we found that a significant difference ($p=0.0243$; Figure 24) existed between the CFD (3.9 mg ±1.45) and HFHS (2.24 mg ±0.34) fed mice which suggested lower estrogen status in the HFHS fed mice (Figure 24) after two weeks of wheel running exposure.

5. DISCUSSION AND CONCLUSIONS

5.1 Overview

This study investigated the effect of chronic overfeeding on physical activity in mice using a high fat (45% fat), high sugar (20% fructose; HFHS) diet. The mice on the HFHS diet showed a significant increase in daily calories consumed, as well as an increase in body weight and body fat. Exposure to the HFHS diet significantly reduced acute running wheel distance in both male (≈ 61%) and female mice (≈ 62%) when compared to mice provided a control fed diet (CFD). This HFHS-induced reduction in acute activity was replicated in Experiment 2. Further, when we increased running wheel exposure time to two-weeks, the daily distance ran for male and female HFHS fed mice remained significantly lower than the CFD mice throughout this time suggesting that having access to exercise was not sufficient to overcome diet-induced reductions to activity. Additionally, the diet-induced increases in body composition (e.g. percent body fat) were not overcome by the two-week period of wheel access. In neither experiment, where measured, were the sex hormones significantly affected by the dietary treatment. Thus, our results indicate that chronic overfeeding reduces physical activity and that this effect is repeatable, in spite of a lack of change in sex hormone levels. These results provide a potential explanation for why some children and adults remain inactive, regardless of having access to activity promoting environments (sidewalks, parks, etc.) and exercise modalities (34, 41, 96).

5.2 Effect of Chronic Overfeeding on Acute Running Wheel Activity

The first component of the current study (Experiment 1) evaluated the effect of chronic overfeeding on acute running wheel activity in mice. Our results indicated significant reductions in distance ran, for both male and female mice. The reduction in daily exercise distance was differentially regulated in the overfed male versus female mice suggesting potentially different regulating mechanisms. In the overfed males, the decreased distance appeared to be a result of a significant reduction in exercise duration. In females, the significant decrease in distance appeared to be the result of a significant reduction in speed of activity ($p=0.006$; Figure 18). These findings tentatively suggest that the decrease in daily activity in males may be regulated by alterations in energy-availability since duration of activity was compromised whereas in females, muscle contractile mechanisms that influence the speed of activity may be inhibited. Further strengthening our conclusion that overfeeding decreased daily activity was through the replication of these results to a similar magnitude in experiment 2. Thus, our observations in both experiment 1 and 2 strongly show that overfeeding significantly reduced daily activity in mice.

Our findings of an overall decrease in activity with overfeeding, are supported by human studies (71, 114) which have indirectly linked caloric excess with reductions in activity. Schmidt et al. (114) showed that chronic overfeeding in male and female obesity-prone and obesity-resistant subjects, significantly reduced spontaneous physical activity in the obesity-prone individuals. Additionally, Levine et al. (71) demonstrated

that free-living walking was significantly decreased when lean and obese subjects were overfed 1,000 kcals/day for nine weeks. However, neither human study suggested potential mechanisms for the observed decreases in activity after overfeeding, nor listed potential medicating factors such as sex. Thus, the sexual-dimorphism we observed in the potential indices that affected wheel running activity in mice is unique to this current study.

Several studies have studied wheel running activity with other various types of diets in mice, such as the specialized 'Western diet' (11, 87, 88) and the 'Very high fat diet' (46) types, though varying activity responses with these diets have been reported. For example, Meek et al. (88) examined the effect of a specialized 'Western diet' (42% fat) on voluntary physical activity in mice selectively bred for high running wheel activity for 52 generations, and found that the diet led to significant increases in running wheel activity, when compared to selected controls. Meek et al. speculated that the heightened wheel running activity in their high runner mice with the Western diet, was due to the excess dietary lipids in the diet being used preferentially in exercise metabolism which allowed for enhanced endurance. Coupled with their observations that the selectively-bred mice had reached a plateau in running distances several generations before the mice used in their study, Meek et al. further speculated that there was an increase in distance with the high fat diet because the mice normally expended their available energy stores on a daily basis (because of their high activity status) and the extra fat provided a more calorically-dense fuel source allowing the mice to run further on a daily basis. Further, in comparison to the current study, Meek et al.

provided both the diets and running wheels together at the beginning of the study (24 days old to 8-weeks of age), whereas in our study, the mice were overfed for a period of nine-weeks before having access to a running wheel. Thus, because in the difference in the genetic make-up and the access to the diet and wheels, it is unclear if the wheel activity response of our mice would have been different if a similar study design as that of Meek et al. (88) was applied to our mice.

Supporting our hypothesis of a decrease in activity with overfeeding in non-specialized mice are at least three studies. Funkat et al. (46), using only male C57BL/6J mice, demonstrated that providing a specialized 'very high fat diet' (60% fat) led to significant reductions (≈ 30% estimated) in acute running wheel activity following a six-week period (8- 14 weeks of age) on the high fat diet. While Funkat, et al.'s study (46) utilized a greater percent fat in the diet than ours, their results support our hypothesis that wheel activity decreases following a period of overfeeding. Additionally, Funkat et al.'s (46) results, suggested that the dietary-induced decrease in activity was strain dependent, with the C57BL/6J mice showing significantly lower wheel running activity when compared to two other strains (DB2 and 129T2) that were provided a high fat diet. A similar study by Bjursell et al. (11) showed that C57Bl/6J mice receiving a Western diet over a 21-day period, had significantly lower home-cage activity when compared to control counterparts. Lastly, while their purpose was not specifically to 'overfeed' mice, a study by Rendeiro et al. (105) provided isocaloric diets consisting of either 18% fructose or 18% glucose to male C57BL/6J mice for 11-weeks and found significant reductions to home cage activity (≈ 20% reduction) following this period in the 18%

fructose fed mice. Thus, together with the current study, the findings from human (71, 114) and rodent studies (11, 46, 105) suggest that chronic overfeeding decreases activity.

5.3 Effect of Chronic Overfeeding on Sex Hormone Levels

The primary sex hormones in males (testosterone) and females (17β-estradiol) have been shown to be potent regulators of physical activity in rodents (14, 16, 17, 49, 110, 132). The separate body of literature that suggests that sex hormones may be markedly altered by chronic overfeeding (13, 112) led us to hypothesize that if overfeeding altered activity levels, this alteration was possibly mediated through a concurrent alteration in sex hormones.

Somewhat to our surprise, we found that the serum levels of testosterone and 17β- estradiol were not significantly altered by the HFHS diet in male or female mice, respectively. We replicated this finding in the male mice in experiment 2. However, the use of uterine horn weights in the experiment 2 female mice as an indirect measure of estrogenic status (due to assay issues) suggested that the estrogen values in the HFHS female mice would have been lower in HFHS fed mice compared to the CFD mice. It is unclear why the uterine horn weights were significantly lower in experiment 2, but we would speculate that this result could likely be due to the additional two-weeks of the HFHS diet exposure as compared to experiment 1. Given uterine horn weight is only an indirect measure of biologically active estrogens (133, 135), we are cautious to conclude that the difference in uterine horn weight between the HFHS and CFD mice was indeed

indicative of significantly lower 17β-estradiol levels, but this observation is worth further investigation.

One additional consideration to our present findings, with regard to the effect of chronic overfeeding on sex hormones levels, is the group sample sizes utilized. In this study, we based our group sample sizes on previous work from our laboratory (65) which suggested a power of 0.80 could be attained through utilizing four to six mice (C57Bl/6J) per group, however, this power analysis was based on wheel running activity and not sex hormone levels. Given the high variability presented in our sex hormone analyses, particularly testosterone, brings forth the question of whether a higher group sample size was needed in order to definitively conclude chronic overfeeding does not alter sex hormone levels in mice. While we did conduct power analyses for the sex hormone assays, the accuracy of the power values are unclear given the variability of our samples. For testosterone concentrations in male mice of this current study, the variability is not surprising given the wide range of testosterone values reported across the literature (36, 63). Through a review of testosterone values reported in adult male C57Bl mice (control animals), values have been reported as low as 0.16 (±0.09 ng/ml) (67) and 4.29 (±1.95 ng/ml) to upwards of 10-14 ng/ml (variability not provided) (36). The reason for the high variability in testosterone values for male C57Bl mice reported in the literature is unclear, but could potentially be related to the different ELISA manufacturer kits and/or an effect of the time of day the samples (serum) was taken. Circadian cycle may have effect on testosterone levels, though presently unclear (6, 83). The aforementioned studies (36, 63, 67) did not state the time point of the light/dark

cycle the samples were taken, and thus, circadian influences may partially explain the high variability of 'normal' testosterone levels, in mice, reported across the literature. In our present study, however, the samples were consistently taken between the hours 9:00-11:00 am in the male mice in order to control for circadian influences. Taken together, the values obtained in our study did fall within normal ranges provided by previous studies (36, 63) of similar age, and same strain, but the known high variability reported in these studies suggest a higher sample size may be required in order to obtain an accurate power value. While a greater sample size for male mice may be needed to analyze for testosterone levels, this was not the case for females when analyzing for 17β-estradiol. The ranges and variability of 17β-estradiol levels in this present study were similar to what is presented in the literature (19, 86), and an increased sample size is not warranted.

In spite of these findings, given that the sex hormones were largely not altered by HFHS exposure (with the exception of the 2 week running female mice), our hypothesis that the sex hormones were direct links between overfeeding and reduced activity was not supported. Non-significant results are often hard to support, especially in this case because measuring sex hormone levels in small samples is a non-trivial procedure (16). However, in this case, we were cautious and redundant with our sex hormone assays. As noted in the Results, the values we obtained from all sex hormone analyses (with the exception of the Experiment 2 females) were within published assay detection limits, the standard curves were normal, and the coefficient of variance within samples (2-3 per sample) were small (<8%). Additionally, we strictly controlled the phase of the

menstrual cycle when samples were taken from the female mice to control possible fluctuations in 17β -estradiol. Further, at least in the males, our results replicated in both experiments. Given these factors, we are confident in the validity of our sex hormone measurements, and given the statistical power of the hormone comparisons, conclude that in our overfeeding model, sex hormone levels were not the responsible mechanism for the overfeeding-induced inhibition of activity.

There remain other possibilities to explain our observation of a lack of change in sex hormones with chronic overfeeding. In our study, we only considered circulating serum-levels of the sex steroids as an indication for overfeeding-induced alterations to sex hormone regulation and function. A recent study by Sato, et al. (112) demonstrated that following 28-days of overfeeding in males (1,000 kcals/day above energy needs), regardless of a family history of type 2 diabetes, that overfeeding did not have an overall effect on serum concentrations of testosterone. However, Sato, et al. (112) showed that skeletal muscle expression of 3β- hydroxysteroid dehydrogenase (HSD) and 17β-HSD enzymes, which are involved in the formation of testosterone, were significantly reduced by overfeeding. Therefore, while local concentrations of testosterone in the skeletal muscle of these men were not assayed in this study, Sato, et al.'s findings that these steroidogenic enzymes were decreased following overfeeding suggest there may have been a local effect of chronic overfeeding on the skeletal muscle that was not indicated by circulating concentrations of testosterone (112).

Another potential explanation for our unexpected findings is a potential for genetic variability in sex hormone response to diet-induced changes In our study, we

chose to utilize a commonly used inbred strain of mice which are genetically homogenous, and thus are presumed to respond similarly to diet-induced changes mouse-to-mouse (136). In a study by Bouchard et al. (13), twin male subjects were overfed by 1,000 kcals/day (above energy needs) for 8 weeks (6 days/week), and found that serum levels of testosterone were only affected in subjects who had the greatest sensitivity to diet according to body composition changes following overfeeding (i.e. higher total body weight, percent body fat, and fat mass). Similarly, Sato, et al. (112), showed greater decreases in serum testosterone with overfeeding in men with a family history of type 2 diabetes when compared to men that did not have that history. Given that there are established differences in susceptibility to diet-induced changes to sex hormone levels due to genetics (13) and familial influences (112), it is possible that using a different strain of mice would have resulted in alterations in sex hormone levels with overfeeding. However, importantly, our results show that even without alterations in sex hormone levels, overfeeding still produced marked and significant decreases in daily activity in both sexes, suggesting that sex hormones are not involved as the primary mechanism for this effect.

5.4 Effect of Two-week Running Wheel Access on HFHS Diet-Induced Alterations to Body Composition and Running Wheel Activity

Given that the first aim of the current study was to evaluate the effect of chronic overfeeding on acute wheel running activity and sex hormone levels, we extended the

experiment to determine if two-weeks of running wheel exposure would prompt an increase in activity in the HFHS animals, and whether any activity alteration would be sufficient to reverse overfeeding-induced alterations to body composition.

Interestingly, we found that the average daily distance for both HFHS fed male and female mice remained significantly lower throughout the two-week period compared to the CFD mice, suggesting that wheel exposure was not of sufficient impact to recover baseline activity levels inhibited by overfeeding. Additionally, the continual low levels of activity exhibited by the HFHS fed mice appeared to be insufficient to alter body composition changes that resulted from overfeeding. Combined, the results of this experiment suggest that in mice, there is an overriding effect of chronic overfeeding on activity and body composition that is not overcome by having access to a running wheel. In humans, objective measurements of daily physical activity show that activity generally remains unchanged following implementation of 'built-in environments' that are geared to promote physical activity (e.g. sidewalks, parks, gyms, etc.; (34, 41, 96). It remains unclear why human activity remains unchanged with ease of access to exercise modalities and safe environments though the results of the current study suggest poor dietary intake may be one activity-inhibiting factor that cannot be overcome by access to activity opportunities. From an evolutionary perspective, the increase in food availability in Westernized societies has been hypothesized to have removed the stimulus to search food, and as such, has contributed to the low levels of activity (76). We would suggest that future work should focus more on factors that may biologically inhibit activity, such as chronic overfeeding, as a means of increasing physical activity

since these biological-inhibitions may not be readily overcome with environmental stimuli.

5.5 Limitations

There are limitations that we have considered when interpreting the results of these investigations. As noted previously, one limitation is that we only considered serum levels of sex hormones to determine the effect of overfeeding. Given that local effects of overfeeding on sex hormone function has been shown and that those effects are not necessarily indicated by serum concentrations (112), it is possible that the sex hormones could play a role between overfeeding and reduced activity, but we could not make that determination due to the lack of local hormone measurements. However, to our knowledge, this limitation extends across all of the human and animal sex hormone literature, and thus, there is little evidence whether this limitation did or did not alter the results of this current study. Additionally, we have a constant concern as to whether mouse results translate to humans. Given that at least two human studies have shown similar reductions in activity with overfeeding, we believe that our model and the results of this study, at least on the whole organism scale, translate into humans.

5.6 Conclusions

Several conclusions can be drawn from the experiments contained in this study. First, we showed and then replicated the observation that chronic overfeeding significantly reduces daily physical activity in mice fed a HFHS diet. Secondly, a new finding unique to this study, was that overfeeding reductions in daily distance ran may be differentially regulated in males and females. Given that a significant effect of chronic overfeeding on reduced daily activity has now been shown in both humans and mice, these results suggest that food can directly affect activity levels and thus, food intake should be considered as an alterable factor in increasing physical activity in humans.

While we hypothesized that the primary sex hormones would be the mediating factors in any overfeeding-related physical activity inhibition, our findings failed to support this hypothesis. While there are potential sex hormone local effects that may be involved, our evidence cannot shed light on this possibility. Thus, while the inhibition of activity by overfeeding is real and repeatable, the responsible mechanism is still unclear.

Taken altogether, the major implications from this study provide a potential explanation for why physical activity levels in both youth and adults have continued to decrease, even when they have access to environments that promote physical activity (26, 34, 41). In this study we have clearly shown that excessive caloric consumption reduces daily activity and our data is supported by existing indirect human studies.

Therefore, as scientists, exercise physiologists, and other health care providers seek ways to promote and increase daily physical activity among U.S. residents, dietary intake should be an integral component to consider as an intervention method. With this in mind, future studies should be geared towards elucidating the mechanistic pathway through which the influence of diet on activity regulation occurs. Identifying this link would initiate a line of further studies to prevent and treat the development of obesity and other chronic health diseases in humans.

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APPENDIX A. FIGURES

Figure 1: Experimental 1 Design

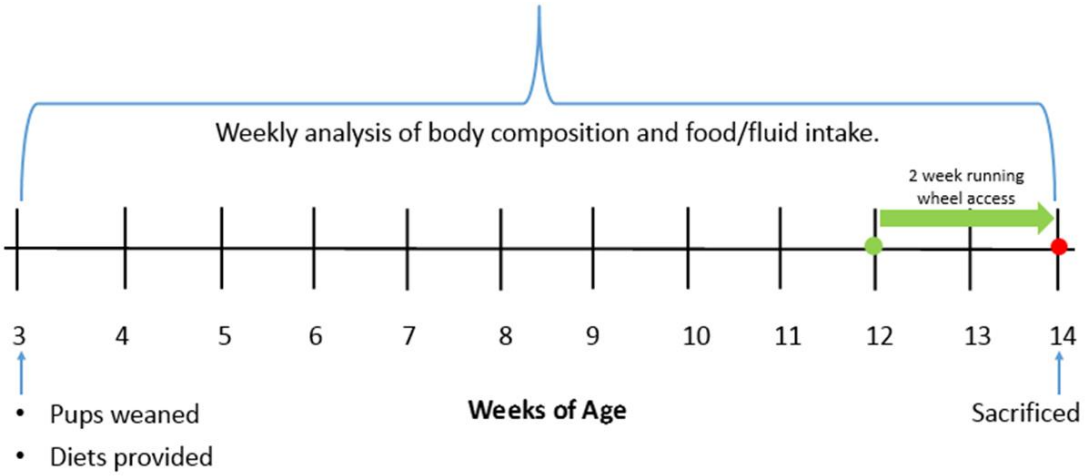
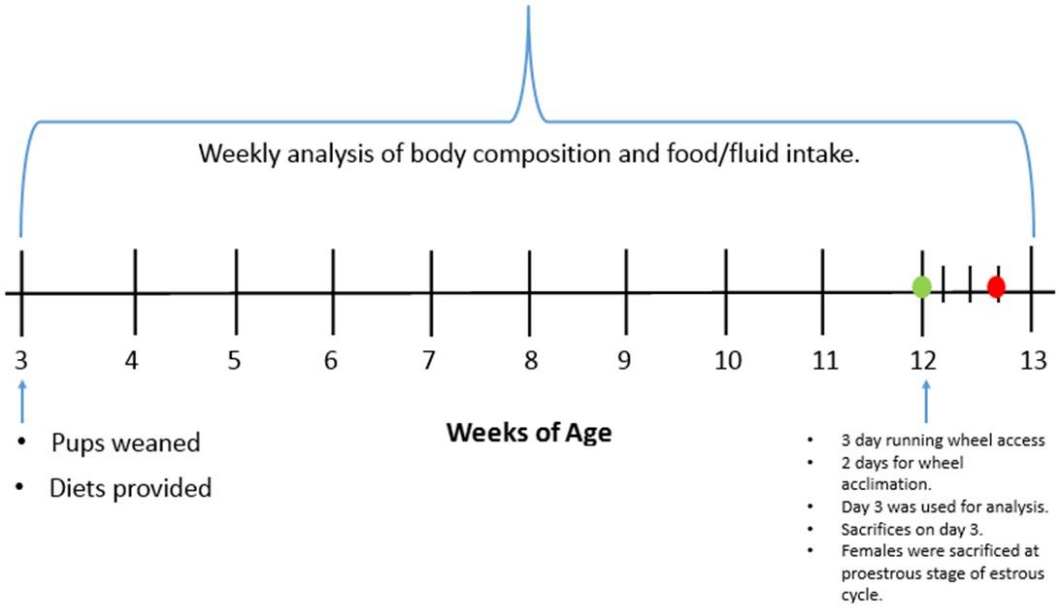


Figure 2: Experimental 2 Design



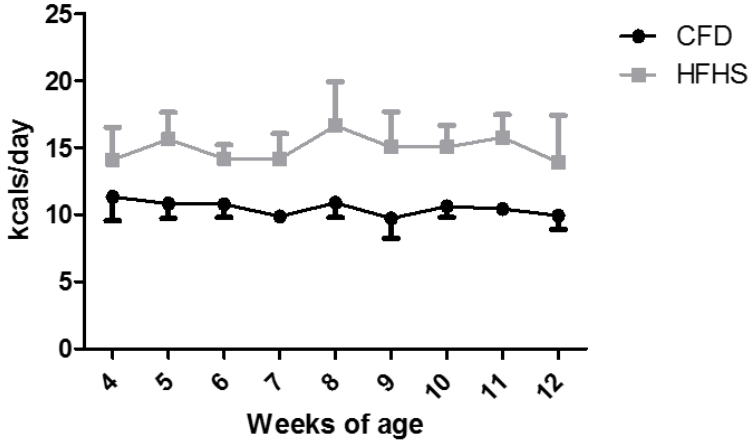


Figure 3: Male Nutrition Intake from 4-12 Weeks of Age Experiment. Average daily caloric intake (kcals/day) was significantly greater in male HFHS fed mice when compared to the CFD male mice from 4 to 12 weeks of age ($p<0.0001$) (Mean ± standard deviation).

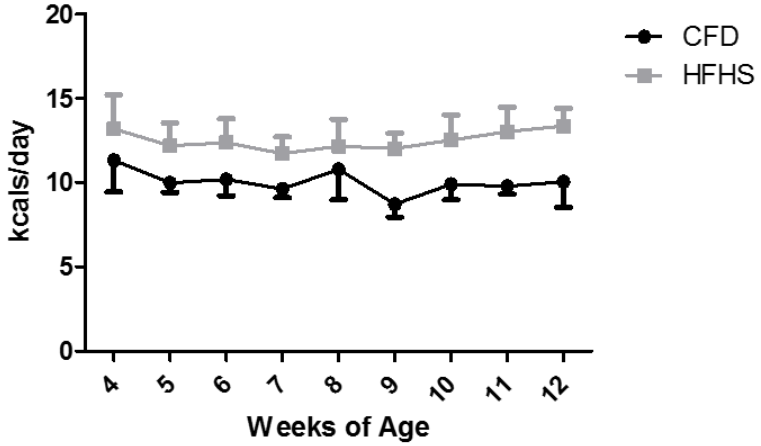


Figure 4: Female Nutrition Intake from 4-12 Weeks of Age Experiment 1. Average daily caloric intake (kcals/day) was significantly greater in female HFHS fed mice when compared to the CFD female mice from 4 to 12 weeks of age ($p<0.0001$).

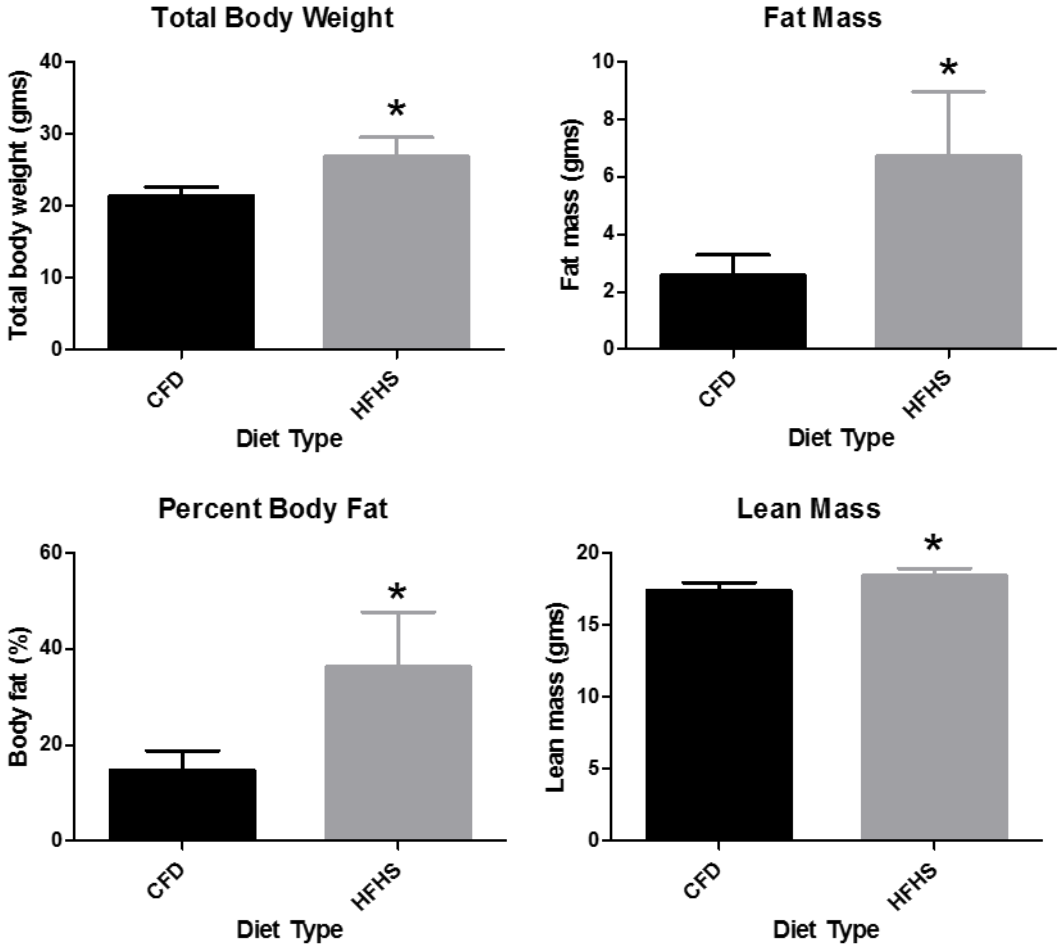


Figure 5: Male Body Composition at 12 Weeks of Age Experiment 1. At 12 weeks of age, the male HFHS fed mice displayed altered body composition measurements through significantly higher increases in total body weight ($p<0.0001$), percent body fat ($p<0.0001$), fat mass ($p<0.0001$), and lean mass ($p=0.0223$).

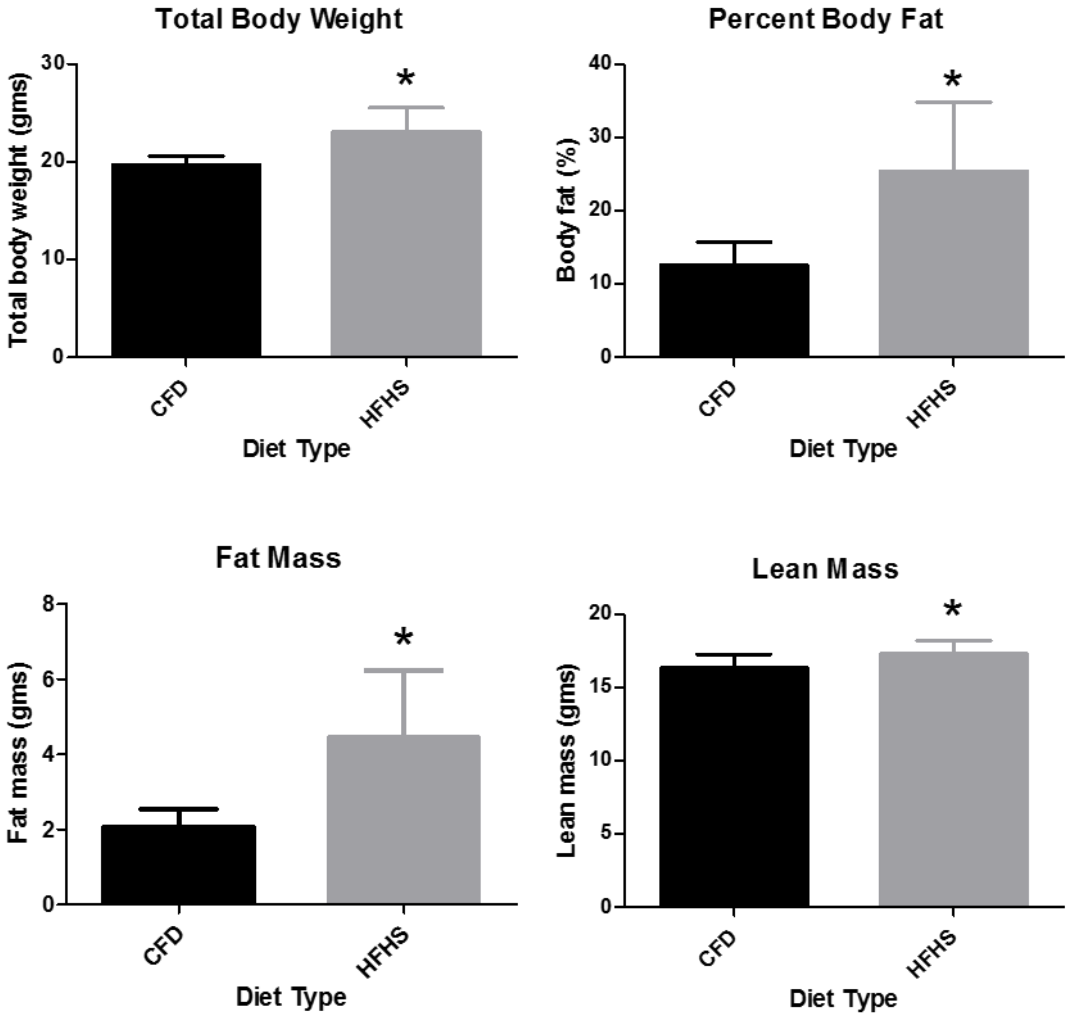


Figure 6: Female Body Composition at 12 Weeks of Age Experiment 1. At 12 weeks of age, the female HFHS fed mice displayed altered body composition measurements through significantly higher increases in total body weight (p=0.0003), percent body fat (p=0.0002), fat mass (p=0.0002), and lean mass (p=0.0181).

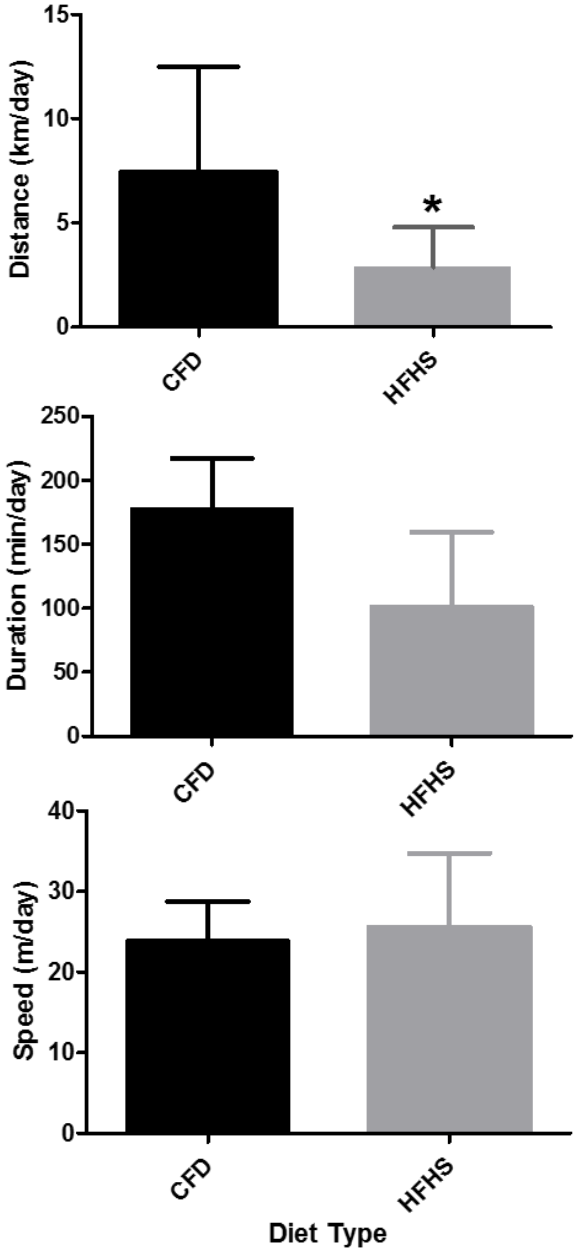


Figure 7: Male Physical Activity Day 3 Data Experiment 1. There was a significant decrease in the distance (p=0.0497) ran on day three of running wheel access. There were no significant differences in the speed (p=0.7392), or duration (p=0.0552; power=0.5048; LSN=10), of activity.

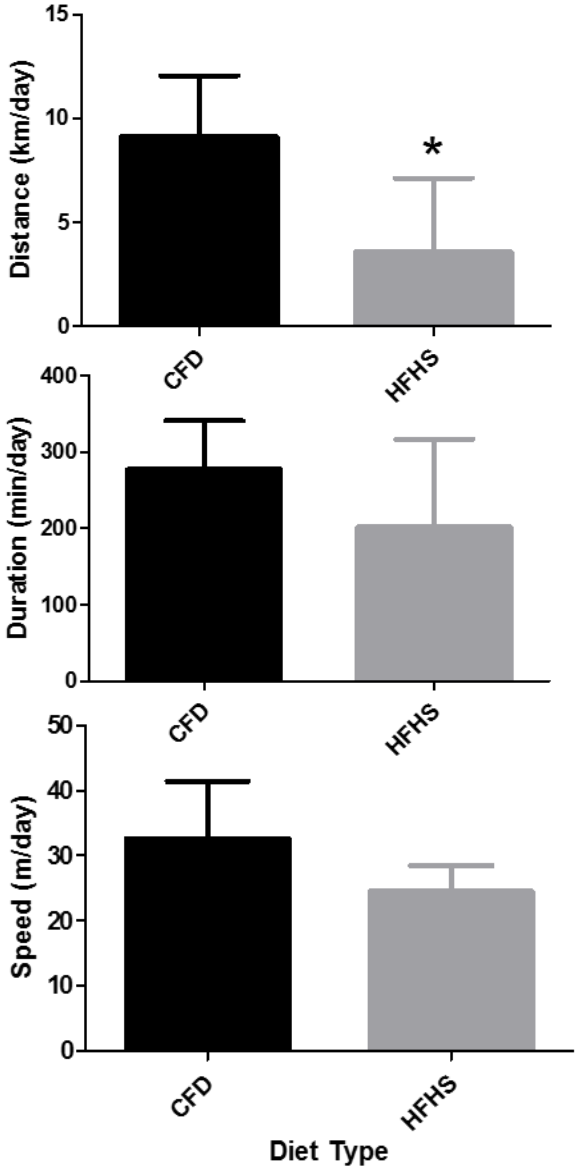


Figure 8: Female Physical Activity Day 3 Data Experiment 1. There was a significant decrease in the distance (p=0.0229) ran on day three of running wheel access. There were no significant differences in the speed (p=0.0715), or duration (p=0.2257), of activity.

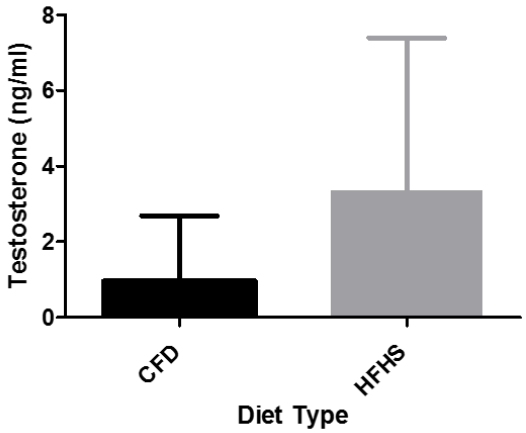


Figure 9: Male Testosterone Concentration Experiment 1. There was not a significant difference in serum testosterone concentration between the CFD and HFHS fed male mice (p=0.2662).

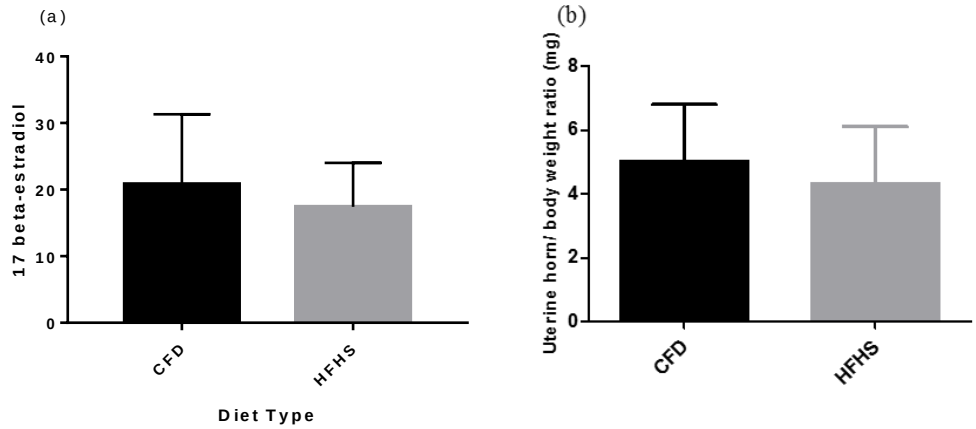


Figure 10 (a): Female 17β-estradiol Concentration Experiment 1. There was not a significant difference in serum 17β-estradiol concentration between the CFD and HFHS fed female mice (p=0.5983).

Figure 10 (b): Uterine Horn Weights Experiment 1 There no significant difference in the uterine horn to total body weight ratio between the CFD and HFHS fed female at 12 weeks of age (p=0.5390).

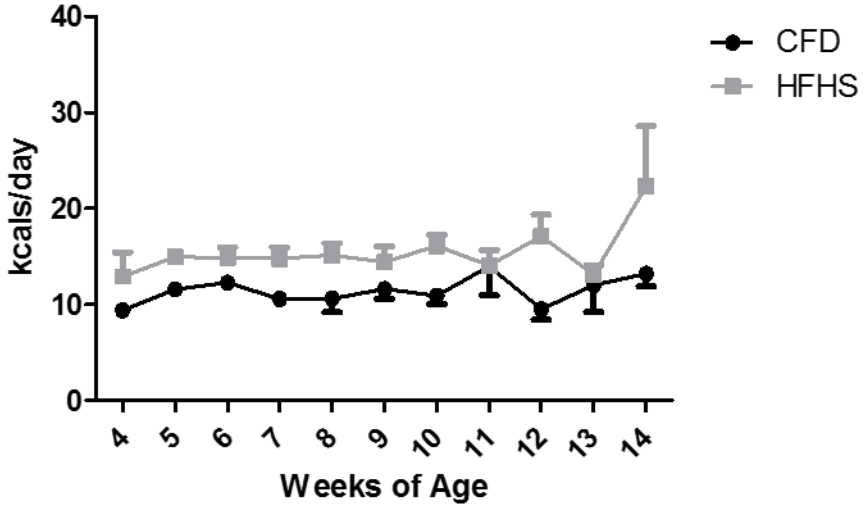


Figure 11: Male Nutrition Intake from 4-14 Weeks of Age Experiment 2. Average daily caloric intake (kcals/day) was significantly greater in female HFHS fed mice when compared to the CFD female mice from 4 to 14 weeks of age ($p<0.0001$).

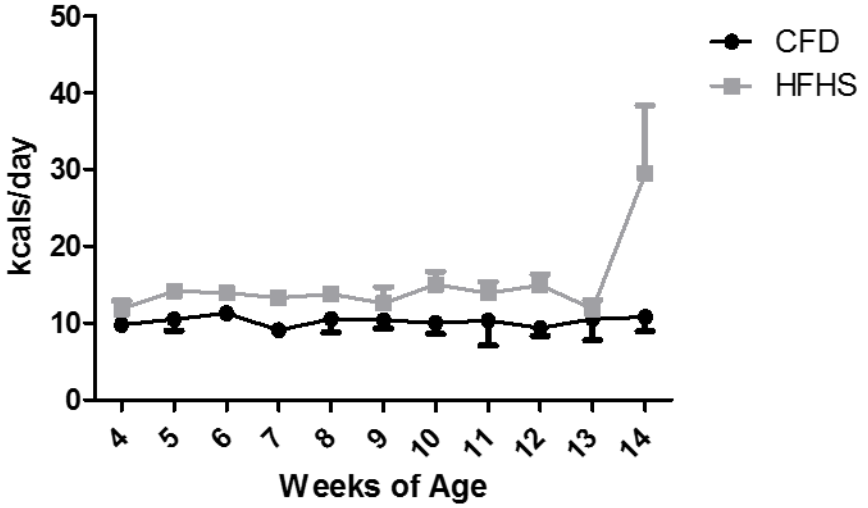


Figure 12: Female Nutrition Intake from 4-14 Weeks of Age Experiment 2. Average daily caloric intake (kcals/day) was significantly greater in male HFHS fed mice when compared to the CFD male mice from 4 to 14 weeks of age ($p<0.0001$).

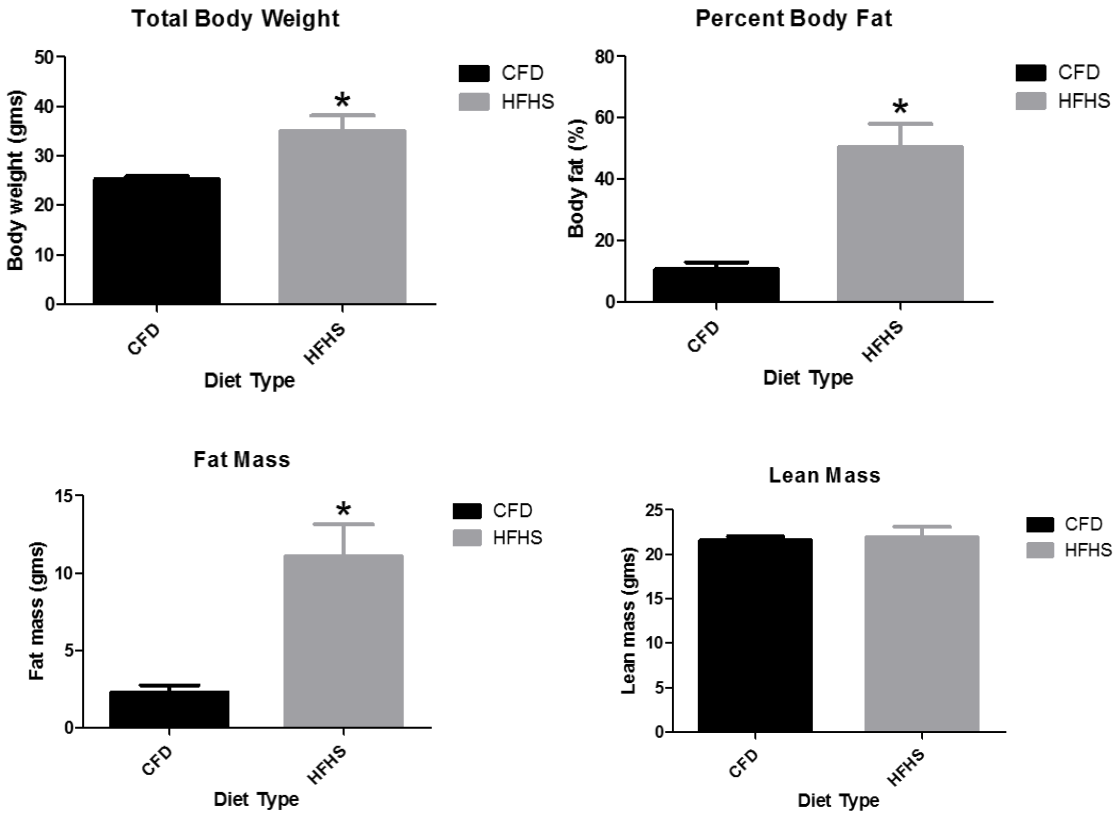


Figure 13: Male Body Composition at 12 Weeks of Age Experiment 2. At 12 weeks of age, the male HFHS fed mice displayed altered body composition measurements through significantly higher increases in total body weight ($p<0.0001$), percent body fat ($p<0.0001$), and fat mass ($p<0.0001$), but not lean mass ($p=0.5510$) compared to control mice.

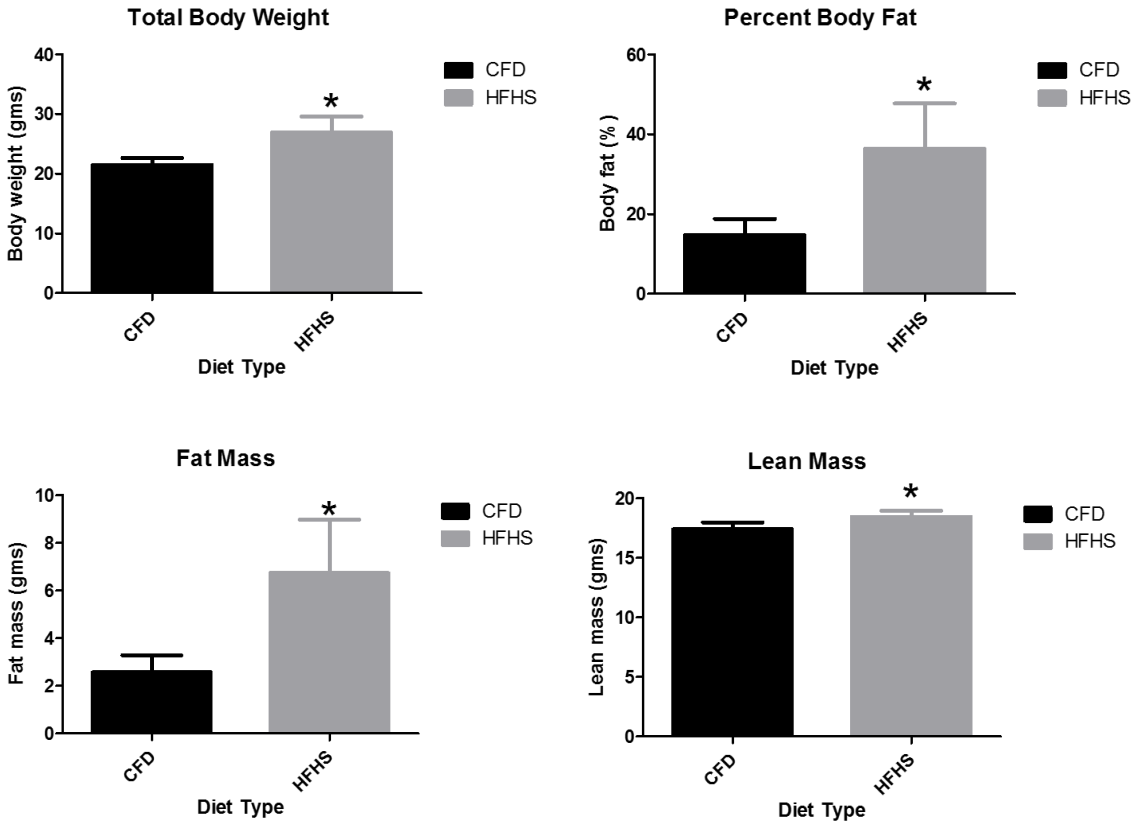


Figure 14: Female Body Composition at 12 Weeks of Age Experiment 2. At 12 weeks of age, the female HFHS fed mice displayed altered body composition measurements through significantly higher increases in total body weight (p=0.0003), percent body fat (p=0.0005), fat mass (p=0.0005), and lean mass (p=0.0043) compared to control mice.

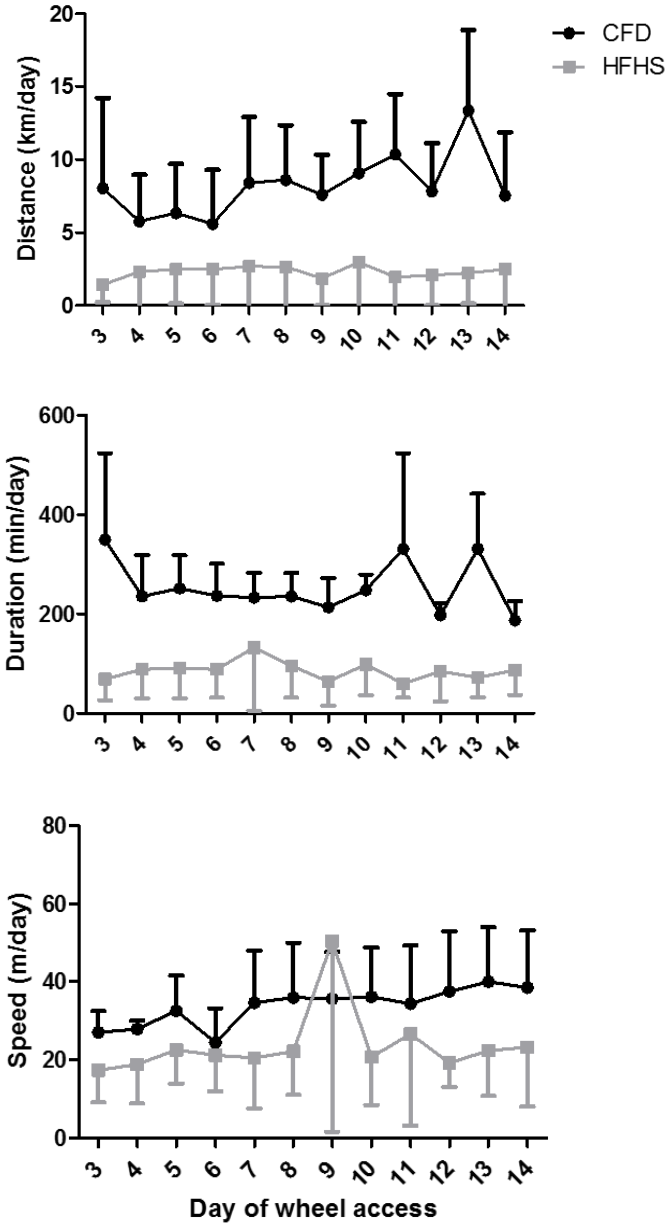


Figure 15: Male 2-Week Physical Activity Data Experiment 2. There was a significant difference in the distance (p=0.0118) ran over a 2-week time period of running wheel access. There were no significant differences in the speed (p=0.4534), or duration (p=0.0556), of activity.

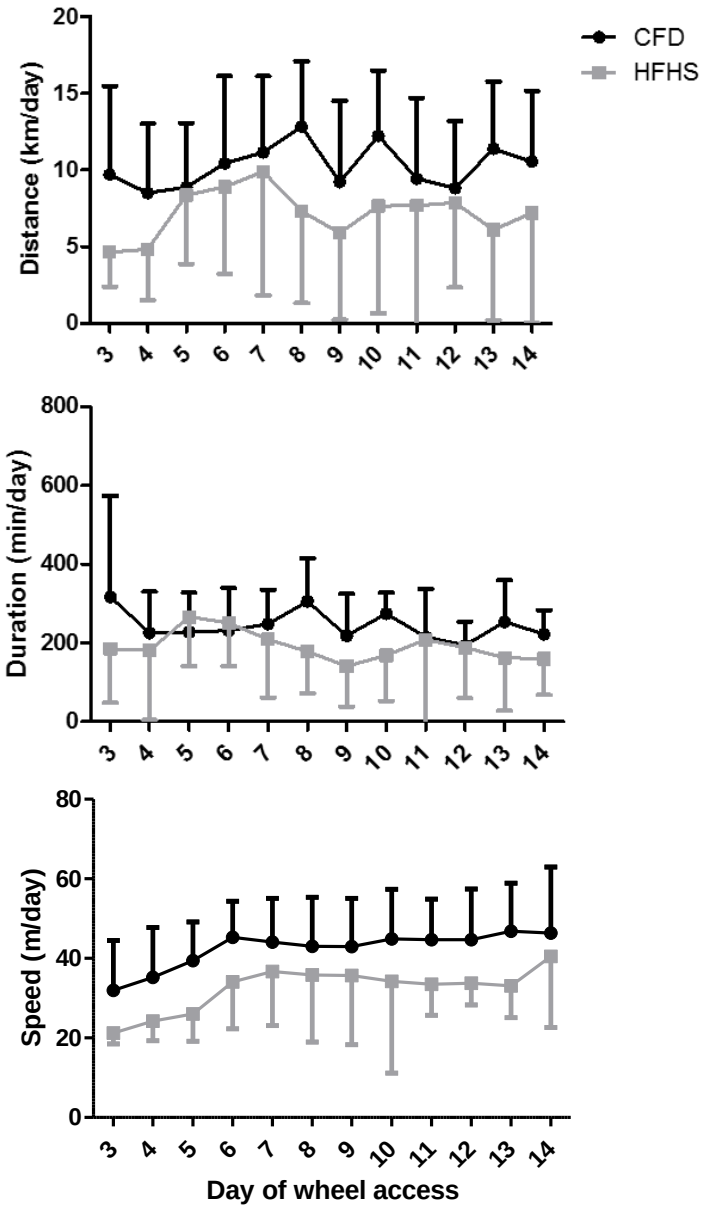


Figure 16: Female 2-Week Physical Activity Data Experiment 2. There was a significant difference in the distance (p=0.0218) ran over a 2-week time period of running wheel access, with the decrease in distance due to decreased speed (p=0.0147), and not duration (p=0.4915) of activity.

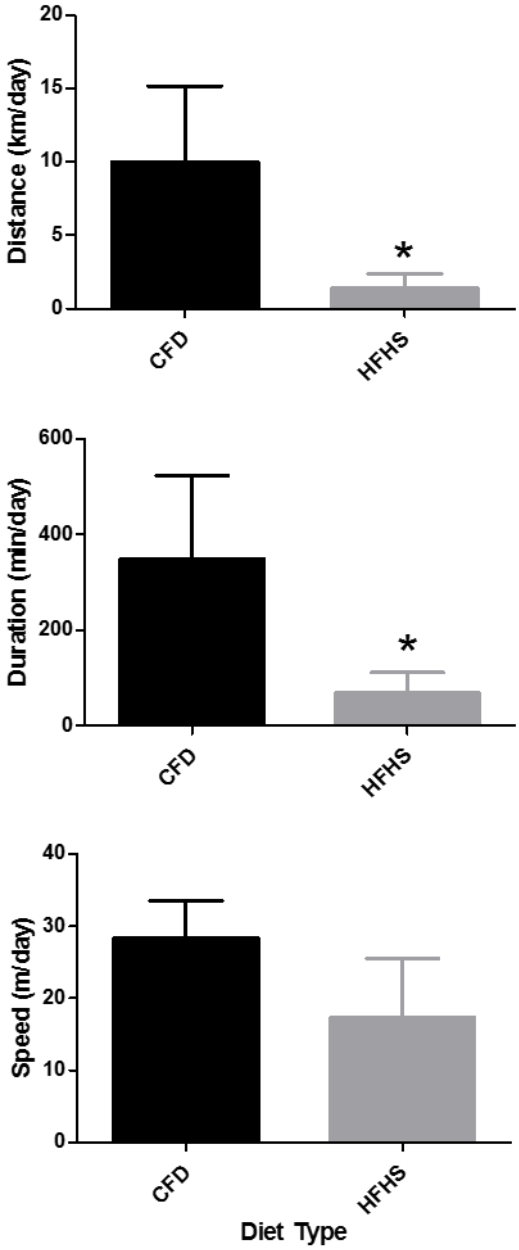


Figure 17: Male Physical Activity Day 3 Data Experiment 2. There was a significant decrease in the distance (p=0.008) and duration ran (p=0.0095) on day three of running wheel access. There was not a significant difference in the speed of activity (p=0.0558).

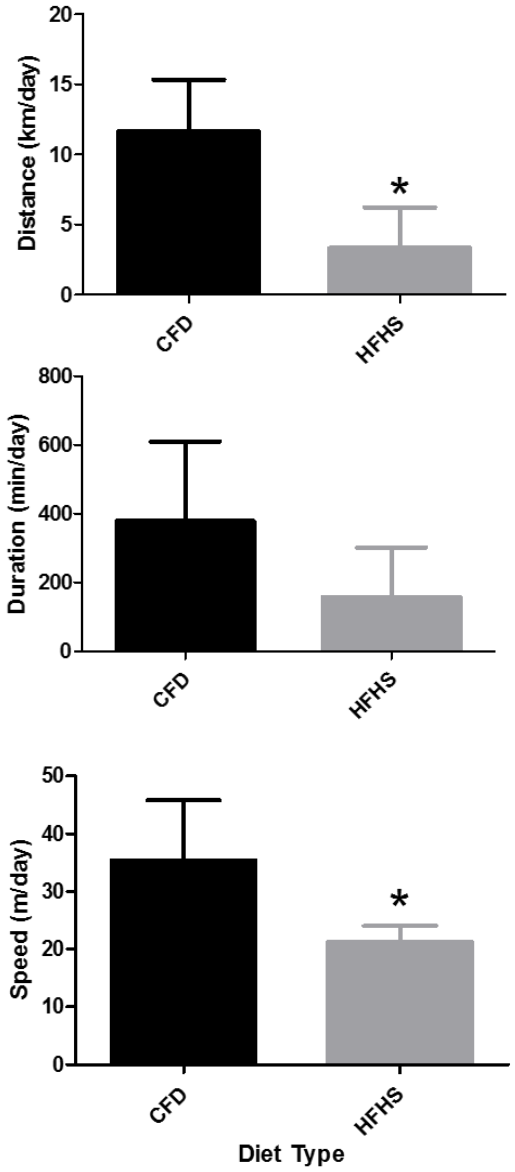


Figure 18: Female Physical Activity Day 3 Data Experiment 2. There were significant decreases in the distance (p=0.0014) and speed (p=0.006) on day three of running wheel access. There was not a significant difference in the duration of activity (p=0.0674).

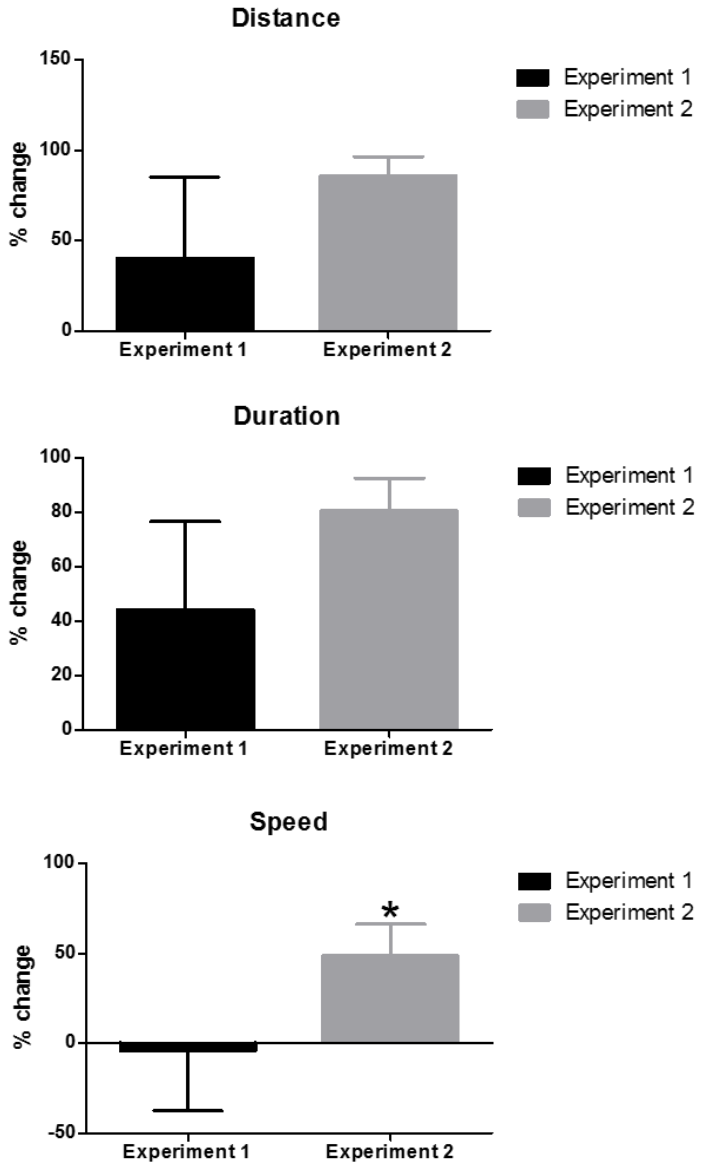


Figure 19: Male Mean Percent Change in Physical Activity Data by Diet Type between Experiments 1 and 2. When analyzing the percent change in acute running wheel activity between male CFD and HFHS fed mice (percent change= μ CFD mice– of each HFHS mouse *100, for distance, duration, and speed) each experiment, we found that the mean percent change in distance (p=0.0896) and duration (p=0.0689) were not significantly different, however there was a significant difference in the percent change in speed of activity (p=0.0211) between Experiments 1 and 2.

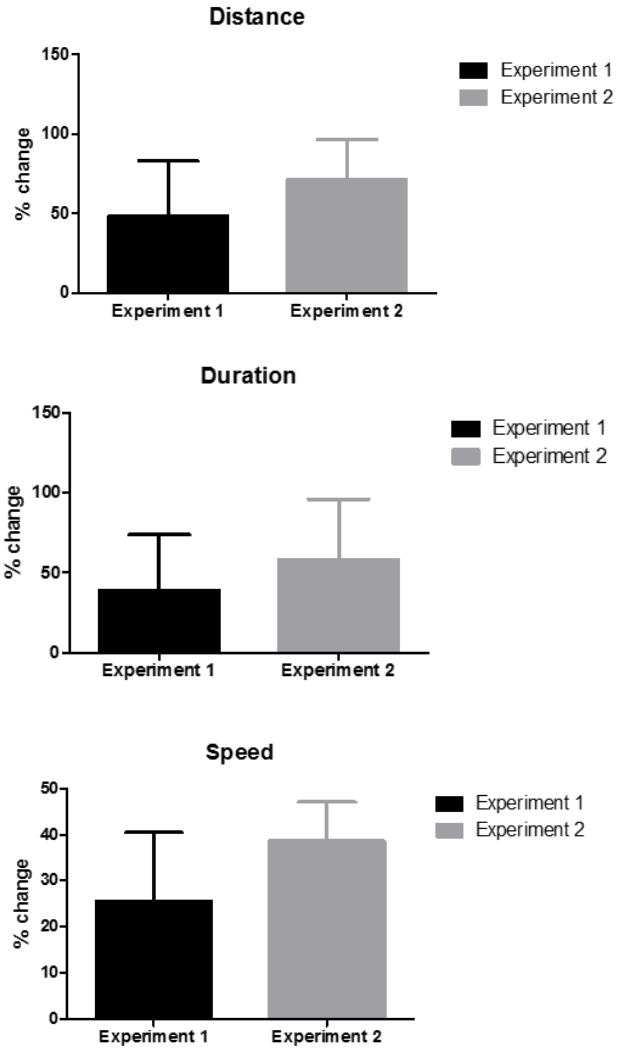


Figure 20: Female Mean Percent Change in Physical Activity Data by Diet Type between Experiments 1 and 2. When analyzing the percent change in acute running wheel activity between female CFD and HFHS fed mice (percent change= μ CFD mice– of each HFHS mouse *100, for distance, duration, and speed) each experiment, we found no significant differences in the mean percent change in distance (p=0.2265), duration (p=0.2674) or speed of activity (p=0.1544) between Experiments 1 and 2.

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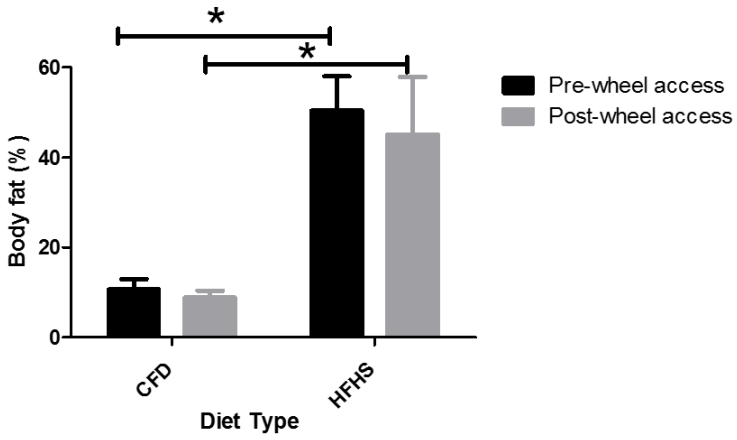

Figure 21: Male Percent Body Fat Pre- and Post-Wheel Running Experiment 2. In male mice, a two-way ANOVA indicated an overall significant difference ($p<0.0001$), and a Student's t test further indicated this difference was due to diet type ($p<0.0001$), and not due body compositional changes with running wheel access ($p=0.2980$).

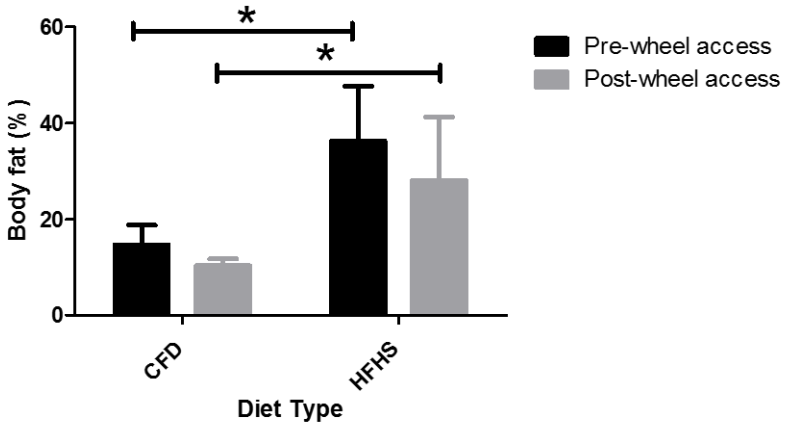

Figure 22: Female Percent Body Fat Pre- and Post-Wheel Running Experiment 2. In female mice, a two-way ANOVA indicated an overall significant difference ($p<0.0001$), and a Student's t test further indicated this difference was due to diet type ($p<0.0001$), and not due body compositional changes with running wheel access ($p=0.0728$). A power analysis provided a power of 0.4370 and a least significant number of 33.26.

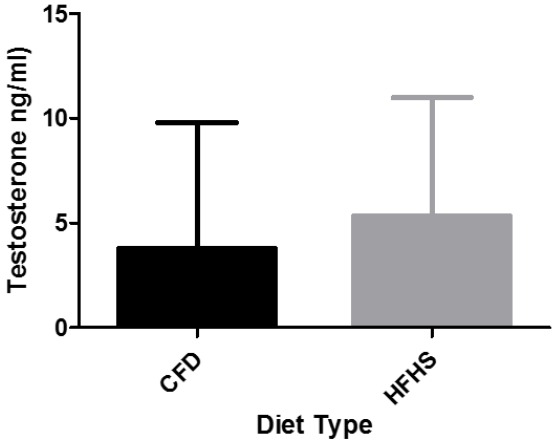

Figure 23: Male Testosterone Concentration Experiment 2. There was not a significant difference in serum testosterone concentration between the CFD and HFHS fed male mice with 2 weeks of wheel running access (12- 14 weeks of age) (p=0.6836).

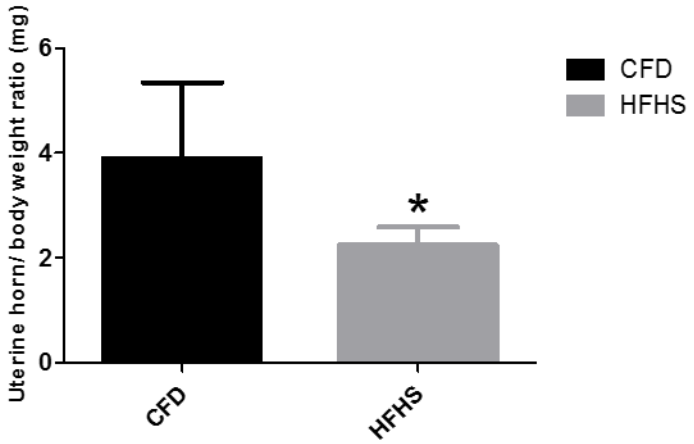

Figure 24: Female Uterine Horn Weight Experiment 2. There was a significant difference in the uterine horn to total body weight ratio between the CFD and HFHS fed female mice with 2 weeks of wheel running access (12- 14 weeks of age) (p=0.0243).

APPENDIX B. TABLES

Table 1: Diet Descriptions for Experiments 1 and 2

Diet Type	Description	Kcals/gram (g)
Normal "chow-like" diet (CFD)	25.2% protein, 39.5% carbohydrate, 3.3% crude fiber, 10.0% neutral fiber, 9.9% Ash	3.0 kcals/g
High fat/high sugar diet (HFHS)	20% protein, 35% carbohydrate (sucrose), 45% fat (6% soybean oil, 39% lard) + 20% fructose solution.	4.73 kcals/g

Table 2: Offspring Demographics for Experiment 1

Sex	Diet Type	Running wheel access? (yes or no)	n
Female n= 25	HFHS	No	6
	CFD	No	7
	HFHS	Yes	6
	CFD	Yes	6
Male n= 22	HFHS	No	5
	CFD	No	6
	HFHS	Yes	5
	CFD	Yes	6

Table 3: Offspring Demographics for Experiment 2

Sex	Diet Type	n
Female n= 14	HFHS	7
	CFD	7
Male n= 10	HFHS	5
	CFD	5