# The role of Fibroblast Growth Factor 2 in Energy Balance and Metabolism

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Shazeen (Sajin) Alam

Department of Neuroscience

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**Abstract**

Recent studies demonstrate fibroblast growth factors act peripherally and centrally to regulate energy homeostasis. Fibroblast growth factor 2 (FGF2) is exclusively expressed by astrocytes and has hypophagic and weight reducing effects. The objective of this study is to further elucidate the role of FGF2 on feeding and energy balance using FGF2 KO animals. To this end, we exposed male and female wild-type and FGF2 KO mice a regular chow or a 60% high fat diet over the course of 6 weeks. We hypothesized FGF2KO animals would exhibit hyperphagia and weight gain, and that this would augment in the presence of a high fat diet. We also hypothesized FGF2KO animals would increase carbohydrate expenditure and decrease energy expenditure, and that there would be differences between the sexes. Our results demonstrate that both WT and FGF2KO animals gained weight similarly and had similar caloric intake when ingesting high fat diet. Carbohydrate expenditure was increased in male KO's and female KO's had increased ambulatory counts during the night phase. Future studies should investigate the effects of stressed FGF2KO animals and challenge them on HFD and subject them to more behaviour testing to calculate energy expenditure and anxiety-like behaviour while challenging them on HFD. Furthermore, they should continue to explore the circuitry involved in energy balance and regulation and elucidate FGF2's role in regulating this circuitry and the HPA-axis.

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**Obesity**

Obesity is a prevalent health condition in North America and the incidence rate has rapidly increased over the past two decades. Obesity increases the risk for multiple chronic illnesses such as diabetes, cardiovascular disease, and cancer ultimately leading to early death. Obesity is often defined in terms of the body mass index (BMI) with a BMI of over 30 being associated with increased risk of disease, and this is coupled with an imbalance between energy intake and energy expenditure (Hill et al. 2012). Although obesity can result from a sedentary lifestyle, genetic mutations, individual differences in metabolic systems, hormonal systems and central nervous system circuitry involving the hypothalamus are also factors that lead to obesity (Hruby & Hu 2015; Han & Lean 2016).

There are several factors that affect body weight and energy balance, mainly revolving energy expenditure and energy intake. Energy expenditure takes account a few components: resting metabolic rate (the amount of energy required at rest which is proportional to body weight), the thermic effect of food (energy cost for metabolizing food), and the energy expended during physical activity (Hill et al. 2012). Energy is obtained from three main sources: carbohydrates, proteins, and fat (Hill et al. 2012). Carbohydrates are the most limited macromolecules and are the most abundant source of energy during exercise (Pendergast et al. 2000). Normally, the body breaks down glucose in order to meet metabolic needs. Excess glucose is then converted into glycogen and stored in the liver (Jensen et al. 2011). Insulin is released by beta- cells in the pancreas to facilitate satiety and glycogen anabolism (Woods et al. 2006). During exercise, the muscles will eventually deplete the glycogen stores leading to fatigue (Constable et al. 1985). Overall, fats contain the most energy, and in instances where there is an excess amount of fatty acids, the liver converts them into triglycerides, and transports them to

adipose tissue to be stored in the form of white fat (Rui 2014). Adipose tissue is not only involved with storage of white fat; it is also involved with regulation of metabolic homeostasis (Coelho et al. 2013). During periods of energy starvation, white adipose tissue will resort to lipolysis, the breakdown of triglycerides into fatty acids and glycerol, to meet metabolic needs of other tissues (Duncan et al. 2010). When there is an imbalance of energy intake and energy expenditure, body weight is subject to change. For example, excess food intake results in an increase in weight, whereas excess of energy expenditure can lead to a decrease in weight (Hill et al. 2012).

**Energy Balance and the Hypothalamus**

The hypothalamus utilizes highly complicated feedback mechanisms in its regulation of energy balance and weight regulation. For instance, the arcuate (ARC) nucleus of the hypothalamus is an integrative center of energy balance, homeostasis and body weight regulation (McNay et al. 2011, Joly-Amado et al. 2014). The ARC nucleus contains neurons that are involved in the facilitation of appetitive behavior or the suppression of food intake and is positioned near the median eminence which contains capillaries to allow transport of peripheral hormonal and nutrient signals (Timper et al. 2017). The pro-appetitive neurons are NPY and AGRP, and the ones involved with suppression of feeding are POMC-containing neurons (Kleinridders 2009).

When hormones such as leptin bind to its receptors on POMC neurons, they stimulate the release of alpha-melanocyte stimulating hormone (alpha-MSH), which then binds to melanocortin-4-receptors (MCR4) in the paraventricular nucleus (Sohn et al. 2015). Human studies have also shown agonists of MC4R such as setmelanotide reduce body weight and

suppress food intake. (Kühnen et al. 2016 & Collet et al. 2017). Because the arcuate nucleus is an integrative center, it is a target of multiple hypothalamic projections and responsible for innervation to other second order hypothalamic neurons in order to regulate energy balance and weight management, containing both pro-appetitive and appetitive suppressing neurons and neurons involved with the facilitation and suppression of energy expenditure (Timper et al. 2017). Such examples of these second order neurons including the paraventricular hypothalamus are the lateral hypothalamus, dorsomedial, ventromedial nuclei of the hypothalamus, (Elmquist et al. 1998; Timper et al. 2017). An example of how interconnected and complex these hypothalamic regions are, NPY and AgRP terminals from the arcuate nuclei are involved with converging on neurosecretory parvocellular neurons in the paraventricular hypothalamus in order to stimulate appetite whereas POMC terminals suppress food intake, while the dorsomedial hypothalamus plays a role in facilitating food intake and is involved with sending inhibitory projections to the POMC neurons of the arcuate nucleus (Cowley et al. 1999; Bellinger et al. 2002; Jobst et al. 2004; Rau et al. 2019).

**Energy Balance and Hormones**

There are several hormones that are associated with the regulation of energy balance that act on the hypothalamus. For instance, adipose tissue is involved with secreting a wide variety of hormones, growth factors, enzymes and cytokines to further maintain metabolic homeostasis in the body and manage food intake and energy expenditure. (Coelho et al. 2013). Leptin is a prevalent adipokine involved in the maintenance of energy homeostasis and body weight by acting as an appetite suppressor (Zhou & Rui 2013). One way for leptin to exert its effects is through binding to specific leptin receptors in POMC containing neurons in the arcuate nucleus

of the hypothalamus and inhibiting NPY/AgRP neurons (Park & Ahima 2015). Rodent models exhibiting mutations in the leptin receptor (db/db) and leptin (ob/ob) show hyperphagia and obesity (Cohen et al. 2001). In contrast, ghrelin is an appetitive stimulating peptide synthesized in the stomach that targets growth hormone secretagogue receptors (GHS-R) in various energy regulating hypothalamic regions such as LH, DMH, VMH and ARC (Wren et al. 2001; Druce et al. 2005; Sato et al. 2005; Sakata & Sakai 2010). Ghrelin administration increases the activity of NPY and AgRP neurons and stimulates food intake while having opposing effects on POMC neurons through inhibition. (Cowley et al. 2003). In addition, ghrelin increases fat tissue and decreases fat oxidization and overall, increases body weight in both humans and rodent models (Meier et al. 2004).

**Sex Differences in Obesity and Metabolic Processes**

Hormonal dysfunctions play a major role in the etiology of obesity. Interestingly the global prevalence of obesity is generally higher in women compared to men (Lovejoy et al. 2009). When accounting body composition, women are generally shorter and weigh less, and proportionally have more fat mass compared to men (Lovejoy et al. 2009). Furthermore, women have lower energy expenditure and tend to be lower compared to males and have a greater increased risk of developing obesity as they age (Lovejoy et al. 2009). Fat storage is also sex specific, where women have increased subcutaneous adipose tissue while males have increased visceral adipose tissue (Lovejoy et al. 2009).

Estrogen exerts its biological functions via estrogen receptors, one of which is estrogen receptor α. Estrogen receptor α is abundant in the key hypothalamic regions involved in energy

homeostasis including the ventromedial hypothalamus and the arcuate nucleus, in which they have been shown to play a role in the regulation of food intake and energy expenditure and (Heine et al. 2000; Musatov et al. 2007; Frank et al. 2014; Liu and Shi et al. 2015). Animal studies involving the knockout of estrogen receptor α showed increased visceral adiposity, elevated insulin levels and impaired glucose tolerance in both male and female mice (Heine et al. 2000). Furthermore, silencing of estrogen alpha receptor α specifically in the ventromedial hypotahalmus resulted in obesity, hyperphagia, impaired tolerance to glucose and reduced energy expenditure in female mice (Musatov et al. 2007). Surprisingly, Xu et al. showed that rodent models of estrogen receptor α deletion in POMC neurons alone had increased hyperphagia but had no effects in energy expenditure or fat distribution (Xu et al. 2011). Next, they knocked out specifically estrogen receptor alpha in SF-1 neurons that are present in the VMH and POMC neurons together, and reported hypometabolism, hyperphagia and increased visceral adiposity (Xu et al. 2011). This suggests that the estrogen receptor α in the ventromedial hypothalamus has a more prominent role in affecting overall metabolism such as adiposity storage and energy expenditure (Musatov et al. 2007; Xu et al. 2011).

**Energy Balance and Astrocytes**

Besides neurons, recent literature also suggests astrocytes play a role in energy balance. Astrocytes are a class of glial cells present in the central nervous system with prominent roles in development, regulation of blood flow, maintenance of homeostasis and blood brain barrier and energy balance (Sofroniew & Vinters et al. 2010). Astrocytes are uniquely positioned and consist of two different processes: fine peri-synaptic processes which are positioned at most synapses (forming the tripartite synapse), and end-feet processes positioned near blood vessels (Bélanger et al. 2012). Peri-synaptic processes express receptors for growth factors and neurotransmitters, as well as multiple ion channels, while the end-feet processes contain various receptors including glucose transporters and growth factor receptors (Bélanger et al. 2012; Alvarez et al. 2013). Although all tissues require the breakdown of glycogen to survive in periods of starvation, the brain contains low levels of glycogen (Brown & Ransom 2007). Astrocytes are the only source of glycogen in the brain and glycogen is believed to be broken down into lactate during periods of hypoglycemia as a glucose substrate (Brown & Ransom 2007).

Recent research demonstrates that astrocytes express leptin receptors, and long-term exposure to high fat diets increase the amount of leptin receptors in the astrocytes present in the arcuate nucleus (Hsuchou et al. 2009; Wang et al. 2015). In addition, selective deletion of leptin receptors in astrocytes has led to fewer numbers and shorter lengths of primary astrocytic projections in the arcuate nucleus (Kim et al. 2014). One study also hypothesized astrocytes are involved in inhibiting AGRP neuron activity, which facilitate food intake under normal conditions (Yang et al. 2015). Yang et al. used clozapine-N-oxide in order to induce activation of astrocytes and reported a reduction in the action potential firing rate of AgRP neurons,

suggesting inhibition of food intake (Yang et al. 2015). Some researchers, using glial-fibrillary acidic protein (GFAP) expression as a marker of astrocytes, have found an increased number of GFAP positive cells after high fat feeding in many rodent models, which they report is indicative of astrocyte reactivity (Buckman et al. 2014; Balland & Cowley 2017; Tsai et al. 2018) However, not all reactive astrocytes express GFAP, and GFAP is not specific to only astrocytes (Sofroniew et al. 2010; Buckman et al. 2014; Balland & Cowley 2017; Tsai et al. 2018).

**Fibroblast Growth Factor 2**

Astrocytes express the growth factor fibroblast growth factor 2 (FGF2), which is a protein belonging to a family of 22 total growth factors found in both the developing and adult brain (Molteni et al. 2001) and is almost exclusively produced in astrocytes. Fibroblast growth factors (FGFs) have varied functions both in development and later in adulthood, such as cellular proliferation, migration and differentiation (Yun et al. 2010). Of interest, FGF2 has extensive functions including development, promotion of neurogenesis, neuroprotection and astrocytic proliferation and maturation (Jin et al. 2005 & Irmady et al. 2011)

Currently, there are five fibroblast growth factor receptors (FGFR) identified. FGF1-4R are receptors for ligands with a tyrosine kinase domain, whereas FGFR5 is a functional receptor lacking this domain (Bikfalvi et al. 1997; Zhang et al. 2006; Tiong et al. 2013). While FGF2 binds to all FGF receptors in a promiscuous way, some studies have suggested that some FGF ligands have greater affinities towards specific FGFR's. For example, ligand-binding specificity of FGFR's is determined by a specific IgIII domain isoform (a,b, or c) and FGF2 binds with a higher affinity to FGFR1 IIIb, FGFRIIIc, and FGFR2 IIIc (Tiong et al. 2013). In the adult brain,

FGF2 is largely expressed by astrocytes throughout the central nervous system including the cortex, hypothalamus, and, at least in rats, within a small sub-population of neurons in the CA2 area of the hippocampus (Eckenstein et al. 1994; Bikfalvi et al. 1997). FGFR's, on the other hand are expressed on all cells types within the brain (Reuss et al. 2003).

Several studies have shown that disruptions in FGF2 function are linked to several mood and anxiety disorders. In human models with major depressive disorder, FGF2 mRNA levels in the cortex and hippocampus downregulated (Evans et al. 2004; Gaughran et al 2006). Similarly, models of social defeat (a stressor) which are also capable inducing depressive-like behaviour in rodents result in downregulated levels of FGF2 in the hippocampus (Turner et al. 2008).

Both acute and chronic injections of FGF2 in animal models were utilized further in existing studies to elucidate FGF2's potential therapeutic role. For instance, several studies have found that FGF2 injected animals exhibited less depressive-like and anxiety-like behaviour compared to the control groups (Turner et al. 2008; Perez et al. 2009; El Sayed et al. 2012; Salmaso et al. 2016). In addition, transgenic rodent models silencing the FGF2 gene or models with the gene knocked out exhibit increased anxiety-like behaviours, and administration of FGF2 rescues this increase to wild-type control levels (Eren- Koçak, et al. 2011; Salmaso et al. 2016).

Interestingly, epidemiological and meta-analytical studies demonstrated that obesity, mood and anxiety disorders are often comorbid (Simon et al. 2006; Gariepy et al. 2009; Luppino et al. 2010). Correlation studies have shown individuals who are most vulnerable to developing obesity are those diagnosed with major depressive and bipolar disorders (Romain et al. 2018). Furthermore, obesity, major depressive and anxiety disorders are all associated with alterations in the HPA axis such as increased salivary cortisol levels indicative of hyperactive HPA activity (Nestler et al. 2002; Jacobson et al. 2014; Dieleman, et al. 2015; Rodriguez et al. 2015).

Recent studies show that FGFs play a role in energy homeostasis both peripherally and centrally. Systems involved with energy balance other than the brain, such as white adipose tissue and the liver, also produce or express FGFs and FGFR's. Adipocytes express receptors such as FGFR1 and FGFR2, whereas the liver contains FGFR4, presumably to regulate glucose levels as shown by administration of FGFs such as FGF1 and FGF21 (Adams et al. 2012; Yang et al. 2012; Suh et al. 2014). FGF1 is produced in several areas including the brain, kidney and adipose tissue and is within the same subfamily as FGF2 but is considered the acidic form (Nies et al. 2015). FGF21 is not thought to be expressed in the brain, but is in several organs and is the only peripheral FGF known capable of diffusing into the circulation to regulate energy homeostasis (Fisher & Maratos-Flier 2015; Nies et al. 2015). FGF19 is expressed in the intestines and can diffuse into the hepatic portal into the liver where it binds to FGFR1 and FGFR4 with the same affinity, whereas FGF21 preferentially binds to FGFR1 more than FGFR4 to exert similar effects of insulin sensitization (Yang et al. 2012; Ryan et al. 2013).

FGFR1 is present in brain regions involved with both energy balance and motivational systems such as the lateral hypothalamus, the arcuate nucleus of the hypothalamus and the ventral tegmental area (Matsuo et al. 1994). Specific blockade of FGFR1 via bilateral infusions of anti-FGFR1 antibody in the lateral hypothalamus increased food intake suggesting FGFR1's role in regulating the satiety signaling (Li et al. 1996). Interestingly, intracerebroventricular (ICV) injections of FGF2 also suppress food intake and reduce body weight in a dose dependent manner (Denton et al 1995; Hotta et al. 2001). Infusions of FGF2 in the lateral hypothalamus has also resulted in a suppression of food intake, and infusions of anti-FGF2 caused an increase of food intake, however, body weight was not reported in this same study (Sasaki et al. 1994).

Although it is well understood that FGF's and specific receptors are involved with energy balance and metabolism, very few studies have explored the effects of FGF2 and its receptors when animal models were fed on high fat diets. For example, high fat diets are used to study obesity and other metabolic disorders such as type 2 diabetes since they reliably promote weight gain, hyperglycemia and insulin resistance (Buettner et al. 2007 & Woods et al. 2003 & Winzell & Ahrén 2004). One study has shown high fat diets reduce FGFR1 expression in the hypothalamus in wild-type animals (Ryan et al. 2013). Another study by Kim et al. 2015 demonstrates that rodents exposed to a high fat diet for 9 weeks had lower levels of FGF2 mRNA in their epidymis adipocytes compared to regular chow control groups and that suppression of FGF2 expression in adipocytes may be necessary for the increase in size and number of adipocytes (Kim et al. 2015). This further suggests FGF2's homeostatic role in energy balance such as maintaining body weight and limiting excess increase of adipocytes.

Despite previous findings of hypophagia in rodents with ICV administration of FGF2, it is unclear whether FGF2 KO animals would manifest the opposite effect and become hyperphagic, and whether these effects would be augmented in the presence of a high fat diet. In addition, there are currently no studies that investigate the effects of body weight and food intake in FGF2 KO animals and there is a lack of understanding pertaining to FGF2's role in energy expenditure and respiratory exchange ratio (RER) values. The respiratory exchange ratio (RER) is an indication of which pre-dominant fuel source is burned by the specific animal, with values closer to 1 indicating more carbohydrate metabolism, while values closer to 0.7 indicating fat metabolism (Ramos-Jiménez et al. 2008). Despite FGF2 and FGFR1 expression in several hypothalamic areas including the ARC nucleus, the effects of FGF2 KO on the ARC nucleus and PVN remains to be explored.

The objective of this study is to elucidate the role of FGF2 on feeding and energy balance using male and female FGF2 KO mice exposed to a normal diet or a 60% high fat diet. If FGF2 is involved in suppressing food intake and maintaining body weight, we hypothesize FGF2 KO animals will have diminished metabolic homeostasis and increased carbohydrate expenditure and decreased energy expenditure in comparison to WT's. In addition, we hypothesize FGF2 KO animals will exhibit hyperphagia and weight gain.

**Table 1.** Summary of hypotheses

| **Weight** | | **Food Intake** | | **Carbohydrate Expenditure** | | **Energy Expenditure** | |
|---|---|---|---|---|---|---|---|
| WT | KO | WT | KO | WT | KO | WT | KO |
| - | ⬆ | - | ⬆ | - | ⬆ | - | ⬇ |

## Methodology

*Experimental animals*

We used 80 mice in total, 43 FGF2 KO mice and 37 black swiss wild-type mice (See Table 2). Two animals had a much higher body weight compared to the rest of their groups and were excluded from the study. All wild-type control mice were littermates that were bred at the University of Ottawa. We used both males and females. Mice were single housed in standard cages with nestlets and a plastic house for enrichment throughout the study. The mice were maintained on a 12-hour light/dark cycle (8am-8pm) in a temperature-controlled environment (21°C) with food and water available ad libidum. Animals were fed standard regular chow or

with 60% high fat diet (Bioserve, 3.93kcal/g). All animal use procedures were approved by the Carleton University Animal Care Committee, and followed the guidelines set by the Canadian Council for the Use and Care of Animals in Research.

**Table 2.** Summary of groups

| **Male WT CHOW** | **Female WT CHOW** | **Male WT HFD** | **Female WT HFD** | **Male KO CHOW** | **Female KO CHOW** | **Male KO HFD** | **Female KO HFD** |
|---|---|---|---|---|---|---|---|
| n=9 | n=9 | n=10 | n=9 | n=12 | n=11 | n=8 | n=12 |

*Genotyping*

Animals were ear-notched and polymerase chain reactions (PCR) were conducted to confirm the presence or absence of the FGF2 gene. Lysis buffer was first prepared in order to lyse the tissue. After samples were collected, 75ml of alkaline lysis buffer (30ml distilled water, 42mL of ETA and 50% NaOH) was added into sample tubes and the buffer was stored at a room temperature. Each sample were then heated for about 30 minutes at 95°C. Afterwards, 75 mL of 40mM Tris HCL was added into each tube and put on ice for about 15 minutes and stored in room temperature. Mastermix was made first before proceeding with the PCR protocol.
The mice were genotyped using custom primers ordered from Thermo Fisher Scientific. The sequence for the forward primer for the wild-type is CGAGAAGAGCGACCCACAC, while the reverse primer sequence is CCAGTTCGGGGGACCCTATT. The sequence for the forward primer for the FGF2KO (mutant) is CAAAGAACTTATAGCCCCCC, while the reverse primer

is TAGCGATGATGAACCAGG. Mastermix contained 6.25 µl of DREAM Mastermix. + 3.25 µl x n of H20 + 2µl of FGF primer (20ml forward, 20ml reverse, 60ml H20 stock). 11.5µl of mastermix was added to each PCR tube and 1µl of lysed DNA to the PCR tube. The tubes will were then spun down. Afterwards, the samples were transferred into a thermocycler with cycling conditions recommended by Jackson Laboratories (see Table 3).

**Table 3.** Cycling Conditions for PCR

| Step # | Temperature (°C) | Time | Note |
|---|---|---|---|
| **1** | 94 | 2 minutes | |
| **2** | 94 | 20 seconds | |
| **3** | 65 | 15 seconds | -0.5°C per cycle decrease |
| **4** | 68 | 10 seconds | |
| **5** | | | Repeat steps 2-4 for 10 cycles |
| **6** | 94 | 15 seconds | |
| **7** | 60 | 15 seconds | |
| **8** | 72 | 10 seconds | |
| **9** | | | Repeat steps 6-8 for 28 cycles |
| **10** | 72 | 2 minutes | |
| **11** | 10 | | hold |

After the cycling conditions, a 2% agarose gel (4g agarose added to 200ml of 1X TAE electrophoresis buffer) was prepared to run the samples and 2µl of ethidium bromide for every 100ml was added in order to stain and visualize the amplified gene expression of each animal

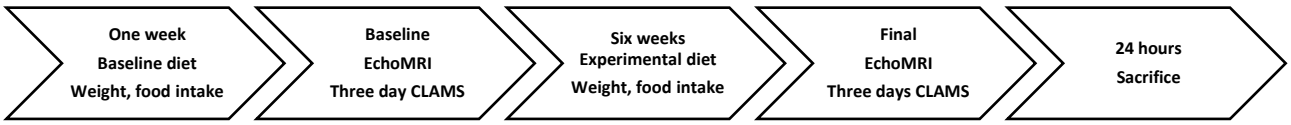
and confirm its genotype. Once mixed together, the gel was poured and left to set with loading wells in place. 12-13ul of samples was added into the gel wells. Each gel contained a positive and negative control in order to ensure there is no contamination. The electrophoresis was run between 20-30 minutes with voltage of about 120V.

*Weight, Food Intake and Metabolic Monitoring, Analysis of Metabolism*

We measured food intake and body weight throughout the experiment by using a plastic container and digital scientific scale (American Weigh Scales Model LB-3000 Digital Scale 3000 x 0.1g) . After single housing the animals for one week, baseline measurements of weight and food intake (standard regular chow) were taken. After one week, the animals were scanned with an ECHOMRI machine to obtain body fat and lean muscle mass composition using nuclear magnetic resonance. The mouse was placed in a tube, which was inserted into the reader for 30 seconds, and their body weight will be entered in the system. After the ECHOMRI scan, raw fat, lean muscle and weight in grams values was exported into a Microsoft excel file and the animals were transferred into the Comprehensive Lab Animal Monitoring System (CLAMS) for 72 hours. The CLAMS contained mashed regular chow and water and obtained readings such as volume of carbon dioxide (CO2) exhaled, volume of oxygen (VO2) inhaled, therefore, calculating the respiratory exchange ratio every 26 minutes. Further measurements were recorded such as beam breaks as an indication of locomotor activity along X, Y and Z axis. Each measurement per mouse was exported into a Microsoft excel spreadsheet. The first 48 hours were used to acclimatize the animals to their environment, and the final 24 hours was recorded. Animals were monitored daily once in the CLAMS.

After 72 hours, the animals were returned to their standard housing conditions. 24 hours after CLAMS, animals began their experimental diet. Half the animals received high fat diet (60% fat, Bioserve), while the other half continued to use regular chow for a period of 6 weeks. After 6 weeks, the animals received a final ECHOMRI scan and placed in the CLAMS system for 72 hours (See Table 4 for Timeline)

**Table 4.** Timeline of Behavioural Analysis




We characterized weight change by subtracting final body weight obtained on the last day of experimental diet from the baseline body weight. In general, weight was averaged and graphed over each week. Food intake was calculated by subtracting present day intake with previous day intake in Microsoft excel. Regular chow contained 2.90 kcal/g and high fat diet contains 3.93kcal/g, therefore, caloric intake was calculated by multiplying the value of food intake in grams by the number of calories specific to each diet. Caloric intake was calculated and averaged across each week, and total average caloric intake was calculated.

The ECHOMRI values was uploaded into Microsoft excel and the document contained the raw values of fat and lean muscle mass for each animal in grams. In order to calculate body fat composition by percentage, animals had their raw body fat value in grams divided by their weight and then multiplied by 100. Body fat composition change was calculated by the final

body fat composition percentage subtracted by baseline body fat composition percentage for each animal.

The CLAMS automatically calculated the respiratory exchange ratio by dividing the volume of carbon dioxide exhaled over the volume of oxygen inhaled. The respiratory exchange ratio (RER) is an indication of which pre-dominant fuel source is burned by the specific animal, with values closer to 1 indicating more carbohydrate metabolism, while values closer to 0.7 indicating fatter metabolism. The RER was graphed across the final 24 hours in the CLAMS with each reading taken approximately every 26 minutes. Average RER values was also calculated, as well as average RER values during the light and dark cycles. Energy expenditure was calculated and graphed across the last 24 hours from CLAMS and was defined as the amount of energy an individual need to carry out a physical function. In order to calculate energy expenditure in kcal/hr/kg, the amount of VO2 (ml/kg/hr) was multiplied by the caloric equivalent (kcal/l) and had appropriate unit conversion. Each RER has its own specific caloric equivalent in kcal/l and must be multiplied by the amount of VO2. (AACU, "Engaging…"). Average energy expenditure during the light and dark cycle was calculated.

The CLAMS automatically calculated the amount of beam breaks across the X-axis, we analyzed the total X-axis ambulatory counts at baseline and after experimental diet. In addition, we calculated the amount of X-axis ambulatory counts during the light and dark cycle. We also graphed the amount of x-ambulatory counts across 24 hours. Due to technical issues with the CLAMS system, 12 animals were excluded for all CLAMS measurements such as RER, energy expenditure and ambulatory counts.

*Animal Sacrifice and Tissue Analysis*

Upon completion of the experiment, 24 hours after they are placed in the CLAMS system, the animals were sacrificed. Half of the animals were given an overdose of 44mg/kg sodium pentobarbital . As soon as it was determined the animal was not sensitive to painful stimulation, the chest was opened, and the animal was perfused with 0.1M PBS followed by cold 4% paraformaldehyde. After the brains were extracted, they were placed in vials containing 30% sucrose, 4% paraformaldehyde solution for 48 hours. The brains were then wrapped individually in aluminum foil and stored into a -80°C freezer and later sectioned on a Fisher cryostat (40uM thick). Five sister sections were obtained from each brain sample, and the sections were placed in PCR tubes containing 1mL of glycerol and 0.1M PBS based cryoprotectant and stored in -20 °C. Tubes from each sister section contained 1mL of cryoprotectant (15g sucrose, 15mL glycerol, 25mL 0.1M PBS stock solution). The sections will then be used for future immunohistochemistry analysis.

The remaining half of the animals were decapitated with brains and trunk blood extracted for future potential hormonal analysis (i.e. leptin levels as measured by ELISA in order to determine hormone level responsivity). During decapitation, trunk blood was collected in Eppendorf tubes containing 10µl of Ethylenediaminetetraacetic acid (EDTA) to prevent blood clotting. The tubes were centrifuged in order to separate the serum. The serum was pipetted out and placed into another Eppendorf tube and then flash frozen and stored in -80°C. The decapitated brains were individually wrapped with aluminum foils and stored in -80°C.

*Statistical Analyses*

All data was analyzed using IBM SPSS Statistics Data Editor. The independent variables will be genotype, sex, diet and time and the dependent variables will be weight, caloric intake, % body fat composition based on weight, RER values, energy expenditure and total ambulatory counts. Analysis of variance was conducted with genotype (WT vs. FGF2KO), sex (male, female) and diet (regular chow vs. high fat diet) for weight change, % body fat composition based on weight, change in body fat %, and total ambulatory counts. Repeated measures analysis was conducted for, weight gain, caloric food intake, RER values over time, energy expenditure over time and ambulatory counts over time. If overall model was significant, then ANOVAs were followed two tailed t-test comparisons assuming unequal variance. Probability values will be considered significant when $p \leq 0.05$. Simple effects using excel were conducted if two-way interactions were obtained.

**Results**

*Weight Gain and Change Results*

In the first analysis, we compared the baseline weight of WT and FGF2KO males and females before the six weeks of experimental diet (Figure 1). There was a significant sex difference ($F_{(1,76)}$=55.487, p<0.001) such that males had higher body weights compared to females, however, no significant main effect of genotype was observed ($F_{(1,76)}$=0.119, p=0.731). Because there were significant sex differences in many of the measures at baseline such as weight and body fat composition, we conducted the weekly average weight after six

weeks of experimental diet separately in males and females and investigated the effects of genotype and diet.

In males (Figure 2A), there was a significant interaction of week by diet ($F_{(6,210)}$=37.213, p<0.001). As expected, males on HFD had higher body weights compared to those fed on regular chow on weeks 2-6 (Week 2:$t_{(37)}$=2.658, p=0.013; Week 3: $t_{(37)}$=3.547, p=0.001; Week 4: $t_{(37)}$=3.892, p=0.01; Week 5: $t_{(37)}$=4.093, p<0.01; Week 6: $t_{(37)}$=3.982, p=0.01).

In females (Figure 2B), there was a significant interaction of week by diet observed ($F_{(6,216)}$=8.320, p<0.001). Not surprisingly, females fed on HFD had higher body weights compared to those fed on regular chow at weeks 3-6 (Week 3: $t_{(39)}$=3.868, p<0.01; Week 4: $t_{(39)}$=3.997, p<0.001; Week 5: $t_{(39)}$=3.929, p<0.001; Week 6: $t_{(39)}$=3.535, p=0.001).

In order to analyze the amount of weight change before and after the experimental diet, we calculated the weight from the final week of experimental diet and subtracted it by the baseline week and compared the average (in grams) across all groups (Figure 3). In males (Figure 3A), there was a significant main effect of diet ($F_{(1,35)}$=66.236, p<0.001) where males fed with high fat diets had increased changes in body weight compared to chow ($t_{(37)}$=7.835, p<0.001), no effect of genotype was observed ($F_{(1,35)}$=0.031, p=0.861).

Interestingly, in females (Figure 3B), there was a significant interaction of diet and genotype ($F_{(1,37)}$=5.459, p=0.025). Female KO mice fed on HFD showed greater weight gain then their regular chow-fed counterparts (p<0.001), while this effect was smaller in WT females with only a trend for significance (0.0819) Moreover, KO females showed less weight-change then their WT counterparts when fed regular chow (p=0.01495).

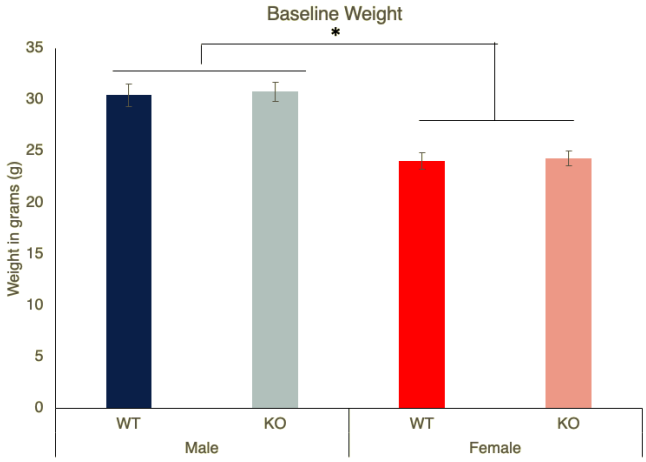



**Figure 1. Baseline weight across all groups before experimental diet.** Graphical representation of baseline weight in grams across male and female WT and FGF2KO's. Bars represent group means and error bars represent +- SEM. There were sex differences such that males overall have higher body weights compared to females. Asterisk denote sex differences ($p<0.001$).

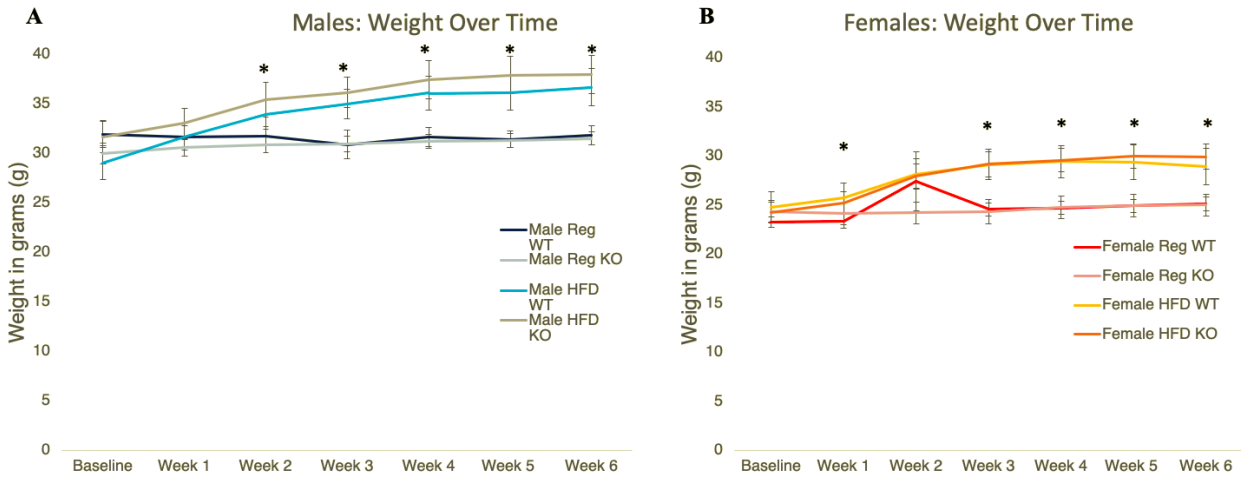



**Figure 2. Weekly average weight gain in grams (g) across weeks.**
Graphical representation of weight in grams from baseline week to 6 weeks of experimental diet. Weight was measured daily. Lines represent group means and error bars represent +- SEM. A) Represents the data in males. There was a significant interaction of week by diet where males fed on HFD had higher body weights compared to their chow counterparts. B) Represents the data in females. There was a significant interaction of week by diet where females fed on HFD had higher body weighs compared to their chow counterparts on weeks Asterisks denote significant main effect of diet at specific time points ($p<0.05$).

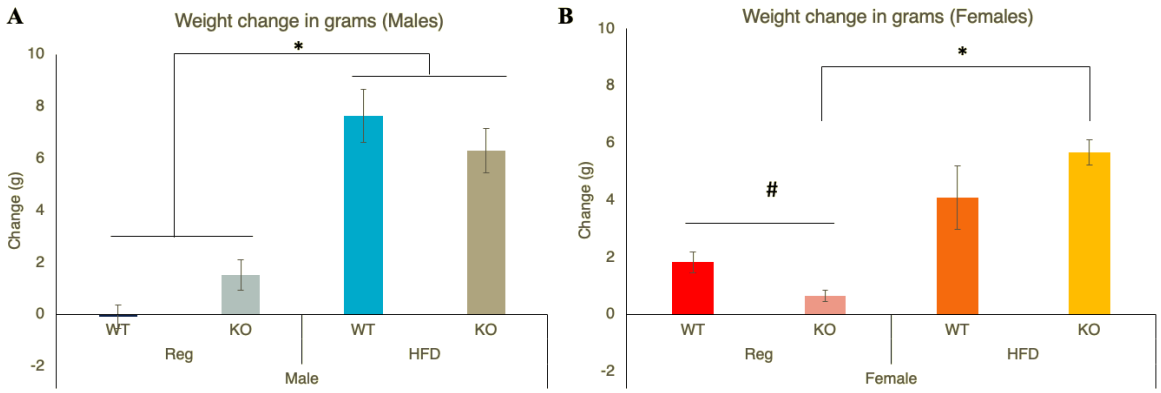



**Figure 3. Change in weight in grams (g) from final experimental week to baseline week.**
Graphical representation of change in weight in grams across all eight groups. Weight from week six subtracted by baseline weight. Bars represent group means and error bars represent +- SEM. A) Represents the data in males, asterisk denote significant main effect of diet ($p<0.001$). B) Represents the data in females, there was a significant interaction of diet and genotype. Asterisk denotes significance of $p<0.05$, KO female HFD animals had increased weight change compared to their chow counterparts. # denotes significance of $p<0.05$, KO's on chow had less weight change compared to their chow counterparts.

*Caloric Intake Results*

Since weight is largely influenced by the balance of energy intake and energy expenditure, we examined the baseline caloric intake across all groups. Weight in grams was initially measured and multiplied by its respective caloric amount in order to calculate calories. Because there were significant sex differences in baseline weight, we analyzed caloric intake in males and females separately.

In males (Figure 4A), there was a significant interaction of week by diet ($F_{(6,210)}$=10.229, p<0.001) where 2-tailed t-tests showed that males fed on HFD had higher caloric intake compared to males fed on chow for week 1 of experimental diet ($t_{37)}$=2.841, p=0.010). However, this was reversed on weeks 3-6, where males on chow had higher caloric intake then their HFD counterparts (Week 3: $t_{37)}$=3.995, p<0.001; Week 4: $t_{37)}$=3.690, p<0.01; Week 5: $t_{37)}$=3.199, p=0.004; Week 6: $t_{37)}$=2.837, p=0.008).

In females, there was a similar significant interaction of week by diet ($F_{(6,216)}$=17.203, p<0.001) and week by genotype ($F_{(6,222)}$=2.488, p=0.024). In the analysis for time and diet, females on HFD had higher caloric intake only at week 1 ($t_{(39)}$=4.129, p<0.001) and this was reversed from Week 2 onwards such that females on chow had higher caloric intakes (Week 2: $t_{(39)}$= 2.060, p=0.046; Week 3: $t_{(39)}$=3.039, p=0.04; Week 4: $t_{(39)}$=2.198, p=0.037; Week 5: $t_{(39)}$=6.130, p<0.001; Week 6: $t_{(39)}$=4.353, p<0.001).

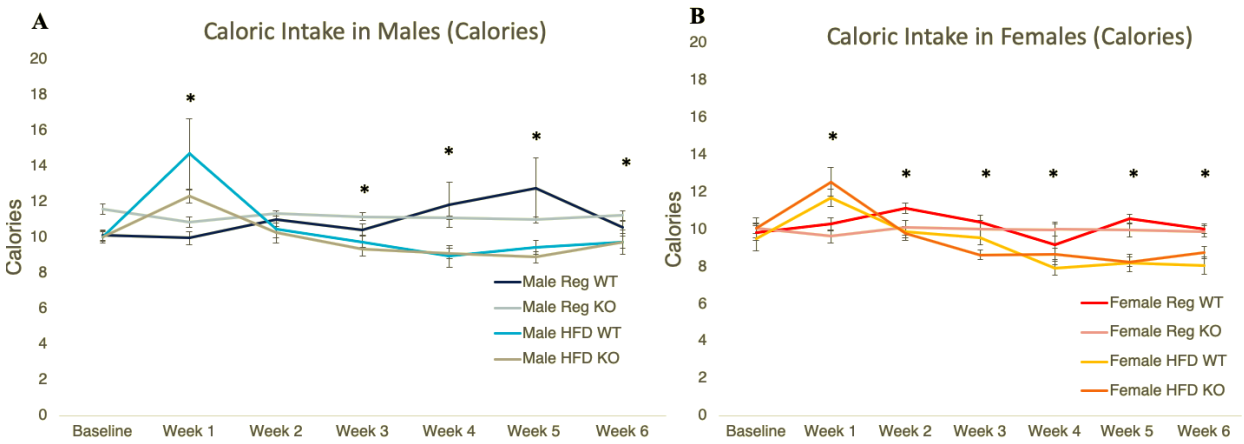



**Figure 4. Weekly Caloric Intake in all groups (regular chow= 2.9kcal/g, high fat diet= 3.93kcal/g).**
Each bar represent group means and error bars represent +-SEM. Food intake was measured daily. A) Represents the data in males. There was a significant interaction of time by diet ($p<0.001$). Asterisks denote significant main effect of diet at specific time points ($p<0.05$). B) Represents the data in females. There was a significant interaction of time by diet. Asterisks denote significant main effect of diet at specific time points ($p<0.05$).

*EchoMRI Results*

Because weight alone does not examine body composition, we examined the amount of body fat using EchoMRI. To truly calculate the body composition of an animal, it is necessary to calculate the of body fat given an animal's weight. Therefore, the amount of body fat in grams is divided by body weight multiplied by 100%.

First, we examined baseline body fat composition before six weeks of experimental diet (Figure 5) and found overall, there was a significant main effect of genotype ($F_{(1,76)}$==4.545, p=0.036), however these were not significant at the level of simple comparisons ($t_{(78)}$=1.698, p=0.094). There were also significant sex differences ($F_{(1,76)}$=23.291, p<0.001) where males had greater body fat compared to females ($t_{(78)}$=4.648, p<0.001).

After six weeks of experimental diet, we examined final body fat separately in males and females (Figure 6). In males (Figure 6A), there was a significant main effect of diet ($F_{(1,35)}$=84.523, p<0.001) where high fat diet fed animals had increased body fat composition ($t_{(37)}$ =8.991, p<0.001). Similarly, in females (Figure 6B), there also was a significant main effect of diet ($F_{(1,37)}$=51.747, p<0.001) where high fat diet fed animals had increased body fat composition ($t_{(34.984)}$=7.428, p<0.001). No effects of genotype were found in either males or females (Males: $F_{(1,35)}$=2.361, p=0.133; Females: $F_{(1,37)}$=1.484, p=0.231).

Change in body fat composition based on weight in percentage was also calculated by final body fat composition/weight subtracted by baseline/weight x 100% (Figure 7). When analyzing males and females separately, males (Figure 7A) had a significant interaction of diet by genotype ($F_{(1,35)}$=4.273, p=0.046), however these were not significant at the level of simple

comparisons (male KO HFD vs male WT HFD p=0.211; male KO chow versus male WT chow p=0.116), suggesting small effect size. In females (Figure 7B), there was a significant main effect of diet ($F_{(1,37)}$=74.142, p<0.001, where high fat fed animals had increased changes than chow fed animals ($t_{(39)}$=8.828, p<0.001).

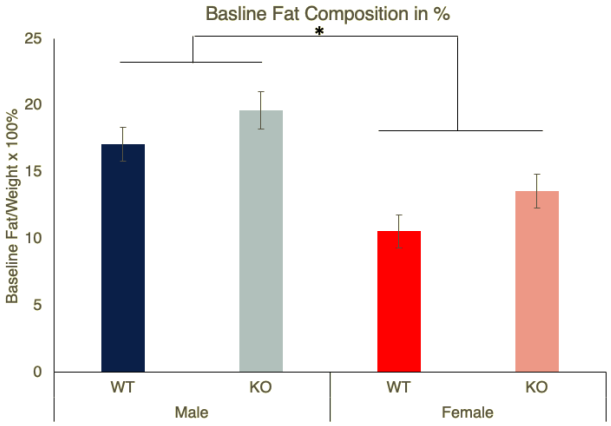



**Figure 5. Baseline fat composition in % based on weight.** Graphical representation of baseline fat composition (grams of fat divided by body weight multiplied by 100%. Bars represent group means and error bars represent +- SEM. There was a significant sex difference in which males overall had higher body fat composition compared to females. Asterisk denotes significant differences compared to female counterparts ($p<0.001$).

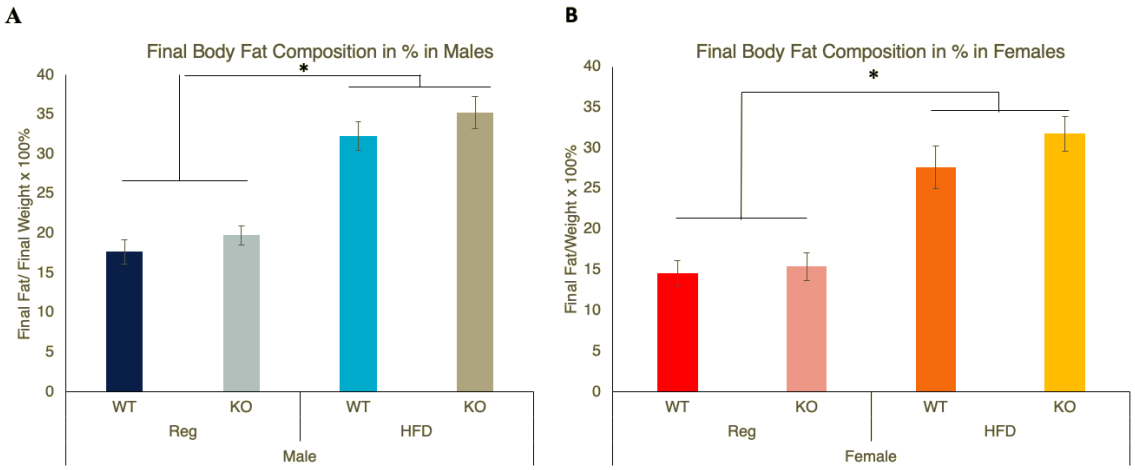



**Figure 6. Final body fat composition in % based on weight (Fat in grams/body weight x 100%)**
Graphical representation of final body fat composition in % after 6 weeks of experimental diet across all groups Bars represent group means and error bars represent +- SEM. There was a significant main effect of diet, where HFD animals had higher body fat composition than their chow counterparts in both males and females. A) Represents the data in males. Asterisks denotes main effect of diet ($p<0.05$). B) Represents the data in females. Asterisk denote main effects of diet $p<0.05$).

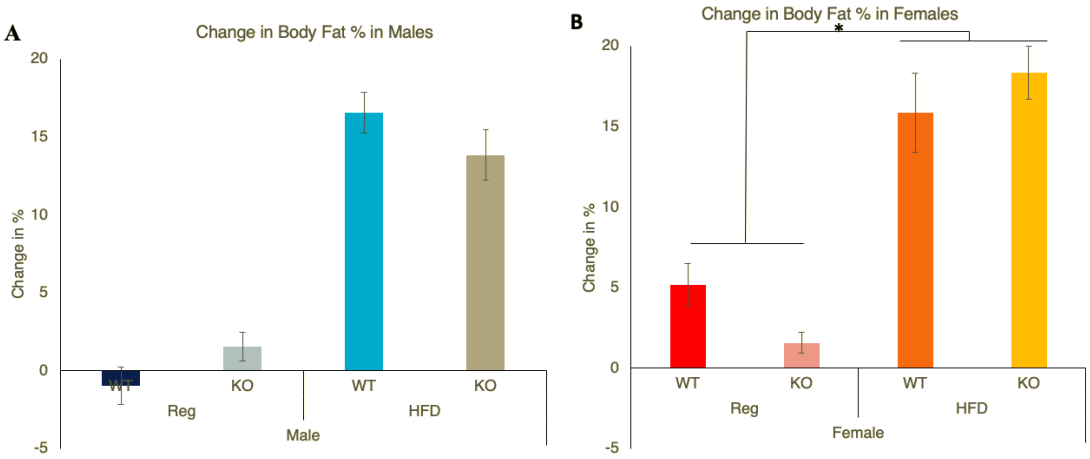



**Figure 7. Final body fat composition in % based on weight (Fat in grams/body weight x 100%)**
Graphical representation of final body fat composition in % after 6 weeks of experimental diet across all groups Bars represent group means and error bars represent +- SEM. A) Represents the data in males. There was a significant interaction between diet and genotype ($p=0.046$), however, these were not significant at the level of simple comparisons. B) Represents the data in females. There was a significant main effect of diet, where HFD females had higher body fat composition than their chow counterparts. Asterisk denote main effects of diet $p<0.05$).

*CLAMS: Metabolic Processes Results*

Respiratory exchange ratio (RER) is an important component of energy expenditure as it is an indicator of the predominant fuel source (fats or carbohydrates) at a given time by an animal. We examined baseline respiratory exchange ratio in male and female WT and FGF2KO animals across 24 hours in CLAMS.

The results (Figure 8A) showed overall, there was a significant interaction of time and sex ($F_{(55,3575)}$=1.944, p<0.001) where males showed higher RERs then females. In order to represent the data an alternative way, we graphed the average RER in 6-hour blocks (Figure 8B).

After establishing the baseline RER, we analyzed the final RER across 24 hours after six weeks of experimental diet (Figure 9A). There was a significant interaction of time by genotype in males ($F_{(55,1485)}$=1.563, p=0.006), where all mice fed HFD had a significantly higher RER then their regular chow-fed counterparts, however, during the day, HFD KO animals had significantly lower RERs compared to their chow counterparts (p=0.007) and WTs on chow (p=0.023), but there were no significant differences when compared to HFD WT's (p=0.310) and this is highlighted in Figure 9B and 9C, which shows the average RER over day and night, respectively. In females, there was a significant interaction of time and diet in females (Figure 10A, $F_{(55,1870)}$=1.924, p<0.001) where those fed on regular chow had higher RER values compared to their chow counterparts, however these were not significant at the level of simple comparisons, Figure 10B is a representation of the data average by day and night.

Using RER values, we analyzed energy expenditure for each animal. First, we examined baseline energy expenditure across 24 hours (Figure 11A). Our results showed there was a significant interaction of time by sex ($F_{(55,3575)}$ =2.580, p<0.001) in which females had higher

energy expenditure compared to males during the light cycle. Figure 11B shows baseline energy expenditure averaged into 6-hour blocks.

Next, final energy expenditure after 6 weeks experimental diet, was analyzed (Figure 12A) and overall, there was only a main effect of time ($F_{(55,1485)}$=13.354, p<0.001) where energy expenditure was greater during the dark cycle. Figure 12B represents the energy expenditure averaged into 6-hour blocks. Similarly, in females (Figure 13A), there was also a significant main effect of time ($F_{(55,1870)}$=13.339, p<0.001). Figure 13B represents the energy expenditure averaged into 6-hour blocks.

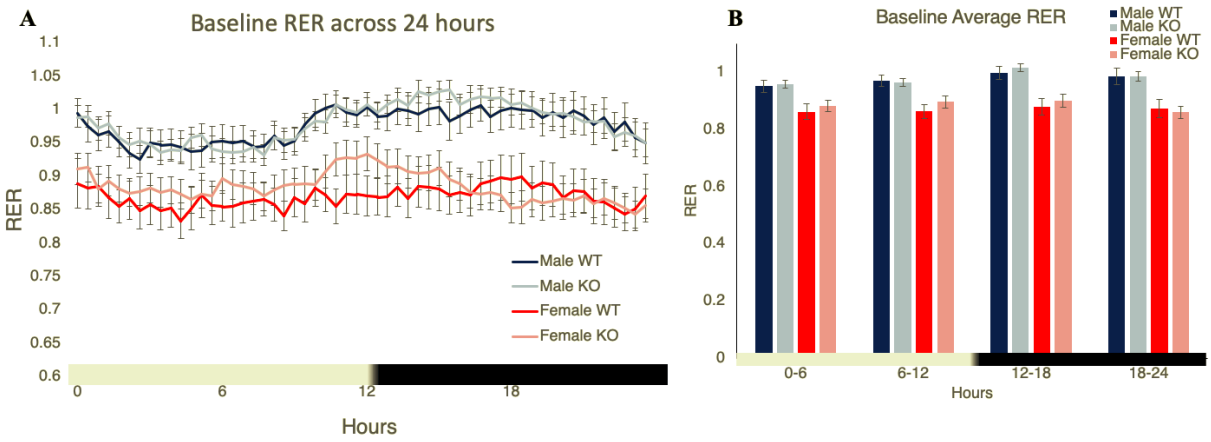



**Figure 8. Baseline respiratory exchange ratio (VCO2/VO2) ratio across 24 hours.**
Graphical representation of baseline respiratory exchange ratio (RER) across 24 hours in the final day of CLAMS in males and female WT's and KO's. Readings were taken approximately every 26 minutes. A) Each point represents group means at every time point across 24 hours and error bars represents +- SEM. The gradient bar represents time of day. There was a significant interaction of time by sex ($p<0.001$). B) Each bar represents group means at specific time intervals, error bars represents +-SEM.

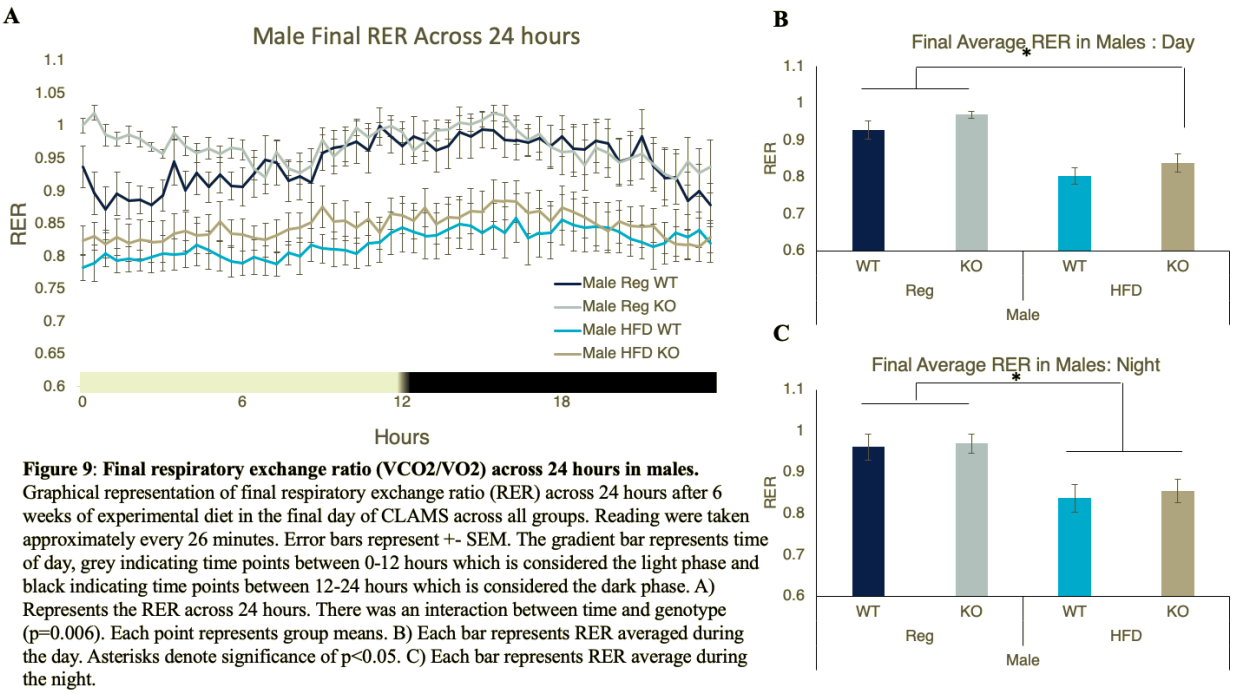



**Figure 9**: **Final respiratory exchange ratio (VCO2/VO2) across 24 hours in males.** Graphical representation of final respiratory exchange ratio (RER) across 24 hours after 6 weeks of experimental diet in the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. Error bars represent +- SEM. The gradient bar represents time of day, grey indicating time points between 0-12 hours which is considered the light phase and black indicating time points between 12-24 hours which is considered the dark phase. A) Represents the RER across 24 hours. There was an interaction between time and genotype ($p=0.006$). Each point represents group means. B) Each bar represents RER averaged during the day. Asterisks denote significance of $p<0.05$. C) Each bar represents RER average during the night.

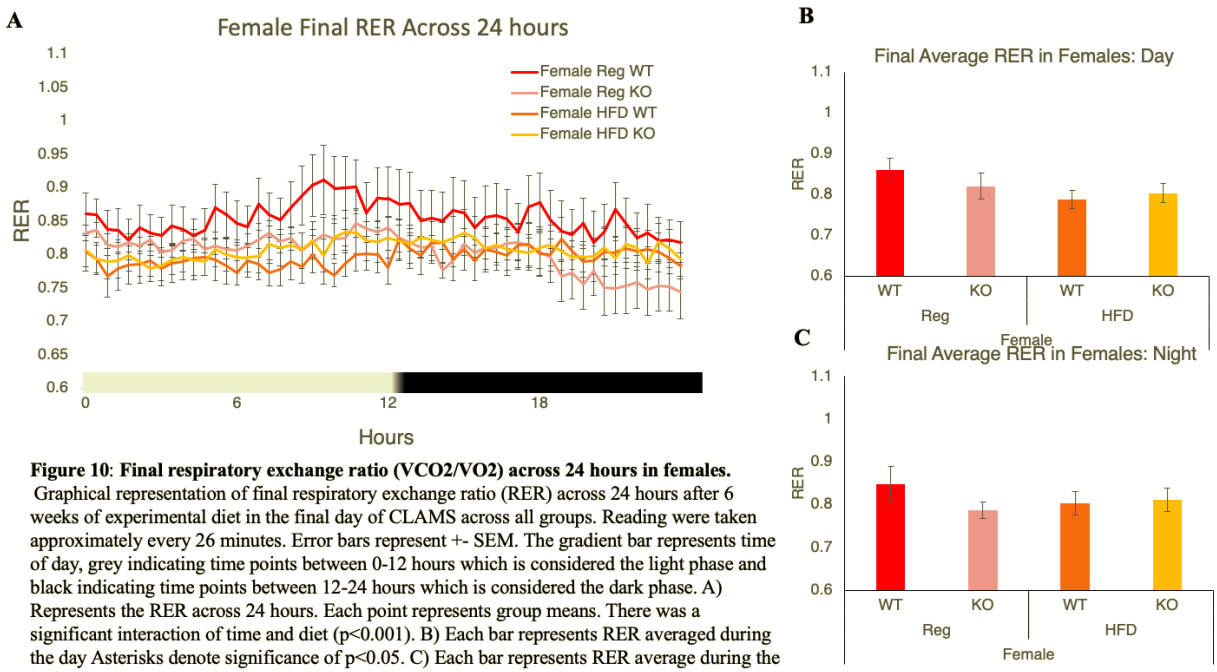



**Figure 10**: **Final respiratory exchange ratio (VCO2/VO2) across 24 hours in females.** Graphical representation of final respiratory exchange ratio (RER) across 24 hours after 6 weeks of experimental diet in the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. Error bars represent +- SEM. The gradient bar represents time of day, grey indicating time points between 0-12 hours which is considered the light phase and black indicating time points between 12-24 hours which is considered the dark phase. A) Represents the RER across 24 hours. Each point represents group means. There was a significant interaction of time and diet ($p<0.001$). B) Each bar represents RER averaged during the day Asterisks denote significance of $p<0.05$. C) Each bar represents RER average during the night.

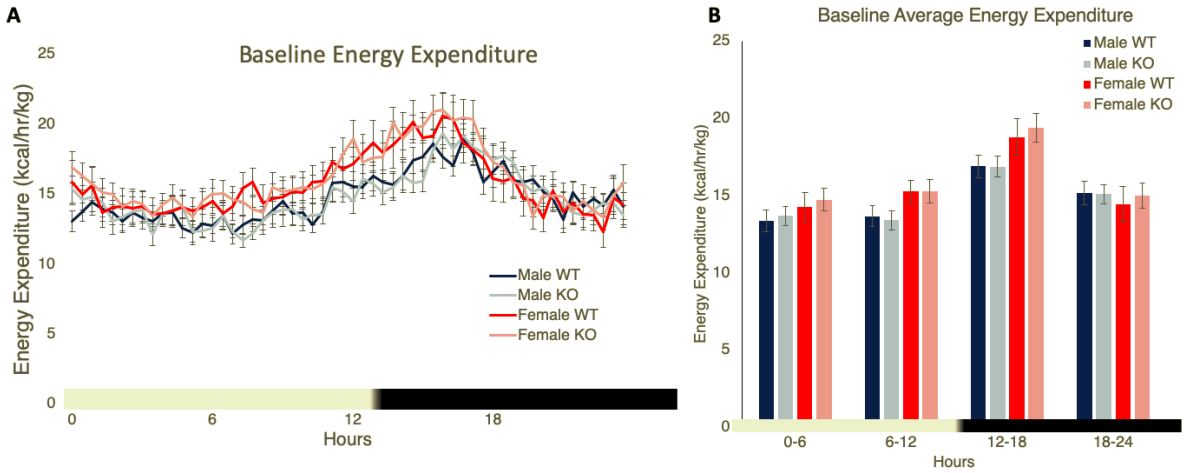



**Figure 11**: **Baseline energy expenditure (kcal/hr/kg) across 24 hours.**
Graphical representation of baseline energy expenditure across 24 hours before 6 weeks of experimental diet in the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. Error bars represent +- SEM. The gradient bar represents time of day, grey indicating time points between 0-12 hours which is considered the light phase and black indicating time points between 12-24 hours which is considered the dark phase. There was an interaction of time by sex. A) Represents the energy expenditure across 24 hours. Each point represents group means. B) Each bar represents energy expenditure averaged in 6 hour intervals.

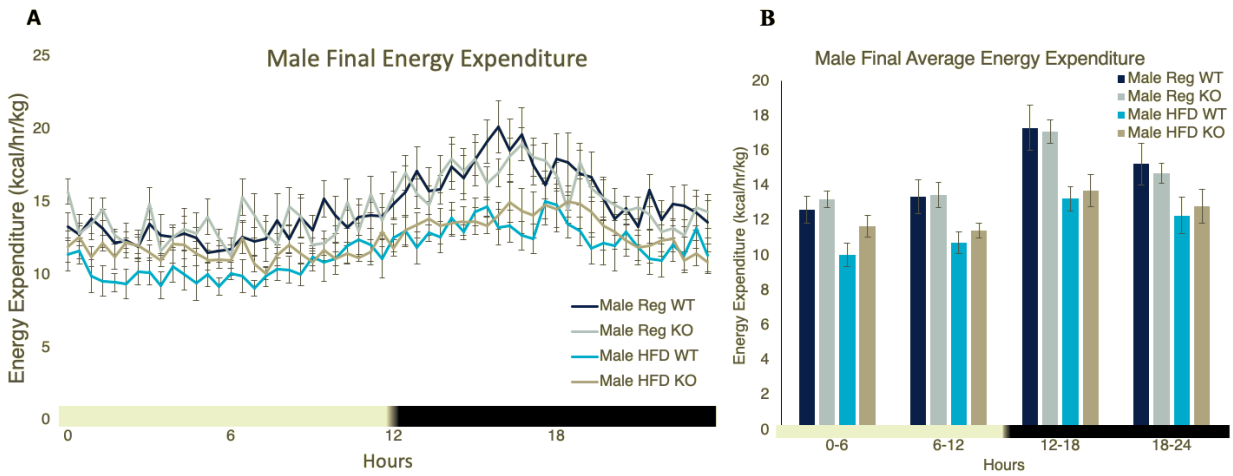



**Figure 12**: **Final energy expenditure (kcal/hr/kg) across 24 hours in males.**
Graphical representation of baseline energy expenditure across 24 hours after 6 weeks of experimental diet in the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. Error bars represent +- SEM. The gradient bar represents time of day, grey indicating time points between 0-12 hours which is considered the light phase and black indicating time points between 12-24 hours which is considered the dark phase. There was a significant main effect of time ($p<0.001$). A) Represents the energy expenditure across 24 hours. Each point represents group means. B) Each bar represents energy expenditure averaged in 6 hour intervals.

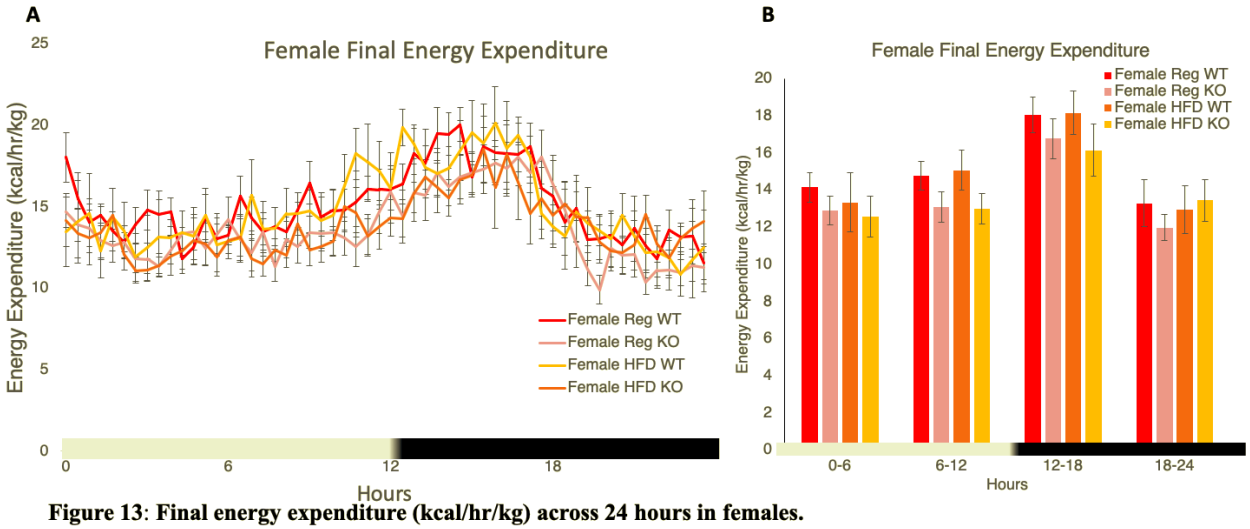



**Figure 13**: **Final energy expenditure (kcal/hr/kg) across 24 hours in females.**
Graphical representation of baseline energy expenditure across 24 hours after 6 weeks of experimental diet in the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. Error bars represent +- SEM. The gradient bar represents time of day, grey indicating time points between 0-12 hours which is considered the light phase and black indicating time points between 12-24 hours which is considered the dark phase. There was a main effect of time ($p<0.001$). A) Represents the energy expenditure across 24 hours. Each point represents group means. B) Each bar represents energy expenditure averaged in 6 hour intervals.

*Ambulatory Counts*

Ambulatory movement is also an important component of energy expenditure. Since FGF2 has also been previously implicated in depressive- like and anxiety like behaviour, we analyzed the ambulatory movement along the X-axis using bream breaks while the animals were in the CLAMS. First, we analyzed their baseline total ambulatory counts over 24 hours (Figure 14A) and found no significant differences (Sex: $F_{(1,63)}$=3.212, p=0.078, Genotype: $F_{(1,63)}$=2.024, p=0.160). When analyzing the data across 24 hours at baseline (Figure 14B), there was a significant interaction of time and genotype ($F_{(55,3465)}$ =1.372, p=0.036). Visibly, female KO's had higher ambulatory counts during the dark cycle.

Next, we analyzed the final total ambulatory counts across all groups in males and females after six weeks of experimental diet (Figure 15A and Figure 16A) and found overall, there was no significant differences across all groups. Finally, we analyzed final ambulatory counts across 24 hours (Figure 15B), and not surprisingly, there was only a significant main effect of time in males ($F_{(1,55)}$=4.745, p<0.001) such that males had higher ambulatory counts during the dark phase. In females, there was a significant interaction of time by genotype (Figure 16B, $F_{(1,55)}$=1.376, p=0.036) by which female KO's had higher ambulatory counts than their WT counterparts during each timepoint.

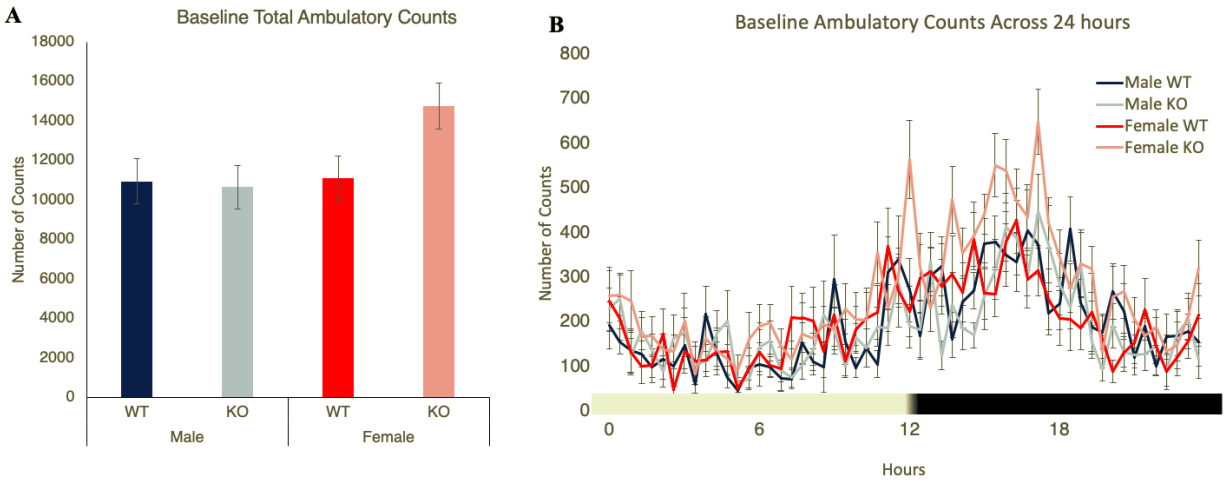



**Figure 14: Baseline total ambulatory counts across 24 hours recorded by amount of ambulatory X axis beam breaks during final day of CLAMS.**

Graphical representation of total ambulatory counts before experimental diet during the final day of CLAMS across all groups. Reading were taken approximately every 26 minutes. A) Represents the total ambulatory counts in 24 hours. Each bar represent group means and error bars represent +-SEM. B) Represents the total ambulatory counts across 24 hours, each point represents group means. There was only a significant main effect of time ($p<0.001$).

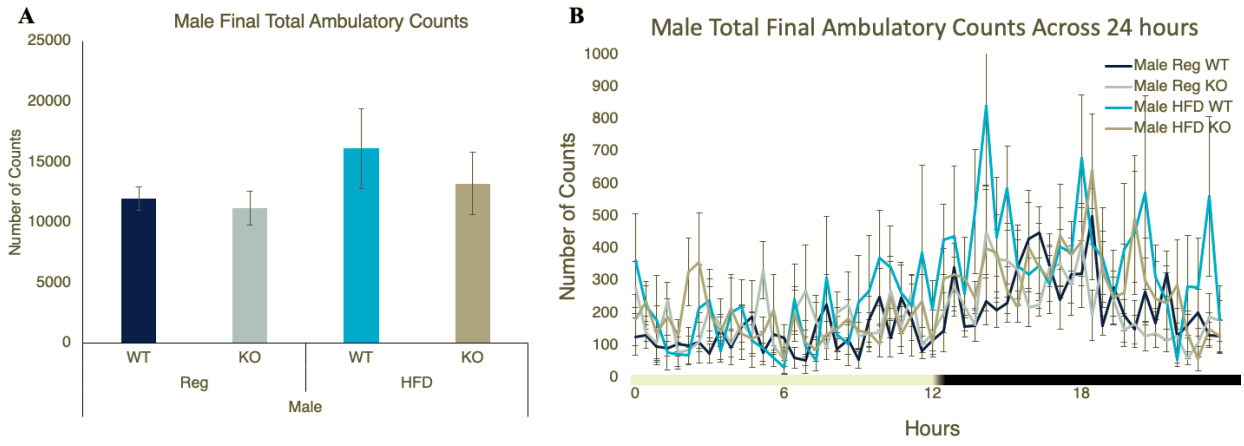



**Figure 15. Total final ambulatory counts across 24 hours recorded by amount of X axis ambulatory beam breaks in males after 6 weeks of experimental diet.**

Graphical representation of total final ambulatory counts. Each measurement was taken approximately every 26 minutes. A) Represents the sum total ambulatory counts. Each bar represents group means and error bars represent +- SEM. B) Represents the ambulatory counts across 24 hours. Each point represents group means. There were no significant differences observed.

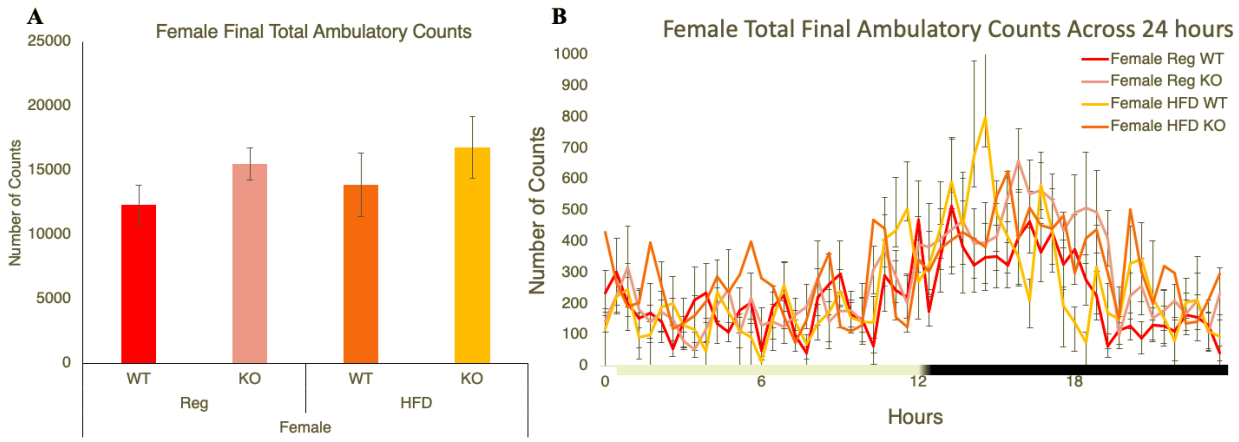



**Figure 16. Total final ambulatory counts across 24 hours recorded by amount of X axis ambulatory beam breaks by in females after 6 weeks of experimental diet.**
Graphical representation of total final ambulatory counts. Each measurement was taken approximately every 26 minutes. A) Represents the sum total ambulatory counts. Each bar represents group means and error bars represent +- SEM. B) Represents the ambulatory counts across 24 hours. Each point represents group means. There was a significant interaction of time by genotype (p=0.026).

## Discussion

The objective of this study was to elucidate the role of FGF2 on feeding and energy balance using male and female FGF2 KO mice exposed to a typical chow diet or a 60% high fat diet. Earlier studies have shown that FGF2 administration and blockade of FGF2 and its receptors with established roles in energy balance are involved in suppressing food intake and maintaining body weight (Matsuo et al. 1994; Sasaki et al. 1994; Denton et al. 1995; Li et al. 1996; Hotta et al. 2001). Therefore, this study initially hypothesized that animals lacking the functional gene for FGF2 would exhibit weight gain and hyperphagia and exhibit changes in metabolism and energy expenditure in comparison to their WT littermates. Furthermore, we hypothesized that these effects would be augmented with a high fat diet and that there would be sex differences.

As expected, the 60% high fat diet was able to induce greater weight gain in both WT's and KO's compared to control chow fed mice. However, FGF2KO mice did not exhibit augmented weight or body fat changes, increased caloric intake or have decreased energy expenditure compared to WT mice in response to the high fat diet as originally hypothesized. Both male and female WT and KO's had increased body weight and exhibited similar changes in body weight on high fat diets compared to their chow counterparts.

Indeed, as hypothesized, there were also sex differences in baseline weight and body fat composition, such that males had increased body weights and body fat composition compared to females. Interestingly in females, WT's on chow had higher body weight changes compared to KO's. Both male and female WT's and KO's ate less calories when consuming high fat diet for majority of the study compared to their chow counterparts. This is surprising, since high fat diets are highly palatable, and multiple rodent studies have demonstrated that those fed with HFD ad

libitium have increased caloric intake compared to regular chow groups (Winzell & Ahrén 2004; Melhorn et al. 2010; Licholai et al. 2018).

When analyzing the metabolic processes such as energy expenditure and respiratory exchange ratio, sex differences and the effects of FGF2KO were more prominent. For instance, after experimental diet, male KO's had higher carbohydrate expenditure compared to WT counterparts. Originally, it was hypothesized KO's would have increased carbohydrate expenditure and decreased energy expenditure compared to WT's. Females only had increased carbohydrate expenditure on regular chow compared to HFD as expected, but there were no significant effects of genotype observed. Furthermore, there were no significant differences observed in the analysis of energy expenditure. Regardless, our baseline results of energy expenditure do not support current literature in which females generally tend to have less energy expenditure compared to males (Lovejoy et al. 2009). However, our results do show that the females weigh less compared to males at baseline (Lovejoy et al. 2009). Future studies should include running wheels for animals in CLAMS to measure physical activity will be pivotal to thoroughly explore energy expenditure in depth. Despite this, there were also prevalent sex differences in the ambulatory analysis. For instance, female KO's had increased baseline ambulatory predominantly during the night phase when they are most active. After experimental diet when analyzing across 24 hours, an interaction of time by genotype was again visualized in female KO's where they had increased ambulatory counts similar to baseline. It is possible this increase contributed to our results of the increase in energy expenditure at baseline in females.

A limitation to our study was the duration and age. We used all adult animals with variable ages on the 60% high fat diet for six weeks. However, it is possible six weeks on high fat diet was not a sufficient challenge for these animals. Therefore, subsequent studies should

aim to use a high fat diet for a longer duration to produce a greater challenge and appropriately age match. Another limitation to our study is our FGF2KO mutant model given that it is a constitutive KO. Previous studies have shown that FGF2 plays a major role in neuronal cellular proliferation and central nervous system development (Dono et al. 1998, Raballo et al. 2000). However, Dono et al. 1998 reports that in FGF2KO embryos, proliferation of neuronal progenitors are normal, but a fraction of them are unable to colonize their target layers in the cerebral cortex, suggesting the possibility that FGF2KO's are functionally compensated by other FGFs in vivo or does not function as a mitogenic signal for neural progenitors (Dono et al. 1998). Therefore, there may be compensatory mechanisms such as the upregulation of other metabolic growth factors (Such as FGF1, FGF21) which may have directly affected the results of the present study. Future studies should then aim to investigate a direct KO of FGF2 in adult animals using anti-FGF2, using inducible effects such as using a viral ShRNA or other uses of silencing the mRNA for FGF2 in adulthood and appropriately age match the animals.

Another broader area future studies should investigate is the metabolic and energy homeostatic effects of FGF2 in stressed models and to carefully examine areas involved in HPA activity on feeding behaviour and metabolic and consider the use of FGF2 administration to further elucidate metabolic effects. Our data has shown ambulatory counts were increased in female KO's. Salmaso et al. 2016 has shown FGF2 KO animals revealed elevated basal serum levels of corticosterone and exhibited an increased number of corticotropin releasing hormone immunoreactive neurons in the paraventricular nucleus of the hypothalamus, suggesting overactivity of the HPA-axis. For FGF2 to exert its therapeutic effects on anxiety-like behaviours in FGF2 KO models, glucocorticoid receptors must be available, suggesting FGF2's

role in the regulation of HPA-axis and it's possible interactions with regulating cortisol activity (Salmaso et al. 2016).

Moreover, it would be interesting to investigate anxiety-like behaviours when FGF2 KO's are on HFD's and/or stressed. In Salmaso et al's study, FGF2 KO male animals showed an increased latency to enter open arms, and a decreased percentage of time spent in open arms which is indicative of an increase of anxiety like behaviour compared to WT's. It would be interesting to explore further anxiety-like and behavioural analysis while also investigating sex differences, as our results have found that FGF2KO females had increased ambulatory counts, and it will be important to examine the effects of HFD to investigate the role of cortisol.

Overall, cortisol is a catabolic hormone that is involved with increasing availability of the prominent fuel sources for the body such as glucose, lipids and amino acids via mitochondrial oxidation and may increase energy expenditure and decrease muscle mass (Christiansen et al. 2007; Macfarlane et al. 2008). Cortisol also have been shown to increase appetite and food intake, and may increase fat mass while also inducing insulin resistance, therefore contributing to obesity and other metabolic disorders (Christiansen et al. 2007; Macfarlane et al. 2008).

Epidemiological and meta-analytical studies have demonstrated that obesity, mood and anxiety disorders are comorbid, and has exhibited obese participants showed an increased risk of developing mood and anxiety disorders or vice versa, in which individuals with depression or anxiety showing an increased risk for developing obesity (Simon et al. 2006; Gariepy et al. 2009; Luppino et al. 2010). Furthermore, obesity, major depressive disorder and anxiety disorders are all associated with alterations in the HPA axis. (Nestler et al. 2002; Jacobson et al. 2014; Dieleman, et al. 2015, Rodriguez et al. 2015). Therefore, future studies should aim to investigate

the brain regions that are involved with HPA activity, FGF2 and feeding when looking at animals on HFD.

The stress response and energy balance share similar circuitry in the brain, specifically revolving the paraventricular nucleus of the hypothalamus. It is well known that the arcuate nucleus of the hypothalamus is a main integrative center to regulate energy balance and expenditure, and it is also involved with second order projections to the paraventricular nucleus Elmquist et al. 1998; Timper et al. 2017) For instance, the stress response initiates with release of corticotropin-releasing factor from the paraventricular nucleus, a region that is also responsible for controlling food intake (Krahn et al. 1986; Heinrichs et al. 1993; Smith et al. 2006). In addition, multiple studies have linked corticotropin releasing factor as both an initiator and suppressor of food intake, mimicking how the body adapts to acute and chronic stress (Dallman et al. 2003; Dallman et al. 2006; Rabasa et al. 2016).

Stress has significant effects on metabolism, specifically in terms of weight and food intake, However, it is difficult to generalize the direction in both humans and animals, given individual differences and the degree of severity and frequency of the stressors (Weinstein et al. 1997; Dallman et al. 2003; Dallman et al. 2006; Sominisky et al. 2014; Rabasa et al. 2016). For instance, many models of acute stress in rodents exhibited instances of hypophagia and weight loss whereas chronic stress models have revealed instances of hyerphagia and weight gain (Rabasa et al. 2016). Hence, future studies should focus on the metabolic effects such as respiratory exchange ratio and energy expenditure on top of weight gain and food intake in varying degrees of stressed animals on high fat diets to further study the phenomena and continue to explore sex differences.

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