Ketogenic Diet-Induced Weight Loss Occurs Independent of Housing Temperature and is Followed by Hyperphagia and Weight Regain After Cessation in Mice

By
Alyssa Jane Weber

A Thesis
presented to
The University of Guelph

In partial fulfilment of requirements
for the degree of
Master of Science
in
Human Health and Nutritional Sciences

Guelph, Ontario, Canada
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ABSTRACT

KETOGENIC DIET-INDUCED WEIGHT LOSS OCCURS INDEPENDENT OF HOUSING TEMPERATURE AND IS FOLLOWED BY HYPERPHAGIA AND WEIGHT REGAIN AFTER CESSION IN MICE

Alyssa Jane Weber
University of Guelph, 2023

Advisor: Dr. David Dyck

Studies in mice demonstrate that KDs reduce food intake, increase energy expenditure and cause weight loss but these studies were completed at room temperature. Adherence to restrictive diets is poor thus it is important to examine effects of cycling from a ketogenic. The current study was tests if housing temperature impacts responses to KD in obese mice and if the mechanisms driving KD-induced weight loss reverse when mice are switched to an obesogenic high fat diet. We demonstrate that KD-induced reductions in food intake, increases in energy expenditure, weight loss and improvements in glucose homeostasis are not dependent upon housing temperature. KD-induced weight loss is largely explained by reductions in caloric intake while cycling mice back to an obesogenic diet following KD leads to hyperphagia-induced weight gain. Our results suggest that prior findings with mice fed a KD at room temperature are likely not an artifact of mice housing.
DEDICATION

For my family
ACKNOWLEDGEMENTS

I must first recognize the support of my supervisor, Dr. David Wright, since this thesis would not have been possible without his mentorship. Thank you for giving me so many opportunities to learn and grow.

To my thesis committee, Dr. Dyck, Dr. Little, and Dr. Robinson, thank you for always being there to help me when I needed it. Of course, past and present members of the MEDD lab who trained me and helped me with the work in this thesis.

Finally, thank you to my Mom, Dad, and Tina for helping me with life so I could focus on my work! And thank you to Winnie and Sadie-Maple for being the best pets in the world!
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<th>Full Form</th>
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<tr>
<td>ACOX1</td>
<td>Acyl-CoA oxidase 1</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related peptide</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of co-variance</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
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<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATGL</td>
<td>Adipose triglyceride lipase</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>βHB</td>
<td>Beta hydroxybutyrate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster differentiation factor 36</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCCAT/Enhancer binding protein homologous protein</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive lab animal monitoring system</td>
</tr>
<tr>
<td>CPT1</td>
<td>Carnitine palmitoyltransferase 1</td>
</tr>
<tr>
<td>ER Stress</td>
<td>Endoplasmic reticulum stress</td>
</tr>
<tr>
<td>eWAT</td>
<td>Epididymal white adipose tissue</td>
</tr>
<tr>
<td>FGF21</td>
<td>Fibroblast growth factor 21</td>
</tr>
<tr>
<td>GDF15</td>
<td>Growth and differentiation factor 15</td>
</tr>
<tr>
<td>GFRAL</td>
<td>GDNF family receptor alpha like</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
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<tr>
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<td>Description</td>
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<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone sensitive lipase</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>iWAT</td>
<td>Subcutaneous inguinal white adipose tissue</td>
</tr>
<tr>
<td>KD</td>
<td>Ketogenic diet</td>
</tr>
<tr>
<td>KO</td>
<td>Knockout</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<tr>
<td>LFD</td>
<td>Low-fat diet</td>
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<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PF</td>
<td>Pair-fed</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>POMC</td>
<td>Pro-opiomelanocortin</td>
</tr>
<tr>
<td>PPARα</td>
<td>Peroxisome proliferator-activated receptor alpha</td>
</tr>
<tr>
<td>PPIB</td>
<td>Peptidyl-prolyl cis-trans isomerase B</td>
</tr>
<tr>
<td>RER</td>
<td>Respiratory exchange ratio</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarco/endoplasmic reticulum Ca2+ ATPase</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signalling 3</td>
</tr>
<tr>
<td>TAG/TAG</td>
<td>Triacylglycerol/triglyceride</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNZ/TN</td>
<td>Thermal neutral zone/thermoneutrality</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>VCO2</td>
<td>Volume carbon dioxide</td>
</tr>
<tr>
<td>VO2</td>
<td>Volume oxygen</td>
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1. INTRODUCTION

1.1. White adipose tissue

White adipose tissue (WAT) is a multifaceted organ that acts to store excess energy to be released in times of need, and also acts as an endocrine and paracrine organ with an essential role in regulating metabolic homeostasis. White adipocytes serve as the primary energy store for excess energy primarily in the form of triacylglycerol (TAG). WAT is composed of mononuclear adipocytes that have a single large lipid droplet that occupies the vast majority of the cell. White adipocytes contain few mitochondria, are not well innervated, and have little vasculature compared to energy expending tissues, such as skeletal muscle or brown adipose tissue (BAT). It should be noted that the structure and function of WAT varies depending on anatomical location. For instance, visceral WAT tends to have larger adipocytes, have higher basal rates of lipolysis, and be more sensitive to stimulated lipolysis compared to subcutaneous WAT.

During energy deficits, like fasting, WAT mobilizes lipids via the multiple enzyme process of lipolysis. Lipolytic enzymes include, adipose triglyceride lipase (ATGL), which removes the first fatty acid from TAG, to form diacylglycerol; hormone sensitive lipase (HSL) removes the second fatty acid from diacylglycerol to form monoacylglycerol; then monoacylglycerol lipase cleaves the final fatty acid off of glycerol. ATGL and HSL represent ~90% of hydrolase activity in cultured mouse WAT. In the fasted state, catecholamines are largely responsible for activating lipolysis by suppressing insulin secretion and through direct effects on WAT. Activation of beta-adrenergic receptors by catecholamines causes a conformational change in their associated G proteins which facilitates activation of adenylyl cyclase and subsequent generation of cAMP, triggering
the activation of protein kinase A (PKA) \(^8\). In adipocytes, PKA signalling is responsible for activating lipolysis by phosphorylating HSL \(^10\) and ATGL \(^11,12\). This stimulates lipolysis to release fatty acids into circulation to provide energy for other tissues \(^9\).

1.2. Brown adipose tissue.
Brown adipose tissue (BAT) is a unique form of adipose tissue that is designed to expend energy. In contrast to WAT, brown adipocytes contain many small lipid droplets (multilocular) and are highly innervated and vascularized \(^13\). BAT exists in small, isolated clusters throughout the body \(^14\) and functions as a thermoregulatory organ, producing heat to maintain body temperature \(^15–17\). Rodents contain relatively large BAT depots, particularly interscapular \(^14\), and infant humans contain substantial active BAT throughout their body. On the other hand, only \(\sim 5\%\) of adult humans possess spontaneously active BAT \(^18\) with minimal energy expending capacity (<20 kcal/day) \(^19\). Nevertheless, numerous papers have reported a positive relationship between BAT content/activity and improved cardiometabolic health, resistance to obesity, and reduced liver fat \(^18,20\). Similarly, in humans there is an inverse relationship between the appearance of cold-induced BAT and body mass index, age, and diabetic status \(^21–23\).

Upon cold exposure, a hypothalamic signalling cascade increases sympathetic tone and the release of catecholamines, predominantly norepinephrine, which bind \(\beta\)-adrenergic receptors within BAT and stimulate mitochondrial oxidation of carbohydrates and lipids \(^24\) to generate heat via uncoupling protein-1 (UCP1), which uncouples electron transport from ATP production \(^15\). Interestingly, intracellular lipolysis of local TAGs is dispensable for BAT thermogenesis, since BAT-specific deletion of ATGL or the ATGL-
regulating protein, CGI-58, does not alter cold-induced BAT thermogenesis \[25,26\]. On the other hand, ablation of CD36, the membrane transporter of long-chain fatty acids, greatly impairs BAT fatty acid uptake and cold-induced thermogenesis \[27\]. Thus, BAT depends on fatty acid uptake from circulation for the generation of heat.

1.3. Beige adipose tissue
Browning involves changing WAT into a more brown-like phenotype, also known as beige or brite adipose tissue. Beige adipose tissue is classically characterized by increased UCP1 content and the appearance of multilocular adipocytes\[28\], along with increased expression of other thermogenic genes, including Cidea, Prdm16, Ppargc1a, and Dio2 \[14,29,30\]. Beige adipocytes are functionally similar to classic brown adipocytes \[31,32\], but unlike classic BAT, beige adipocytes are inducible and reversible \[29\]. Importantly, browning primarily occurs primarily in subcutaneous, not epididymal/intra-abdominal, WAT of rodents, at least in males\[33,34\].

Activation of thermogenesis and the conversion of white to beige adipose tissue is potently activated by adrenergic stimulation. Hence, pharmacologic activation of the \(\beta-3\) adrenergic receptor, which is predominantly expressed in adipose tissue, with the agonist CL316,243 can increase thermogenic gene expression in as little as 3 hours in mice\[35\] and chronic (7-10 days) adrenergic activation causes massive browning in rodents \[36-38\]. Chronic treatment (10 weeks) with the \(\beta-3\) adrenergic agonist mirabegron also causes WAT browning in obese humans\[39\].

WAT browning is associated with improved metabolic health. For instance, repeated cold exposure improves systemic glucose metabolism and leads to browning in
rodents and humans\textsuperscript{21,40–42}. Rodent studies consistently demonstrate a strong relationship between beige adipose tissue, reductions in body weight, and resistance to high-fat diet-induced metabolic disturbances\textsuperscript{43}. However, even in the presence of marked browning, either in response to chronic CL316,243 treatment or cold exposure, there is no difference in basal or acute CL-induced glucose and lipid uptake, nor increased metabolic activity in eWAT or iWAT\textsuperscript{36}, suggesting that browning of WAT does not alter its metabolic activity.

1.4. Thermoneutrality
By the 18\textsuperscript{th} and 19\textsuperscript{th} centuries it was already known that energy expenditure increased when mammals were in cold and hot environments\textsuperscript{44}, clearly showing that ambient temperatures have important effects on whole body energy regulation. The ambient temperature at which energy expenditure is at a minimum is considered the ‘thermoneutral zone’ (TNZ)\textsuperscript{16,44}. Importantly, humans and rodents, like laboratory mice, have very different TNZs. Traditionally laboratory mice have been housed at ambient temperatures of \textasciitilde{}22°C for the comfort of the humans researching them, but this is well below thermoneutrality for mice which is \textasciitilde{}30°C\textsuperscript{45}. There is some debate surrounding the exact ambient temperature at which mice are at thermoneutrality, as conditions, like the type of bedding and enrichments or huddling if mice are group housed, can affect thermal regulation and body temperature in rodents. Temperatures between 25-30°C can represent thermoneutrality for mice, but regardless of debate, temperatures closer to thermoneutral are preferable to traditional room temperature\textsuperscript{46}.
Currently the definition of thermoneutrality is based on whole body energy expenditure \(^{44}\). At the thermoneutral zone, at which point metabolic rate and energy expenditure are minimal and constant \(^{44}\) heat loss is controlled by the regulation of blood flow to superficial sites, like the tail in rodents \(^{47,48}\). At ambient temperatures below the TNZ, metabolic rate and energy expenditure increase linearly reflecting the amount of energy required to maintain core body temperature \(^{44}\). This is known as cold-induced thermogenesis.

Below the TNZ mice initially utilize energetically efficient means of maintaining body temperature, including vasoconstriction and huddling, before initiating more energetically expensive processes, predominantly skeletal muscle shivering thermogenesis \(^{48}\). Shivering is essentially a short-term mechanism due to its energetic cost and muscle fatigue \(^{49}\). One of the main processes responsible for long-term cold acclimation is non-shivering thermogenesis via BAT \(^{48}\). In response to cold environments, adrenergic activation drives thermogenesis via UCP1 in BAT thereby dissipating energy as heat \(^{48}\).

UCP-1 knockout (KO) mice represent an important model for understanding the physiological significance of thermogenesis \(^{32,48,50}\). At thermoneutrality, UCP-1 KO mice display no metabolic differences compared to their wild-type counterparts, although these mice do experience a general reduction in mitochondrial and electron transport chain proteins in BAT \(^{51}\). Surprisingly, UCP-1 KO mice actually demonstrate an obesity-resistant phenotype at room temperature \(^{52}\). However, when UCP-1 KO mice are transferred to a cold temperature (4°C), they are unable to utilize non-shivering thermogenesis and are therefore unable to maintain body temperature and eventually
succeed to the cold. However, if gradually acclimated to mild cold temperatures (~18°C) UCP-1 KO mice can survive and maintain a normal body temperature and oxygen consumption rate when exposed to cold (4°C). These data could be explained by UCP1-independent thermogenesis and other non-canonical forms of thermogenesis, including creatine- and SERCA-dependent thermogenesis.

Considering BAT and beige adipose tissues' role in thermogenesis, it is not surprising that mice housed at TNZ have substantially reduced BAT mass and thermogenic gene expression. Similarly, thermogenic gene expression is greatly reduced in subcutaneous inguinal white adipose tissue. The reduced thermogenic activation is most likely the result of suppressed catecholamine levels in mice housed at TNZ.

Ambient temperature effects numerous physiological processes in mice, from energy expenditure, energy intake, glucose metabolism, heart rate, sleeping patterns, and the adaptations to exercise. Mice housed at room temperature have an ~50% increase in energy expenditure compared to those at TNZ which necessitates a large increase in food intake. Interestingly, although mice at TNZ consume less food, they still gain more weight and accumulate greater fat mass either on chow or high-fat diet (HFD), although this is not a universal finding. Nevertheless, mice housed at thermoneutrality on a HFD have increased liver weight, hepatic fat accumulation, and greater non-alcoholic fatty liver disease score compared to mice housed at room temperature. Similarly, the development of atherosclerosis is exacerbated in rodents housed at thermoneutrality. This is particularly important since under standard room temperature conditions mice do not develop atherosclerosis or the progression of non-
alcoholic fatty liver disease that occurs in humans, reflecting the importance of housing
temperature in mimicking human physiology in health and disease.

Most humans in the developed world spend a majority of their time in TNZ thanks to indoor climate control and clothing. Considering mice are currently the most used animal model to study human diseases, basic biology, and the substantial effects of housing temperature on energy metabolism, it is important that animals be studied under conditions that most appropriately mimic human physiology. Because of this, there is currently extensive research and debate surrounding the best housing temperature for rodents to translate to humans.

1.5. Obesity and weight loss interventions
Obesity is among the most prevalent health problems world-wide. In the modern world, accessibility and abundance of calorically dense and palatable foods make maintaining a healthy diet extremely difficult. This is crucial considering obesity is highly associated with the development of many health disorders, including metabolic syndrome, insulin resistance, Type 2 Diabetes, cardiovascular complications, sleep disorders, as well as hepatic and renal dysfunction.

Obesity is the result of a chronic imbalance in caloric intake and energy expenditure. Under a chronic positive energy balance, in which energy intake is greater than energy output, the leftover energy is converted to TAG, stored in adipocytes in WAT and can spill over to other tissues, including muscle, liver, and pancreas. A chronic positive energy balance can lead to the unhealthy expansion of WAT, in which adipocytes undergo hypertrophy and hyperplasia. Excessive WAT expansion activates various
intrinsic signals, including hypoxia and mechanical stress, thereby initiating an inflammatory response, ultimately leading to immune dysregulation and chronic low-grade inflammation. This intrinsic dysfunction affects WAT’s endocrine profile, leading to secretion of inflammatory cytokines, like interleukin-6 and TNFα, contributing to impaired systemic metabolism.

At the same time, when adipocytes become enlarged, they also become insulin resistant, and concomitantly undergo an increased rate of basal TAG hydrolysis and release of fatty acids into circulation; activated lipolysis is blunted in obesity due to inflammation. This leads to ectopic TAG accumulation in tissues not designed to store large amounts of fats, notably the liver, skeletal muscle, heart, and pancreas. This fat deposition, commonly termed lipotoxicity, in tissues that contribute to the regulation of metabolic homeostasis can lead to further cellular dysregulation and functional impairments in tissues. This ultimately contributes to insulin resistance in numerous metabolic tissues, perpetuates metabolic dysfunction, and can further the pathogenesis of obesity and its associated risk factors.

Due to the numerous metabolic health consequences resulting from obesity, it is crucial to find ways to combat weight gain or promote weight loss. In the USA, 30-45% of adults are trying to lose weight, but in spite of this obesity rates have doubled in only 20 years. Although there are currently several pharmacologic means of reducing body weight, these produce only <10% reduction in body weight, and often have unwanted side-effects. The most promising anti-obesity therapies at the moment are glucagon-like peptide-1 analogues which work mainly by reducing food intake. Bariatric surgery is extremely effective at reducing body weight and improving systemic glucose and lipid
metabolism, but surgery is highly invasive, expensive, and comes with considerable risk \cite{76-78}. Lifestyle modifications, including increased exercise and reduced caloric intake, can produce significant improvements in overall health, but generally produce quite minimal weight loss. Lifestyle interventions’ slow and modest weight loss can be frustrating which often leads to poor adherence to programs \cite{79}. Moreover, even in those individuals who attain their desired weight loss, most people will eventually regain the weight they lost \cite{79}.

The reasons why weight loss is difficult is complicated but is likely at least partly due to physiological responses that are in place to maintain body mass. From an evolutionary standpoint maintaining energy stores would be critical for survival in times of limited food availability \cite{80}. For example, the anorexigenic hormone leptin circulates in amounts proportional to the amount of fat stored in the body \cite{81}. Leptin communicates peripheral energy storage to the brain and adjusts food intake and energy expenditure to maintain a body weight ‘set-point’ \cite{80,81}. Thus, when one loses fat mass, leptin levels decrease, which can limit its anorexigenic drive, facilitating increased food intake and reducing energy expenditure \cite{80,81}. At the same time, diet-induced weight loss leads to marked elevations in circulating ghrelin, an orexigenic hormone, that promotes hunger. Indeed, one reason diet interventions are particularly ineffective at reducing body weight, and especially maintaining the lost weight, is because of a reduced energy expenditure. Thus, as one loses weight, energy expenditure also decreases, thereby hindering weight loss efforts. Therefore, interest has turned to finding interventions that maintain or increase energy expenditure while promoting weight loss.
1.6. Ketogenic diet

Traditionally, diet programs recommended for weight management were high carbohydrate, low fat, energy reduced diets. Recently low carbohydrate, high protein, and high fat diets have become increasingly popular. One example of the low carbohydrate high fat diet is the Ketogenic diet (KD), which requires very low carbohydrate consumption (<5% total caloric intake), low protein (~15%), and very high fat (80%). Ketones, predominantly acetoacetate and β-hydroxybutyrate, are alternative fuel sources that can replace carbohydrates as the primary fuel for peripheral tissues such as the brain and heart.

Ketones are produced in the liver in states of reduced carbohydrate availability, including prolonged fasting, starvation, and diabetes. During these times, lipolysis is activated and circulating fatty acids are increased which are substrates for hepatic ketone production. Specifically, fatty acids are brought into mitochondria and through beta-oxidation are converted to acetyl-CoA. In the liver, acetyl-CoA has two main fates, either oxidation in the citric acid cycle or conversion to ketones, primarily acetoacetate or β-hydroxybutyrate. When acetyl-CoA production via beta-oxidation exceeds citric acid cycle activity they are shunted toward ketogenesis. Finally, ketones are exported and upon reaching extrahepatic tissues they are converted back to acetyl Co-A and oxidized via the citric acid cycle to provide fuel for the carbohydrate deprived tissue.

In humans, KDs produce significant weight loss, visceral fat reduction, and improve glucose tolerance and insulin sensitivity. A metanalysis by Bueno et al. analysed randomized controlled trials that assigned adults to a very low carbohydrate KD or a more traditional weight loss regimen of a low-fat diet (LFD) with a minimum 12 month follow up period. In total, 13 studies with 1577 participants were analyzed. It was seen that
individuals that were assigned a KD lost significantly more weight and had a significantly greater reduction in serum TAGs than their LFD counterparts. However, a significantly greater increase in HDL and LDL cholesterol were seen in the KD compared to the LFD groups. Along these lines, in mice, KD feeding produced substantial weight loss, improved systemic insulin sensitivity, and increased energy expenditure associated with brown adipose tissue thermogenesis.

An important caveat to these health promoting effects of KDs, is that they have been shown to cause rapid and substantial hepatic lipid accumulation following as little as 3 days to 5 weeks. This lipid accumulation can lead to hepatic insulin resistance and increased hepatic glucose output, thereby contributing to hyperglycemia. Importantly, these liver defects are observed even in the face of increased energy expenditure and reduced body weight.

1.7. Possible mechanisms for KD-induced weight loss
While KD consistently leads to weight loss in rodents and humans, the cause of this weight loss is not entirely clear. A possible explanation for weight loss seen with a KD is appetite suppression and reduced food intake resulting from ketosis. Ketone bodies exhibit strong anorexigenic properties via a decrease in circulating ghrelin (an orexigenic signal), reduced cerebral neuropeptide Y, and increased cholecystokinin (CCK) meal response, which can all lead to a reduction of perceived hunger and food intake. This is thought to be one of the mechanisms that can account for the weight loss and tolerability of a KD although studies of KDs do not always account for the reduced food intake, as with pair-feeding experiments, so this remains to be determined.
In addition to reductions in food intake, KD-induced weight loss may result from increased energy expenditure through adrenergic activity and thermogenesis. For instance, mice lacking all beta receptors gained weight and accumulated WAT when placed on a KD, rather than losing weight like wildtype mice\(^95\); interestingly, hepatic effects of KD were independent of adrenergic signalling suggesting the hepatic lipid accumulation observed during KDs may be dietary and not indirect via increased WAT lipolysis. Nevertheless, in beta-less mice, KD feeding was still able to activate iWAT browning, including similarly increased UCP1 mRNA expression\(^95\). However, unlike in iWAT, KD-induced thermogenic gene expression was completely absent in BAT of beta-less mice, and this was associated with reductions in KD-induced increase in whole body energy. Crucially, all previous studies investigating KD-induced weight loss, and mechanisms thereof, were performed in rodents at room temperature. Thus, it remains possible that these adrenergic and thermogenic effects of KD result from interactions with the sub-thermoneutral housing, as has been shown with exercise\(^56,57\).

Mechanistically, KD-induced increases in energy expenditure and weight loss have been shown to require liver-derived fibroblast growth factor 21 (FGF21)\(^96\), a signalling factor which increases energy expenditure\(^97\) and is linked to increases in adipose tissue thermogenesis\(^98,99\). FGF21 also regulates food preferences, specifically suppressing intake of sugars and promoting intake of protein\(^99,100\). Indeed, FGF21 is markedly increased by low-protein diets and is critical for driving metabolic adaptations to a low-protein diet, including increased food and protein intake and increased energy expenditure\(^99\).
A hormone related to FGF21, growth a differentiation factor 15 (GDF15), also controls food preference, in this case suppressing the intake of high fat foods\textsuperscript{101,102}. By reducing food intake, GDF15 has been shown to contribute to weight loss in rodents and non-human primates\textsuperscript{102,103}. Interestingly, the liver is one of the predominant tissue sources of both FGF21 and GDF15 and both are regulated by the integrated stress response, a cellular stress mechanism that involves ATF4 and CHOP\textsuperscript{104}. FGF21 and GDF15 have also been shown to work synergistically to reduce liver steatosis\textsuperscript{104}. Thus, it is possible these hormones could contribute to the metabolic adaptations observed with KDs.

1.8. Weight regain following ketogenic dieting
Diet-induced weight loss is often transient, and people regain most, or all, of the weight they lose while dieting \textsuperscript{105}. This phenomenon has been coined "yo-yo" dieting to characterize the repeated ups-and-downs in body weight. Powerful physiological mechanisms are involved in weight regain, including alterations in several appetite-regulating hormones that lead to increased feelings of hunger\textsuperscript{80,106}. Importantly, a recent meta-analysis of perceived appetite during KDs reported that participants who followed this diet were less hungry and had a reduced desire to eat, despite weight loss \textsuperscript{107}. Thus, authors attributed the clinical benefit to KDs being in their ability to cause weight loss without an increase in perceived appetite, possibly due to ketosis \textsuperscript{107}.

What is much less clear is how people respond after abandoning a KD. One study put individuals with obesity on a very-low energy diet (~600 kcal per day for females with a macronutrient composition of carbohydrates 42%, protein 36%, fat 18% and fibre 4%) and tracked perceived appetite and various appetite-regulating hormones over 8 weeks
and a 4-week refeeding period afterward \(^{108}\); although the diet composition is not strictly ketogenic, the very-low caloric content made the participants ketogenic after 3 days as confirmed by circulating \(\beta\)-hydroxybutyrate. During the diet, there was a transient increase in perceived appetite at 3 days, which then receded, without any change in appetite regulating hormones throughout the very-low energy diet. However, upon refeeding, when participants were weight stable and no longer ketogenic, there was a large increase in perceived hunger and acylated ghrelin, an appetite stimulating hormone \(^{108}\). Both of these responses would be expected to stimulate appetite and food intake and conceivably lead to weight regain over time. However, it is unclear how body weight responds to a traditional high-fat and high-sugar diet following adaptations to a ketogenic diet.
2. THESIS OBJECTIVE AND HYPOTHESES

Taken together, interactions between WAT and liver are responsible for ketogenesis which serves a crucial role in times of starvation. There is considerable evidence in humans that ketogenic diets can lead to rapid and significant weight loss in individuals with obesity, but there is little mechanistic evidence to explain how this occurs. Moreover, the research that suggests a role for thermogenesis in ketogenic diet-induced weight loss is confounded by sub-thermoneutral housing temperatures. Lastly, the effects of switching from a KD back to a less restricted dietary pattern on weight loss/regain, and the underlying mechanisms have not been adequately studied. Thus, the aim of the current thesis was to 1) compare the mechanistic underpinnings of ketogenic diet-induced weight loss at room temperature and thermoneutrality and 2) determine the effects, and underlying mechanisms, of switching from a KD to a high fat and sugar diet, on weight regain in mice.

Hypotheses:

1) KD-induced weight loss will be attenuated at thermoneutrality.

2) KD-induced weight loss and improvements in glucose tolerance will be driven largely by reduced food intake.

3) Mice will rapidly regain body weight after switching to a high-fat and high-sugar diet following KD.
3. Ketogenic Diet-Induced Weight Loss Occurs Independent of Housing Temperature and is Followed by Hyperphagia and Weight Regain After Cessation in Mice

As published:

Weber AJ¹, Medak K¹, Townsend LK², Wright DC¹. Ketogenic Diet Induced Weight Loss Occurs Independent of Housing Temperature and is Followed by Hyperphagia and Weight Gain Following Diet Cessation in Mice. The Journal of Physiology, DOI: 10.1113/JP283469

Serum analyses, liver TAGs, and qPCR were performed by KM.

Affiliations: ¹Department of Human Health and Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada; ²Centre for Metabolism, Obesity, and Diabetes Research and the Department of Medicine, McMaster University, Canada
3.1 Introduction
Very low carbohydrate, high fat ketogenic diets (KDs) have become an increasingly popular nonpharmacological tool for improving metabolic health. Diet trials lasting from several weeks to months have reported KD-induced weight loss and improvements in indices of glucose metabolism in participants with overweight or obesity. In rodents, KDs increase energy expenditure, reduce food intake, and cause weight loss. Mechanistically, KD-induced increases in energy expenditure and weight loss have been shown to require liver-derived fibroblast growth factor 21 (FGF21), a signalling factor which increases energy expenditure, and the presence of β-adrenergic receptors.

Previous studies examining the mechanistic underpinnings of KD-induced weight loss have housed mice at room temperature (RT). While this is comfortable for staff working with animals it is below the thermal neutral zone for mice and requires the engagement of energy producing processes to maintain core temperature. This can have a profound impact on mouse physiology as shown by the >50% increase in energy expenditure and food intake when compared to mice housed at thermal neutrality (TN). As β-adrenergic signalling and FGF21, reputed mediators of KD-induced energy expenditure and weight loss, are increased with cold, this could be a significant confounding factor in previous investigations examining the effects of KDs in mice housed at RT. In this regard, sub-TN housing conditions could be resulting in an artifactual increase in energy expenditure with KDs and could potentially explain the larger and more consistent increases in energy expenditure in rodents compared to humans (as reviewed in).
As adherence to diets is typically poor\textsuperscript{119}, it would be expected that individuals consuming KDs would, at some point, revert to a less restrictive dietary pattern. Hypocaloric diets causing weight loss result in adaptations leading to compensatory reductions in energy expenditure \textsuperscript{120}, an effect that has been suggested to predispose to weight regain \textsuperscript{121}. Conversely, KDs increase energy expenditure \textsuperscript{89,92,95}, and currently it is not known if cycling animals from a ketogenic to an obesogenic high fat diet (HFD) would result in alterations in energy expenditure, or if predicted increases in weight regain would be driven by hyperphagia. The purpose of the current investigation was two-fold. First, we wanted to determine the impact of housing temperature on the effects of a KD in mice with pre-existing obesity. We hypothesized that housing mice at TN would attenuate KD-induced increases in energy expenditure and weight loss. Second, we wanted to examine the effects of cycling mice from a KD to an obesogenic HFD. We postulated that this would lead to a rapid regain of weight driven by increases in food intake in mice housed at TN.
### 3.2. Methods

**Ethical approval and anaesthesia**

All experiments were approved by Animal Care Committee at the University of Guelph and followed Canadian Council on Animal Care guidelines for the care and use of laboratory animals (AUP no. 3864). The authors fully understand the ethical principles under which The Journal of Physiology operates and confirm that this work complies with its animal ethics checklist. Mice were sedated by injection of sodium pentobarbital (60 mg/kg body weight (bw) i.p.) and killed by exsanguination of the heart.

**Diet interventions**

Eight-week-old male C57BL/6J mice (stock no. 000664) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were allowed 1 week to acclimate to our facility at either room temperature (RT; 22°C) or thermoneutrality (TN; 29°C) before experiments began. During acclimation mice were single housed in shoebox cages with unrestricted access to a standard chow diet (Teklad 7004; Envigo, Indianapolis, Indiana, USA) and water. Mice were on a 12:12-h light–dark cycle. Following acclimation, mice were given free access to a HFD (45%, 35% and 20% kcal from fat, carbohydrate and protein, respectively, 4.73 kcal/g, Research Diets, New Brunswick, NJ, USA; cat. no. D12451) for 5 weeks to induce obesity.

**Temperature comparison**

Following 5 weeks of the HFD, half of the mice at each temperature were switched to a KD for 3 weeks. The composition of the KD was ~93.4%, 4.7% and 1.8% kcal from fat, protein and carbohydrate, respectively, with 7.24 kcal/g (F3666, Bio-Serv, Frenchtown,
NJ, USA). This diet has previously been shown to induce weight loss, increase energy expenditure and reduce food intake\textsuperscript{89,92,95,122}. We chose this duration of KD feeding as a prior study has reported increases in energy expenditure and reductions in food intake in obese mice within this time frame\textsuperscript{89}. Throughout the KD feeding experiments, food was measured and replaced daily and body weight recorded every 3 days. At the end of the 3-week KD intervention, mice were sedated by injection of sodium pentobarbital (60 mg/kg bw) and epididymal and inguinal white adipose tissue depots (eWAT and iWAT, respectively), interscapular brown adipose tissue depot (iBAT) and liver were excised, quickly weighed, snap-frozen in liquid nitrogen and stored at −80°C. Blood was allowed to clot for \(\sim\)15 min then stored on ice until it was centrifuged at 1000 g for 10 min and the serum was aliquoted and stored at −80°C. Tissue collection was performed at the same temperature (e.g. RT or TN) that the mice were housed at.

**Pair feeding at RT and TN**

To assess the importance of food intake on the effects of KD, we performed pair-feeding experiments. Following the HFD, mice either remained on the HFD, or were given free access to the KD, or were given a HFD with calories matched to the average intake of the KD group (HFD pair-fed; HFD-PF). To do this, KD food was weighed each morning and caloric intake determined (kcal/g of diet \(\times\) food consumed (g)) and mice in the HFD-PF group were given an amount of food equal to that number of calories such that mice in the HFD-PF group were 1 day ‘behind’ the *ad libitum* group. As before, food was weighed and KD replaced daily with body weight assessed every 3 days. Tissue harvest was completed as described above.
**Weight regain experiments**

To determine the impact of cycling mice from a KD to a HFD we repeated the previous KD intervention. As the effects of the KD were similar at both RT and TN, these experiments were only conducted at TN. Following the initial KD intervention, the KD mice were returned to the HFD for another 3 weeks, during which time food intake and body weight were assessed every 3 days. This experiment was repeated, but another group previously fed the KD were pair-fed, as described earlier, to mice that were continually provided with the HFD. Tissue harvest was completed as described above.

**Glucose tolerance**

Three days prior to tissue harvest, glucose tolerance was assessed. Mice were fasted for 6 h (starting at ~08.00 h) and given an oral gavage of glucose (2 g/kg bw). Blood glucose was taken from a small drop of blood from the tail vein using a handheld glucometer and glucose strips (Freestyle Lite; Abbott Laboratories, Abbott Park, Illinois, USA) prior to and 15, 30, 60, 90 and 120 min after the glucose gavage and the glucose area under the curve (AUC) calculated.

**Comprehensive lab animal monitoring system**

To assess energy expenditure in response to the KD, mice were housed at either RT or TN and placed on a HFD for 5 weeks then switched to a KD for 10 days before being placed in a Comprehensive Lab Animal Monitoring System (CLAMS) at either RT or TN.
Mice were moved to the CLAMS at the beginning of their light phase for ~24 h to acclimate to the system. Following acclimation, respiratory exchange ratio (RER; VCO2 /VO2), energy expenditure, calculated using the modified Weir equation (Weir, 1949: \(3.9 \times \text{VO2} + (1.1 \times \text{VCO2})\)), and physical activity (beam breaks) were measured over one full light and dark cycle.

To determine if changes in energy expenditure explained weight regain following the consumption of a KD, we repeated the weight regain experiment and measured RER, physical activity and energy expenditure in HFD controls or in mice previously fed a KD and then switched back to a HFD with food provided ad libitum or pair fed to the control mice that had received the HFD throughout. These experiments were initiated on day 10 of the terminal diet switch.

**Liver triglyceride content**

To quantify liver triglyceride (TAG) content, snap-frozen liver was chipped into ~30 mg pieces, homogenized in 1 ml of 1:2 methanol:chloroform and agitated overnight at 4°C. The following day, 1 ml of 4 mM MgCl was added, vortexed and centrifuged for 1 h at 1000 g at 4°C. The infranatant was extracted, evaporated overnight and reconstituted in a 3:2 butanol-Triton X-114 mix. TAG content was measured with a commercially available kit (Sigma-Aldrich, St Louis, MO, USA, cat. no. F6428) in duplicate.

**qPCR analysis**

Gene expression was determined as we have described previously \(^{123}\). Liver (10–40 mg) was homogenized in 1 ml of QIAzol (cat. no. 15596018; Thermo Fisher Scientific,
Waltham, MA, USA) in a bead mill followed by RNA extraction using Bio Basic EZ-10 Spin Column Total RNA Miniprep Super Kits (cat. no. BS784; Bio Basic, Markham, Ontario, Canada) including DNase free treatment with a commercially available kit (cat. no. AM1906; Thermo Fisher Scientific). Complementary DNA was synthesized from total RNA using Superscript II (cat. no. 4368814, Thermo Fisher Scientific). A quantitative reverse transcription–PCR was run in 96-well plates on a CFX Connect system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green Supermix (cat. no. 1725271; Bio-Rad Laboratories). All markers are expressed relative to Ppib, which did not change between groups. Relative differences in mRNA expression were determined using the $2^{-\Delta\Delta CT}$ method and normalized to the respective control group $^{124}$. Primer sequences are given in Table 1.

**Serum analyses**

Serum NEFA (HR series; Wako Diagnostics, Richmond, VA, USA), glycerol (cat. no. F6428; Sigma-Aldrich) and β-hydroxybutyrate (βHB; cat. no. 700190, Cayman Chemical Co., Ann Arbor, MI, USA) were measured on 96-well plates using colorimetric assays. Circulating insulin (cat. no. 10-1247-01; Mercodia, Winston Salem, NC, USA), FGF21 (cat. no. MF2100; R&D Systems, Minneapolis, MN, USA) and growth differentiation factor 15 (GDF15; cat. no. DY6385, R&D systems) levels were measured by enzyme-linked immunosorbent assay as per the manufacturer’s instructions. All assays were conducted in duplicate and in accordance with the manufacturer’s instructions.

**Statistical analysis**
Statistical tests were completed using Graph Pad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) or for ANCOVA SPSS Statistics version 26.0.0.0 (IBM Corp., Armonk, NY, USA). Data were compared by unpaired two-tailed Student’s t-test (HFD vs. HFD–KD–HFD), one-way ANOVA (HFD vs. HFD–KD–HFD–PF vs. HFD–KD–HFD), two-way ANOVA (e.g. HFD vs. KD at TN and RT) or repeated measures ANOVA (body weight over time). Energy expenditure data was also analysed by one- or two-way ANCOVA with body weight as a covariate. Post hoc tests with Tukey’s or Šidák’s (repeated measures ANOVA) correction were completed when a significant interaction was identified. There were no a priori exclusion criteria for animals and outliers were defined as points outside 2 standard deviations away from the group mean. Significance level was set at P < 0.05. Main effects are designated by a solid bar over the figure while significant interactions are shown by individual bars connected by a line. Data are available upon request.
Table 0-1. Primer sequences used for qPCR analysis

References are given in parentheses

<table>
<thead>
<tr>
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<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
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<td>TTGACGGTAACTGACTCCAGC</td>
</tr>
<tr>
<td>Atf4</td>
<td>CCTTCGCCACAGTCGGTTTG</td>
<td>CTGTCCCGGAAAAGGCATCC</td>
</tr>
<tr>
<td>Chop</td>
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3.3. Results

KD-induced weight loss and differences in glucose tolerance occur independent of housing temperature.

To test the impact of housing temperature on the effects of KD, mice were fed a HFD for 5 weeks before switching half of the mice in each temperature group to a KD for 3 weeks (Figure 1A). Prior to the initiation of the KD body weights were not different between groups (RT HFD 35.4±2.3, RT KD 34.1±2.7, TN HFD 35.1±4.2, TN KD 35.6±2.0 grams, mean±SD n=10/group, main effect of diet p=0.6713, main effect of temperature p=0.4760, interaction effect p=0.3388). We confirmed that this diet induced ketosis by measuring circulating β-Hydroxybutyrate, and, interestingly, mice on a KD at TN had significantly (p=0.0198) greater circulating levels compared to those at RT (Table 2). Mice on the KD were lighter (Figure 1B) (main effect of diet p<0.0001) and gained less weight (main effect of diet p<0.0001) than HFD controls (Figure 1C). Reductions in body weight with the KD were paralleled by smaller eWAT and iWAT fat depots, whereas there was a main effect of temperature to increase liver and BAT weight (Table 2). There were main effects of temperature (p=0.0130) and diet (p<0.0001) to reduce food intake (Figure 1D) and there was a significant negative correlation between food intake and weight loss in mice given the KD (Figure 1E).

The KD mediated reductions in body mass were mirrored by significantly improved glucose tolerance compared to HFD mice, independent of housing temperature (Figure 1F & G) (main effect of diet p=0.0006). Similarly, circulating insulin levels were below the level of detection in mice provided the KD, whereas there were no differences between groups in regards to serum NEFA concentrations (Table 2). FGF21 mediates some of the adaptations to a KD and in the current study circulating concentrations were
increased with the KD (main effect p<0.0001) independent of housing temperature. GDF15 is a stress sensitive hormone that is implicated in the control of fuel metabolism and has previously been shown to be increased by a KD. In the current study we found that there was an interaction between diet and temperature with serum GDF15 levels being increased in the KD groups at both RT (p<0.0001) and TN (p<0.0001) with GDF15 being greater in the KD group at TN compared to RT (p<0.0001). Collectively these data indicate that the beneficial effects of KDs on weight loss and glucose metabolism in conditions of pre-existing obesity are not dependent on housing temperature.
Figure 0-1: The effects of a ketogenic diet occur independent of housing temperature.

Male C57BL6/J mice were housed at either room temperature (RT) or thermal neutrality (TN) and fed a high fat diet (HFD) for 5 weeks. Half of the mice in each temperature group were then switched to a ketogenic diet (KD) (A) and body weight (B), changes in weight (C), food intake (D, E) and glucose tolerance (F, G) determined. Data are presented as means±SD with individual data points shown when possible (n=10/group). Data were analyzed using a two-way ANOVA (diet by temperature). diet = main effect of diet, temperature = main effect of temperature as shown by a bar over the figure p<0.05.
Table 0-2. The impact of housing temperature and ketogenic diet induced on tissue weights and serum metabolites and hormones.

<table>
<thead>
<tr>
<th></th>
<th>RT-HFD</th>
<th>RT-KD</th>
<th>TN-HFD</th>
<th>TN-KD</th>
<th>diet</th>
<th>temp</th>
<th>interaction</th>
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<tbody>
<tr>
<td>Liver (g)</td>
<td>1.37 ± 0.22</td>
<td>1.14 ± 0.14</td>
<td>1.40 ± 0.24</td>
<td>1.45 ± 0.34</td>
<td>0.2557</td>
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<td>.0771</td>
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<td>eWAT (g)</td>
<td>2.49 ± 0.45</td>
<td>0.75 ± 0.18</td>
<td>2.25 ± 0.54</td>
<td>0.82 ± 0.14</td>
<td>&lt;<strong>0.0001</strong></td>
<td>0.4778</td>
<td>0.2036</td>
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<td>iWAT (g)</td>
<td>1.11 ± 0.17</td>
<td>0.28 ± 0.04</td>
<td>1.15 ± 0.37</td>
<td>0.28 ± 0.07</td>
<td>&lt;<strong>0.0001</strong></td>
<td>0.7629</td>
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<td>iBAT (g)</td>
<td>0.14 ± 0.05</td>
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<td>0.21 ± 0.09</td>
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<td>0.8276</td>
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<td>0.1819</td>
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<td>serum NEFA (mM)</td>
<td>0.65 ± 0.12</td>
<td>0.69 ± 0.11</td>
<td>0.77 ± 0.11</td>
<td>0.89 ± 0.24</td>
<td>0.0951</td>
<td><strong>0.0017</strong></td>
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<td>serum insulin (pM)</td>
<td>709 ± 287</td>
<td>ND</td>
<td>718 ± 275</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>serum FGF21 (pg/ml)</td>
<td>734 ± 137</td>
<td>15278 ± 1332</td>
<td>990 ± 223</td>
<td>20018 ± 2590</td>
<td>&lt;<strong>0.0001</strong></td>
<td>0.0821</td>
<td>0.0875</td>
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<td>serum GDF15 (pg/ml)</td>
<td>173 ± 65</td>
<td>344 ± 38*</td>
<td>215 ± 37</td>
<td>502 ± 100*#</td>
<td>&lt;<strong>0.0001</strong></td>
<td>&lt;<strong>0.0001</strong></td>
<td>0.0106</td>
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<td>serum βHB (mM)</td>
<td>0.18 ± 0.07</td>
<td>1.96 ± 0.86*</td>
<td>0.17 ± 0.05</td>
<td>3.14 ± 1.29*#</td>
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<td>liver TAG (mg/g wet weight)</td>
<td>79 ± 11</td>
<td>143 ± 16</td>
<td>231 ± 38#</td>
<td>75 ± 9*</td>
<td>0.0521</td>
<td>0.0739</td>
<td>&lt;<strong>0.0001</strong></td>
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</table>

Data are presented as means ± SD for n=10/group. Data was analyzed using a 2-way ANOVA and main effects of diet, temperature (temp) and interactions are shown. * indicates a significant (p<0.05) difference between high fat diet and ketogenic diet group within the same temperature, # significant difference (P<0.05) between the same diet groups at different temperatures, as determined using a Tukey post hoc analysis. RT-HFD = room temperature high fat diet, RT-KD = room temperature ketogenic diet, TN-HFD = thermal neutral high fat diet, TN-KD = thermal neutral ketogenic diet.
KD increases indices of liver inflammation and ER stress compared to HFD mice

Prior work has demonstrated that the consumption of a KD increases markers of oxidative metabolism and endoplasmic reticulum stress and inflammation, reputed mediators of insulin resistance, in mouse liver. We wanted to determine if this response was impacted by housing temperature. We first assessed liver TAG accumulation and found a significant diet by temperature interaction such that liver TAG concentration was greater in livers from HFD mice at TN compared to RT (p=0.0002) and that TAG accumulation was less in livers from mice fed a KD compared to HFD at TN (p=0.0001) (Table 2). We next measured the expression of genes encoding proteins involved in fatty acid oxidation (Cpt1α, Acox1) and the transcriptional regulation of oxidative metabolism (Pparα). As shown in Figure 2A there was a diet by temperature interaction (p=0.0019) with the mRNA expression of Cpt1α being decreased in livers from HFD mice at TN compared to RT (p=0.0118), while the KD increased Cpt1α expression only under TN conditions (p<0.0001). There was an interaction between temperature and diet when measuring Acox1 expression with the expression of this gene being lower in liver from the KD compared to HFD group at RT (p=0.0049) and in livers from the HFD TN compared to HFD RT group (p=0.0175). There were main effects of both diet (p=0.0407) and temperature to reduce the mRNA expression of Pparα (p=0.0001).

As a next step we assessed indices of inflammatory genes in liver and found a main effect of the KD to increase (p=0.0035) and TN housing (p=0.0088) to reduce the expression of Il6, and a main effect of KD to increase the mRNA expression of Socs3 (p<0.0001) and Tnfα (p<0.0001) (Figure 2B). When examining indices of ER stress, there were main effects of KD and TN housing to increase the expression of Chop (diet
p<0.0001, temperature p=0.0420) and Xbp1s (diet p<0.0001, temperature p=0.0084) and a main effect of the KD to increase Atf4 expression (p<0.0001) (Figure 2C). There was an interaction between diet and temperature (p=0.0304) with the KD increasing Atf3 expression under both RT (p=0.0360) and TN conditions (p<0.0001) and Atf4 expression being greater in livers from KD mice at TN compared to RT (0.0129) (Figure 2C). Taken together these findings demonstrate that despite causing weight loss and improving glucose homeostasis relative to HFD mice, KD increases indices of liver inflammation and ER stress which is moderately, at least in the case of ER stress markers, potentiated in mice housed at TN.
Figure 0-2: Ketogenic diet and housing temperature alter liver gene expression.

Male C57BL6/J mice were housed at either room temperature (RT) or thermal neutrality (TN) and fed a high fat diet (HFD) for 5 weeks. Half of the mice in each temperature group were then switched to a ketogenic diet (KD). At the end of the 3-week diet intervention livers were harvested and the expression of oxidative (A), inflammatory (B) and ER stress (C) genes were measured. Data are presented as mean±SD with individual data points shown (n=9-10/group). Data was analyzed using a two-way ANOVA (diet by temperature). diet = main effect of diet, temperature = main effect of temperature as shown by a bar over the figure p<0.05. * indicates a significant (p<0.05) difference between groups joined by a bar as determined using a Tukey post hoc analysis.
The effects of a KD on weight loss and glucose tolerance are largely explained by reductions in food intake.

Since mice given a KD ate less food than HFD controls, we performed pair-feeding (PF) experiments to determine whether the metabolic effects of KD feeding could be explained in part by reductions in energy intake (Figure 3A). There was a main effect of diet (p<0.0001) and temperature (p=0.0220) on body weight with KD mice weighing less (p<0.0001) than HFD and HFD-PF mice (p<0.0001), while HFD-PF mice were lighter than HFD controls (p=0.0351). Similar results were found when analyzing weight gain with main effects of diet (p<0.0001) and temperature (p=0.0459) noted. Mice fed the KD gained less weight than HFD (p<0.0001) and HFD-PF animals (p<0.0001) with HFD mice gaining more weight than HFD-PF mice (p=0.0003). There were main effects of diet (p<0.0001) on eWAT and iWAT mass with both weighing less in KD compared to HFD (p<0.0001) and HFD-PF (p<0.0001) groups (Table 3). The pair feeding manipulation resulted in the expected changes in food intake with main effects of diet (p<0.0006) and temperature (p<0.0001) (Figure 2D). Cumulative food intake was greater in HFD compared to KD (p=0.0295) and HFD-PF (p=0.0004) groups. Oral glucose tolerance followed the weight gain data with main effects of diet (p<0.0001) and temperature (p=0.0009). The glucose AUC was greater in the HFD compared to KD (p<0.0001) and HFD-PF (p<0.0001) mice (Figure 2E-G), with the glucose AUC being greater in the HFD-PF compared to KD mice (p=0.0013). The findings from this experiment provide evidence that a portion of the beneficial effects of a KD on weight loss and glucose homeostasis is likely a function of reductions in food intake.
Figure 0-3: Reductions in food intake explain a portion of ketogenic diet induced weight loss.

Male C57BL6/J mice were housed at either room temperature (RT) or thermal neutrality (TN) and fed a high fat diet (HFD) for 5 weeks. Mice in each temperature group were then given either a ketogenic diet (KD), were maintained on a HFD or were provided with a HFD pair fed to the caloric intake of the KD group (HFD-PF) (A) and body weight (B), change in weight (C), food intake (D) and glucose tolerance (E, F, G) determined over the intervention. Please note that the glucose tolerance tests at RT and TN were performed at the same time but the glucose curves are shown on different graphs for clarity. Data are presented as means±SD with individual data points shown when possible (n=9-10/group). Data was analyzed using a two-way ANOVA (diet by temperature). diet = main effect of diet, temperature = main effect of temperature as shown by a bar over the figure p<0.05. * indicates a significant (p<0.05) difference between groups joined by a bar as determined using a Tukey post hoc analysis.
Table 0-3. The effect of pair feeding and a ketogenic diet on tissue weights in mice housed at room temperature or thermal neutrality.

<table>
<thead>
<tr>
<th></th>
<th>RT-HFD</th>
<th>RT-KD</th>
<th>RT-PF</th>
<th>TN-HFD</th>
<th>TN-KD</th>
<th>TN-PF</th>
<th>diet</th>
<th>temp</th>
<th>interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>1.15±</td>
<td>1.21±</td>
<td>0.97±</td>
<td>1.32±</td>
<td>1.12±</td>
<td>1.22</td>
<td>0.488</td>
<td>0.239</td>
<td>.0099</td>
</tr>
<tr>
<td>eWAT (g)</td>
<td>1.77±</td>
<td>0.65±</td>
<td>1.73±</td>
<td>2.09±</td>
<td>0.83±</td>
<td>1.83±</td>
<td>&lt;0.0001</td>
<td>0.068</td>
<td>0.7604</td>
</tr>
<tr>
<td>iWAT (g)</td>
<td>0.82±</td>
<td>0.21±</td>
<td>0.62±</td>
<td>0.92±</td>
<td>0.28±</td>
<td>0.87±</td>
<td>&lt;0.0001</td>
<td>0.053</td>
<td>0.5443</td>
</tr>
<tr>
<td>iBAT (g)</td>
<td>0.13±</td>
<td>0.06±</td>
<td>0.11±</td>
<td>0.21±</td>
<td>0.07±</td>
<td>0.18±</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0366</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD for n=9-10/group. Data was analyzed using a 2-way ANOVA and main effects of diet, temperature (temp) and interactions are shown. RT-HFD = room temperature high fat diet, RT-KD = room temperature ketogenic diet, RT-PF = room temperature pair fed, TN-HFD = thermal neutral high fat diet, TN-KD = thermal neutral ketogenic diet, TN-PF = thermal neutral pair fed.
KD increases energy expenditure compared to HFD mice independent of housing temperature.

As restricting food intake in HFD mice did not completely recapitulate the effects of the KD we wanted to determine if the KD was impacting indices of substrate oxidation and energy expenditure and if this effect was dependent upon housing temperature. Consequently, we measured RER, energy expenditure, and physical activity for one full light and dark cycle in mice housed in metabolic caging maintained at either room temperature or thermal neutrality. As shown in Figure 4A, there was a diet by temperature interaction (p=0.0117) in the light phase with RER being lower in HFD mice at TN compared to room temperature (p=0.0047) and RER in KD mice lower than HFD mice at room temperature (p<0.0001). In the dark phase (Figure 4B) there was a main effect (p<0.0001) of the KD to reduce RER. We next assessed absolute energy expenditure. As presented in Figure 4C, in the light phase there was a main effect of temperature (p<0.0001) with total energy expenditure being greater in mice housed at room temperature compared to TN. In the dark phase (Figure 4D), there were main effects of diet (p=0.0058) and temperature (p<0.0001) with energy expenditure being greater at room temperature and in KD fed mice. When examining 24-hour energy expenditure (i.e. light phase + dark phase) (Figure 4E) there was a main effect of TN housing (p<0.0001) to reduce energy expenditure. As body weights were different between diet groups, we ran an additional analysis using ANCOVA with body weight as a co-variate. Somewhat similar results were found in the light phase (main effect temperature p<0.0001, main effect of diet p=0.0140, interaction p= 0.5910), dark phase (main effect temperature p<0.0001, main effect of diet p<0.0001, interaction p= 0.0860) and 24-hour energy
expenditure (main effect temperature $p<0.0001$, main effect of diet $p<0.0010$, interaction $p=0.7550$). In the light phase there was an interaction between temperature and diet ($p=0.0309$) with activity levels being higher in the HFD mice at thermal neutrality compared to room temperature ($p=0.0306$) (Figure 4F). In the dark phase physical activity was lower (main effect of diet $p=0.0255$) in KD compared to HFD mice (Figure 4G). Taken together these findings provide evidence that KD marginally increases energy expenditure in the dark cycle, independent of housing temperature or increases in physical activity.
Figure 0-4: Ketogenic diet alters substrate oxidation and energy expenditure independent of housing temperature.

Male C57BL6/J mice were housed at either room temperature (RT) or thermal neutrality (TN) and fed a high fat diet (HFD) for 5 weeks. Half of the mice in each temperature group were then switched to a ketogenic diet (KD). Ten days after the diet switch mice were housed in metabolic caging and RER (respiratory exchange ratio) (A, B), total energy expenditure (TEE) (C, D, E) and physical activity (F,G) were measured during the light (A, C, F) and dark (B, D, G) phases. Data are presented as mean±SD with individual data points shown (n=8/group). Data was analyzed using a two-way ANOVA (diet by temperature). diet = main effect of diet, temperature = main effect of temperature as shown by a bar over the figure p<0.05. * indicates a significant (p<0.05) difference between groups joined by a bar as determined using a Tukey post hoc analysis.
Mice rapidly regain weight following the cessation of a KD.

Adherence to weight loss inducing diets is typically poor \(^{129,130}\) and thus we wanted to determine what would occur following the KD intervention if mice were cycled back to a HFD. As the response to a KD in regards to weight loss and glucose homeostasis were not impacted by housing temperature we completed these experiments at TN only. As in the initial experiment, mice were fed a HFD for 5 weeks to induce obesity and then half were switched to a KD (Figure 5A). As shown in Figure 5B, there was a time by diet interaction (p<0.0001) such that body weight in the HFD control mice was greater than that in mice previously fed a KD at day 0 (p<0.0001), 2 (p<0.0001), 4 (p<0.0001), 9 (p=0.0011), 13 (p=0.0031) and 19 (p=0.0312) following the diet switch. The total amount of weight gained following the diet switch was significantly (p<0.0001) greater in mice previously fed the KD (Figure 5C) and these mice had reduced eWAT and iWAT mass compared to HFD controls (Table 4). The greater weight gain in mice previously provided with the KD was paralleled by increases in food intake (p<0.0001) (Figure 5D) and weight re-gain with the diet switch was positively associated with caloric intake (Figure 5E). Despite being hyperphagic and gaining significantly more weight than HFD controls over the 3-week diet switch period, glucose tolerance was slightly, but significantly (p=0.0238) better in mice previously fed the KD diet (Figure 5 F & G). Outside of Atf3 which was reduced, the expression of genes in livers from KD fed mice cycled back to a HFD were not different than HFD controls (Table 5). Taken together this experiment provides evidence that the beneficial effects of a KD on weight loss are largely reversed when mice are switched back to an obesogenic HFD and this is strongly associated with increases in food intake.
Figure 0-5: Mice rapidly regain weight following the cessation of a KD.

Male C57BL6/J mice were housed at thermal neutrality and fed a high fat diet (HFD) for 5 weeks. Half of the mice were then switched to a ketogenic diet (KD) for 3 weeks, followed by a switch back to the HFD for an additional 3-week period (A) body weight (B), changes in weight (C), food intake (D, E) and glucose tolerance (F, G) determined. Data are presented as means±SD with individual data points shown (n=10/group) when possible. Data was analyzed using a repeated measures ANOVA (B) or an unpaired student’s t-test (C, D & G). * p<0.05 compared to the HFD control.
Table 0-4. The impact of switching mice from a ketogenic diet to a high fat diet on tissue weights.

<table>
<thead>
<tr>
<th></th>
<th>HFD</th>
<th>HFD-KD-HFD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>1.63 ± 0.35</td>
<td>1.49 ± 0.23</td>
<td>0.2939</td>
</tr>
<tr>
<td>eWAT (g)</td>
<td>2.48 ± 0.23</td>
<td>2.06 ± 0.57</td>
<td><strong>0.0466</strong></td>
</tr>
<tr>
<td>iWAT (g)</td>
<td>1.28 ± 0.26</td>
<td>0.79 ± 0.22</td>
<td>&lt;<strong>0.0001</strong></td>
</tr>
<tr>
<td>iBAT (g)</td>
<td>0.21 ± 0.06</td>
<td>0.22 ± 0.08</td>
<td>0.5940</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD for n=10/group. Data were analyzed using an unpaired students t-test with p-values provided in the far right hand column. HFD = high fat diet, HFD-KD-HFD = high fat diet -ketogenic diet – high fat diet.
Table 0-5. The effect of switching mice from a ketogenic diet to a high fat diet on the expression of oxidative, inflammatory and ER stress related genes in the liver.

<table>
<thead>
<tr>
<th>gene</th>
<th>HFD</th>
<th>HFD-KD-HFD</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpt1α</td>
<td>1.00 ± 0.22</td>
<td>1.03 ± 0.</td>
<td>0.8493</td>
</tr>
<tr>
<td>Acox1</td>
<td>1.00 ± 0.19</td>
<td>0.89 ± 0.37</td>
<td>0.4501</td>
</tr>
<tr>
<td>Ppara</td>
<td>1.00 ± 0.20</td>
<td>0.85 ± 0.30</td>
<td>0.2260</td>
</tr>
<tr>
<td>IL6</td>
<td>1.00 ± 0.58</td>
<td>0.82 ± 0.78</td>
<td>0.5888</td>
</tr>
<tr>
<td>Socs3</td>
<td>1.00 ± 0.59</td>
<td>0.22 ± 0.46</td>
<td>0.7537</td>
</tr>
<tr>
<td>Tnfa</td>
<td>1.00 ± 0.30</td>
<td>0.84 ± 0.30</td>
<td>0.2712</td>
</tr>
<tr>
<td>Chop</td>
<td>1.00 ± 0.20</td>
<td>1.00 ± 0.20</td>
<td>0.9599</td>
</tr>
<tr>
<td>Atf4</td>
<td>1.00 ± 0.14</td>
<td>1.05 ± 0.37</td>
<td>0.7168</td>
</tr>
<tr>
<td>Xbp1s</td>
<td>1.00 ± 0.37</td>
<td>0.94 ± 0.55</td>
<td>0.8036</td>
</tr>
<tr>
<td>Atf3</td>
<td>1.00 ± 0.17</td>
<td>0.50 ± 0.33</td>
<td><strong>0.0262</strong></td>
</tr>
</tbody>
</table>

Data are presented as mean±SD for n=10/group. Data were analyzed using an unpaired students t-test with p-values provided in the far right hand column. HFD = high fat diet, HFD-KD-HFD = high fat diet -ketogenic diet – high fat diet.
Weight gain after the cessation of a KD is partly explained by increases in food intake.

Having demonstrated that weight gain following the cessation of a KD is associated with increases in food intake we wanted to examine the causality of this relationship. To do so we repeated the diet switch experiment, but this time restricted the caloric intake of half the mice that had previously been fed a KD to that of the HFD controls (Figure 6A). Prior to the diet switch, mice fed the KD weighed significantly less (p<0.0001) than HFD controls (KD 25.0.7±3.3 grams, HFD 35.7±5.3 grams). Mice given ad libitum access to a HFD following the KD intervention gained more weight than either the HFD controls (p<0.0001) or mice that were pair fed to the HFD control group (p<0.0001) (Figure 6B). The HFD pair fed group gained more weight than HFD controls (p=0.0017). Pair fed mice weighed significantly less than HFD controls (p=0.0021) and mice previously fed a KD that were then given ad libitum access to the HFD (p=0.0336). eWAT mass was less (p=0.0110) in pair fed compared to HFD control mice, while iWAT mass was reduced in pair fed (p=0.0002) and HFD ad libitum (p=0.0165) compared to HFD controls (Table 6). By design total caloric intake was not different (p=0.7824) between HFD and HFD pair fed mice whereas energy intake was greater in ad libitum fed mice compared to the HFD control (p<0.0001) or pair fed (p<0.0001) groups. Glucose tolerance was improved in the pair fed compared to HFD control (p=0.0006) and HFD ad libitum (p=0.0362) fed mice. Together, the findings from this experiment provide evidence that weight gain and reversal of the improvements in glucose tolerance following the cessation of a KD are explained in large part by hyperphagia.
Figure 0-6: Increases in food intake partly explains weight gain following the cessation of a KD.

Male C57BL6/J mice were housed at thermal neutrality and fed a high fat diet (HFD) for 5 weeks. Two thirds of the mice were then switched to a ketogenic diet (KD) for 3 weeks, followed by a switch back to either the HFD, or the HFD pair fed to the same number of calories as the HFD controls for an additional 3-week period (A) and changes in weight (B), body weight (C), food intake (D) and glucose tolerance (E, F) determined. Data are presented as means±SD with individual data points shown (n=9-10/group) when possible. Data was analyzed using a one-way ANOVA. * indicates a significant (p<0.05) difference between groups joined by a bar as determined using a Tukey post hoc analysis.
Table 0-6. The impact of restricting food intake on weight regain in mice previously fed a ketogenic diet.

<table>
<thead>
<tr>
<th></th>
<th>HFD</th>
<th>HFD-KD-HFD</th>
<th>HFD-KD-HFD-PF</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver (g)</td>
<td>1.38 ± 0.34</td>
<td>1.46 ± 0.20</td>
<td>0.95 ± 0.23</td>
<td>0.2585</td>
</tr>
<tr>
<td>eWAT (g)</td>
<td>1.83 ± 0.63</td>
<td>1.50 ± 0.45</td>
<td>0.96 ± 0.36*</td>
<td>0.0131</td>
</tr>
<tr>
<td>iWAT (g)</td>
<td>0.96 ± 0.36</td>
<td>0.64 ± 0.15*</td>
<td>0.44 ± 0.05*</td>
<td>0.0002</td>
</tr>
<tr>
<td>iBAT (g)</td>
<td>0.26 ± 0.10</td>
<td>0.21 ± 0.10</td>
<td>0.21 ± 0.03</td>
<td>0.1963</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD for n=9-10/group. Data were analyzed using a one-way ANOVA. * indicates a significant (p<0.05) difference compared to HFD group. HFD = high fat diet, HFD-KD-HFD = high fat diet - ketogenic diet – high fat diet, HFD-KD-HFD-PF = high fat diet - ketogenic diet – high fat diet pair fed.
Weight gain following the cessation of a KD is not caused by reductions in energy expenditure.

To further confirm a primary role for hyperphagia in mediating weight gain following the cessation of a KD we completed metabolic caging experiments in HFD mice or in mice previously fed a KD and then switched to an ad libitum HFD or a HFD with calories matched to the HFD control group. There were no differences in RER between groups in either the light (p=0.0918) (Figure 7A) or dark phase (p=0.2505) (Figure 7B). In the light phase (Figure 7C), total energy expenditure was reduced in pair fed mice previously on a KD compared to HFD controls (p=0.0255) and previously KD fed mice provided a HFD ad libitum (p=0.0007). Dark phase (Figure 7D) (p=0.0430) and 24-hour energy expenditure (Figure 7E) were lower (p=0.0040) in pair fed compared to ad libitum fed mice previously on a KD. As body weights were different between groups, we also analyzed energy expenditure using an ANCOVA with body weight as a covariate. In the light phase, energy expenditure was reduced (p=0.0130) in pair fed compared to ad libitum fed animals previously fed a KD, while in the dark phase (corrected model p=0.120) and 24-hour energy expenditure (corrected model p=0.050) there were no significant differences between groups (corrected model p=0.120). Physical activity levels were not different between groups in either the light (p=0.5005) (Figure 7E) or dark (p=0.5010) (Figure 7F) phase. The data from this experiment indicate that reductions in energy expenditure do not mediate increases in weight gain following a switch from a KD to an obesogenic HFD.
Figure 0-7: Increases in energy expenditure do not explain weight gain following the cessation of a KD.

Male C57BL6/J mice were housed at thermal neutrality and fed a high fat diet (HFD) for 5 weeks. Two thirds of the mice were then switched to a ketogenic diet (KD) for ~3 weeks, followed by a switch back to either the HFD, or the HFD pair fed to the same number of calories as the HFD controls for an additional 10-day period at which point RER (respiratory exchange ratio) (A, B), total energy expenditure (TEE) (C, D, E) and physical activity (F, G) were measured over the light (A, C, F) and dark (B, D, G) phase. Data are presented as mean±SD with individual data points shown (n=4-6/group). Data was analyzed using a one-way ANOVA. *indicates a significant (p<0.05) difference between groups joined by a bar as determined using a Tukey post hoc analysis.
3.4. Discussion
Ketogenic diets have gained increasing attention as a potential non-pharmacological approach to treat and/or prevent obesity and impairments in glucose homeostasis. Several groups have shown that feeding mice a KD results in weight loss in parallel with an \( \sim 10-15\% \) increase in whole body energy expenditure \cite{89,92,95}. Similar to preclinical findings, KDs are effective in causing weight loss in humans, though the reported effects on energy expenditure are less consistent and more subtle (as reviewed in \cite{118}). Previous preclinical studies examining the effects of KDs on energy expenditure in mice have been completed at sub-thermal-neutral housing temperatures, i.e. room temperature \cite{89,92,95,96}. This is a well-documented stress that induces a number of compensatory responses including increases in sympathetic outflow, energy expenditure and food intake \cite{48,116}.

In the current study the ability of a KD to cause weight loss and improve glucose homeostasis was not impacted by housing temperature and seemed to be largely explained, at least with the duration of feeding examined, by reductions in food intake and marginal increases in energy expenditure. The housing temperature-independent effects of a KD are in contrast to a growing body of evidence demonstrating that housing temperature impacts a wide range of experimental endpoints including diet-induced weight gain \cite{116}, the development of inflammation and atherosclerosis \cite{59}, tumour development \cite{131}, the response to food allergens \cite{132}, pesticide-induced obesity \cite{133}, longevity \cite{134}, and metabolic adaptations to exercise \cite{56,135,136}.

In the current investigation we found that the KD reduced caloric intake, a finding consistent with several recent studies in mice with pre-existing metabolic dysfunction \cite{89,112,113}, and that there was a significant negative correlation between food intake and weight loss. Moreover, when matching the caloric intake of HFD to that of KD mice, similar
effects, though of a lesser magnitude, on weight loss and glucose tolerance were noted. While we noted higher energy expenditure in KD mice, this was not explained by differences in physical activity that were reduced in KD mice, a finding consistent with some\textsuperscript{137}, but not all\textsuperscript{92,112} previous studies. Collectively our findings provide evidence that during the initial transition from an obesogenic HFD to a KD, weight loss would appear to be primarily driven by reductions in food intake. With a longer duration of a KD, increases in energy expenditure might play a more prominent role in causing or maintaining weight loss, though this needs to be tested under thermal neutral conditions.

As adherence to restrictive diets is typically poor, we wanted to determine the effects of cycling mice back to an obesogenic HFD following a period of KD-induced weight loss. Prior work has demonstrated that consuming a KD for 1 week leads to muscle atrophy in mice\textsuperscript{114} while longer duration KD feeding in mice reduces lean mass, as determined using dual X-ray absorptiometry\textsuperscript{89}. As there is a close correlation between lean body mass and energy expenditure\textsuperscript{138} we reasoned that the prior consumption of a KD could predispose mice to weight regain secondary to reductions in energy expenditure. Contrary to this, we found that mice fed a KD and then provided with an obesogenic HFD were hyperphagic and there was a strong association between weight gain and calories consumed. Similarly, when we pair fed mice previously provided a KD with the same amount of calories as the HFD controls, this prevented a portion of the regain in weight. This finding is likely confounded, however, by the fact that TEE was reduced in pair fed mice, perhaps due to reductions in the thermogenic effect of food, thus leading to an underestimation of the role of hyperphagia in weight regain following the cessation of a KD. The role of hyperphagia in mediating weight re-gain following a
period of reduced caloric intake and depletion of white adipose tissue, as in the current study, is in keeping with pioneering work from Kennedy\textsuperscript{139}, Hervey\textsuperscript{140} and Friedman \textsuperscript{141} who posited, and subsequently demonstrated, the ‘lipostatic’ regulation of body weight.

Our findings in a preclinical model are consistent with prior work that demonstrated slight weight regain in individuals with obesity following sequential 10 day cycles of ketogenic enteral nutrition\textsuperscript{142}. While the consumption of KDs would appear to have clear metabolic benefit in conditions of obesity, the restrictive nature of these diets likely makes long term adherence difficult and subsequent weight regain driven by increases in food intake a concern.

There are several caveats with the current investigation that need to be addressed. First, the amount of protein in KDs, at least in animals housed at room temperature, would appear to be an important determinant of how effective these diets are at preventing weight gain or inducing weight loss. In this regard, the degree of weight loss or prevention of weight gain is attenuated with KDs containing a higher proportion of protein (∼10–20% vs. 5% protein by kcal)\textsuperscript{143,144}. Likewise, there is good evidence that the restriction of methionine with lower protein KDs could be a key factor in mediating weight loss\textsuperscript{145}. The KD used in the current study, while relatively low in protein, was chosen based on prior investigations using this diet that demonstrated weight loss, increases in energy expenditure and reductions in food intake\textsuperscript{89,92,95,96,145}. Within this framework future work is needed to examine if housing temperature impacts the effects of KDs with higher percentages of protein. A second consideration is the duration of the diet intervention used. In the present study mice were fed a KD for 3 weeks as prior studies have demonstrated reductions in food intake\textsuperscript{114,115} and increases in energy expenditure\textsuperscript{89}.
temperature dependency with either of these endpoints, it is possible that a longer period of KD feeding could be required to uncover an effect. Lastly, while previous work has shown similar effects of a KD on reducing food intake and body weight in male and female rats\textsuperscript{115}, sexually dimorphic responses to temperature have been reported in mice fed a high fat and sugar diet\textsuperscript{116}. Given this, future investigations should explore the impact of housing temperature on KD-induced adaptations in female mice.

In summary we have made the novel observation that housing temperature does not impact the beneficial effects of a KD on weight loss or glucose homeostasis in mice with pre-existing metabolic dysfunction. We further provide several lines of evidence that weight loss during the consumption of a KD, and weight regain when mice are cycled back to an obesogenic HFD, are explained in large part by food intake. The importance of housing mice under thermal neutral conditions is increasingly being recognized as an important step to increase clinical translatability. The current findings, at least with the endpoints examined, highlight that results from previous studies completed at room temperature are not an artifact of thermal stress and perhaps suggest that the elegant approaches taken to uncover the mechanisms mediating KD-induced improvements in metabolic health\textsuperscript{95,96} may hold some degree of relevance to humans. That said, it is our contention, along with others\textsuperscript{64}, that whenever possible thermal neutral housing should be used.
4. INTEGRATIVE DISCUSSION

Low carbohydrate-high fat diets have become increasingly popular for weight loss. In particular, the Ketogenic diet (KD), which involves very low carbohydrate intake (<5% total caloric intake), low protein (~15%), and very high fat (80%), is gaining popularity in the general population. In rodents and humans, KD can lead to weight loss and improvements in systemic glucose tolerance and insulin sensitivity. However, KDs are also linked to hepatic steatosis following as little as 3 days which is associated with hepatic insulin resistance.

In the current work, KDs led to weight loss and improvements in glucose tolerance but also marked increases in hepatic inflammation and endoplasmic reticulum stress. This is significant since both inflammation and ER stress are known to contribute to metabolic dysfunction, like insulin resistance, and even some cancers. Our KD intervention was relatively short-term, so the question remains how long-term KDs will affect liver health. Considering the crucial role the liver plays in maintaining systemic metabolic health it is unclear how these hepatic issues might translate to systemic health in long-term interventions. At the same time, we only assessed these markers in the liver, but it would be interesting to test how other tissues, like muscle, fat, and the brain, might be responding to KDs at the cellular level.

The current KD derived most of its fat content from lard, composed predominantly of saturated fat, which disproportionately contributes to the development of systemic and liver impairments, including ER stress. However, here inflammation and ER stress occurred in the face of reduced hepatic lipid content. It is generally accepted that hepatic lipid accumulation (ie. steatosis) is the driving force behind inflammation and ER stress.
so how this occurs during a KD will need to be explored. Another recent study used a KD derived largely from cocoa-butter which has an especially high content of saturated fatty acids, even compared to lard as in the current study, and observed distinct responses from those in the current work, including no hypophagia or improvement in glucose tolerance. Although this was attributed to the higher protein content of the diet, the fat content could also be contributing. Unfortunately, liver inflammation and endoplasmic reticulum stress was not assessed. When looking at the effects of saturated vs polyunsaturated fats as the main source in a KD, Furhlein et al. demonstrated that polyunsaturated fats are more effective at inducing ketosis and do not adversely affect lipid metabolism, including serum LDL, total serum TGs, and total serum cholesterol, all of which were increased by a KD high in saturated, but not unsaturated fat. Thus, considering the type of fats in KD and HFDs is important to help interpret these findings and should be considered when designing diets for humans.

Aside from potential liver complications, prior work has demonstrated that consuming a KD for 1 week leads to muscle atrophy in mice while longer duration KD feeding in mice reduces lean mass. The mechanism through which KDs lead to muscle loss could involve reduced caloric intake or protein content but maintaining muscle mass in the face of weight loss is very important for maintaining proper health and avoiding yo-yo dieting. This is particularly true for older individuals where muscle loss can be devastating for quality of life. When protein content was matched between diets there was no loss of lean mass during a KD suggesting sufficient protein could prevent muscle loss during a KD. However, a recent meta-analysis found that adherence to a KD led to significant loss of body weight, fat mass, and fat-free mass in humans. This
analysis also looked at whether the loss of fat-free mass could be prevented by combining KD with resistance exercise. Unfortunately, resistance training was not able to prevent the loss of fat-free mass seen during KD.\textsuperscript{153}

Despite their popularity, the mechanism behind KD-induced weight loss remains unclear. One possible explanation for weight loss observed with a KD is reduced food intake, which has been reported in humans \textsuperscript{93} and rodents \textsuperscript{89,112,113}. When matching the caloric intake of HFD to that of KD mice, similar effects, though to a lesser magnitude, on weight loss was observed. These results demonstrate that during the initial transition from a HFD to a KD that weight loss is primarily driven by reduced food intake.

The mechanism by which KDs reduce food intake remains to be determined. In humans, diet-induced weight loss leads to elevations in circulating ghrelin, which promotes hyperphagia and weight regain \textsuperscript{154,155}. However, in a previous study comparing mice fed either chow, high-fat diet, KD, and a pair-fed group for 9 weeks the pair-feeding produced large increases in hypothalamic AgRP and NPY expression with a concomitant suppression of POMC, all of which would be expected to stimulate hunger and food intake \textsuperscript{89}. Conversely, there was no change in NPY or AgRP but a similar reduction in POMC in the KD fed group \textsuperscript{89}. This is consistent with evidence showing that ketone bodies can directly decrease circulating ghrelin and cerebral NPY levels \textsuperscript{87,94}. Another possibility is that macronutrients have distinct effects on appetite regulating hormones \textsuperscript{156,157} so the high fat content might produce distinct hormonal profiles that do not promote hunger the same way as other hypocaloric diets. All of these mechanisms support the evidence suggesting KDs are associated with reduced perceived appetite during the diet \textsuperscript{107}.
Another possibility is that KD produced a large increase in circulating GDF15. GDF15 is a cytokine secreted from nearly every tissue, but most highly from the liver and kidney, and signals through the GFRAL receptor that is located exclusively in the brainstem. Currently the most consistent physiological effect of GDF15 is to suppress food intake, specifically high-fat foods. The elevated GDF15 seen during KD is likely liver-derived and in response to increased endoplasmic reticulum stress, as observed here. The appetite-suppressive effect of GDF15 has led to it being explored as a possible therapeutic option for obesity, but it is important to emphasize that GDF15 is generally considered a stress response cytokine acting as a sentinel for cellular distress. This can be seen in the markedly elevated GDF15 levels in numerous pathological states, including cancer, obesity, kidney and cardiac failure, and inflammatory conditions. Indeed, GDF15 causes aversive conditioning, malaise, nausea, and emesis which makes elevated GDF15 potentially problematic for long-term interventions, like KDs. Interestingly, some of these conditions, including lethargy and vomiting/emesis, are reported in humans adhering to a KD. Thus, it is possible that GDF15 could drive the nausea and malaise during KD and ultimately contribute to the poor adherence to KDs. With all of this in mind, it would be interesting to explore how mice that lack GDF15, or its receptor, GFRAL, respond to a KD.

Rodent studies suggest that KDs can lead to increased energy expenditure via adrenergic activity-induced thermogenesis in white and brown adipose tissue. Crucially, all previous pre-clinical studies investigating KD-induced weight loss, and mechanisms thereof, have been performed in rodents housed at RT which can confound many outcomes, including thermogenesis. A study by Douris et al. showed that KD-
induced weight loss and increase in BAT UCP1 protein content depends on beta-
adrenergic receptors. However, this study was done at room temperature \(^95\). This led us
to hypothesize that metabolic responses to KD would be blunted at TN since adrenergic
and thermogenic activity are reduced and the response to interventions is often blunted
at TN \(^{56,136}\). However, our results strongly suggest that thermogenesis is not involved in
the metabolic adaptations to KD since they occurred independent of housing temperature.
Indeed, we observed only minor effects of KD on whole body energy expenditure at RT
or TN making it unlikely that energy expenditure contributes much to KD-induced weight
loss, as observed in human interventions.

Taken together, this work shows that weight loss during the consumption of a KD,
and weight regain when cycling back to an obesogenic HFD, are explained in large part
by changes in food intake with minimal changes in energy expenditure. However,
understanding the mechanisms behind KD-induced hypophagia will require additional
works. Since KDs are becoming very popular in the general population, the severe liver
defects observed here are concerning and require long-term studies to understand their
significance.
5. REFERENCE LIST


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