THE METABOLIC EFFECTS OF LINOLEIC ACID VERSUS SATURATED FAT
IN MALE MICE, FEMALE MICE, AND OFFSPRING EXPOSED MATERNALLY

by

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And approved by

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ABSTRACT OF THE DISSERTATION

The metabolic effects of linoleic acid versus saturated fat in male mice, female mice, and offspring exposed maternally

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The obesity epidemic is receiving research attention, but that attention may be targeted incorrectly. The biggest change in American diet during the 20th century was a replacement of saturated and monounsaturated fats with linoleic acid in the form of industrial seed oils. In order to determine whether fatty acid profile is important for producing obesity I fed wild-type C57BL6/J mice high-fat diets with either high concentrations of saturated fat or linoleic acid. In addition to body weight, I performed metabolic assays and collected hypothalamic tissue for measuring gene expression, targeting the mechanism of hypothalamic inflammation. These experimental diets were fed to in males, ovariectomized females with and without estrogen treatment, and to breeder dams to expose their offspring prenatally. I found that in males, linoleic acid contributed to a small but significant increase in body weight compared to saturated fat, but that all high-fat diets produced obese mice. The biggest difference between groups was insulin resistance in the linoleic acid-fed mice. Gene expression evidence of hypothalamic inflammation was unclear. In female mice, estrogen conferred
protection from obesity caused by all experimental high-fat diets. Without estrogen, female mice were equally obese from saturated fat and linoleic acid. Glucose metabolism, however, was also impaired by linoleic acid, and expression of hypothalamic genes for metabolism and inflammation were highly variable. In offspring exposed to maternal high-fat diet, females were again protected but not males. Male mice weaned onto a high-fat diet gained more weight when exposed to linoleic acid through maternal feeding than saturated fat. A similar effect on glucose metabolism was seen in male and female offspring as in the first two experiments, where linoleic acid feeding impaired glucose clearance during glucose or insulin challenge. My conclusion is that, in the mouse, linoleic acid is slightly more obesogenic than saturated fat, but effects glucose metabolism much more potently. The effects on obesity, but not glycemia, are partially protected in female mice by estrogen. Due to a lack of clear hypothalamic inflammation biomarkers, these effects are likely occurring in the periphery.
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CHAPTER 1: INTRODUCTION AND BACKGROUND
1 Introduction and background

1.1 Obesity and metabolic syndrome: A problem for health, a difficulty for research

Figure 1: Rates of adult American overweight and obesity from 1960-2008. Overweight is defined as a BMI between 25 and 30, obese as a BMI between 30 and 40, and extremely obese as a BMI of 40 and above. Source: CDC/NCHS [1]

The American rate of obesity exceeds one third in most age groups, and overweight is an additional third [2,3]. Related to obesity is the so-called metabolic syndrome, a collection of signs and symptoms including insulin resistance and elevations in fasting blood glucose, blood pressure, and blood lipids. The components of metabolic syndrome are used as factors in risk assessment of diabetes mellitus and heart disease [4]. Obesity, since it is the most visible aspect of this syndrome, has come to symbolize it. Correlation of body weight versus mortality indicates that a BMI of 30-35, defined as moderately
obese, is associated with a 2-4 year reduction in lifespan; severe obesity, a BMI of 40-45, reduces expected lifespan by 8-10 years [5]. Although some individuals are considered fat yet fit [6], the BMI is largely predictive of metabolic syndrome, which is largely predictive of comorbidities. As high rates of obesity move into the lower age ranges [7] it is appropriate that a tremendous focus has been placed on researching causes and treatments.

More concerning than the increased rates of obesity and related disease is the lack of results for prevention and treatment. The NIH reports $3.405 billion USD awarded for obesity research during the years 2012-2015 and another $1.862 billion projected for the 2016 and 2017 budgets [8]. Francis Bacon argued in the 17th century that government funding of science is the most effective means for spurring inventions and discoveries, yet these sums would surely astound him. This increase in research activity as measured by dollars spent has not resulted in the Baconian model of progress, as clearly shown by the rates of obesity and related diseases. Sustained weight loss through diet, exercise, or both has been largely ineffective at the clinical and population levels [9–11]. On the pharmacological front, legacy obesity drugs have a long history of safety concerns, while the promise of safer and more effective new drugs has yet to materialize [12,13]. There are so many explanations for obesity, from the sociological ones of wealth-induced higher energy intake and sedentarism, to physiological ones such as low-grade inflammation and microbiome changes, that in many ways confusion about its cause appears to have increased.
1.2 Progress in basic science poorly translates into application

Important insights have been gleaned from obesity research, such as the discovery of leptin in 1994, the gene responsible for producing the genetically obese mouse (14). The genetic deletion of leptin, the ob gene, or its receptor, the db gene, produce two of the most widely used animal models for obesity and diabetes, respectively. Leptin is an adipostatic peptide secreted primarily by white adipose tissue, like adiponectin and resistin, and it performs an endocrine signaling function in the brain [15]. Its complete absence seems to centrally communicate a total lack of adipose tissue, which the brain then attempts to rectify through actions that establish chronic positive energy balance, those being the duel fronts of hyperphagia and reduced energy expenditure. In congenital leptin deficiency patients, recombinant leptin injections reduce obesity and insulin resistance [16]. There have only been a few dozen clinical cases of this condition, however, and the prevalence is estimated at < 1/1,000,000 [17].

Paradoxically, patients with common obesity present with increased leptin at both the adipocyte mRNA and the serum levels, compared to normal weight individuals [18]. Furthermore, while producing modest weight loss in the obese, lean subjects lose more weight from leptin treatment than do obese ones [19]. Leptin and the development of leptin resistance associated with excess weight gain highlights the complex metabolic pathology associated with obesity. Interplay of factors, rather than a direct relationship between something like leptin
and weight gain, has to be understood in order to translate basic molecular research into useful treatments and advice.

1.3 Diet energetics and energy expenditure, what might be missing?

The most basic nutritional explanation for the increase in obesity is that people are consuming more calories than they were before, above their requirement. Indeed, during the most relevant period of the obesity epidemic, roughly the 1970s through the mid to late 1990s, there was an estimated average increase in caloric intake of ~200-250 kilocalories (kcals)/day [20]. This increase, coupled with a decrease in voluntary energy expenditure, can partially account for the increased body weights seen in the late 20th and early 21st century. This explanation cannot, however, escape a question of causation: did obesity increase because people ate more, or did people eat more because they became obese?

While respecting the ($E = (\text{calories in}) - (\text{calories out})$) equation, there is a good deal of possible effectors of those terms, (calories out) in particular. The thermic effect of food, and by extension the anti-thermic effect of fasting, is one basic way that the body alters its use of calories in the face of abundance versus scarcity; there is evidence this effect is blunted in obesity [21]. This impaired thermoregulation of eating and metabolism alone suggests that impairment in the organism’s ability to accurately assess energy status could inappropriately activate energy replete or energy deficient activities.
The suppressed resting metabolic rate during calorie restricted weight loss in obese individuals can be reversed by administration of exogenous thyroid hormone [22]. This suggests a physiological basis for altered adaptive thermogenesis in weight loss. There is also evidence that macronutrient ratio, while maintaining the same caloric deficit, can cause different levels of weight loss [23]. Within macronutrient categories, some argue that different forms of macronutrients affect energy production and preferentially shunt calories into fat storage, decreasing satiety even as energy storage is excessive [24]. This has been countered by showing that other than small changes in energy expenditure, weight loss was equivalent from a low or high carbohydrate isocaloric diets in metabolic ward conditions [25]. In addition to weight, altering macronutrient ratio can decrease body fatness during energy balance and deficit [26].

This conceptual distinction is exemplified in the elevation of the lay question “is a calorie a calorie?” to a serious review in the American Journal of Clinical Nutrition [27]. A chicken and egg problem is inherent in this concept, as ultimately thermodynamic equilibrium will assert itself. Does the child grow because their brain signals to eat more food, or do hormonal signals cause growth that uses large amounts of energy and increases appetite as an effect? Is adult obesity also a hormonally driven process that causes increased food intake as a result, or are calories in excess of requirement the preceding event?

1.4 Dietary fat

Table 1: Intake of total fat from 1920-1985 and total fat, SFA, MUFA and PUFA
The first suspect in the investigation of specific obesogenic nutrients was dietary fat, since it is the macronutrient with the highest energy density. The Select Committee on Nutrition and Human Needs began advising Americans in the 1970s to avoid dietary saturated fatty acids (SFA) for heart disease prevention; this advice expanded from heart health to general health. The committee head, Senator George McGovern, had recently gone through Nathan Pritikin’s low-fat weight loss program, a factor likely important in the committee’s perspective [29]. There did exist, and continues to be produced, evidence from animal experiments that dietary fat contributes to obesity [30]. It would be several decades until much care would be taken about the particular fats being studied, but fat in general was obesogenic in the lab.

The most common fat used in these experiments was lard, which was described as primarily SFA. Although large-scale population data on obesity from SFA consumption was not available, vegetarian weight loss programs such as Pritikin’s were popular and effective, and anecdotally vegetarians have always had the reputation of weighing less than meat eaters. The scientific evidence that

<table>
<thead>
<tr>
<th>Number of studies</th>
<th>Number of subjects</th>
<th>Fat</th>
<th>Saturated fatty acids</th>
<th>Monounsaturated fatty acids</th>
<th>Polyunsaturated fatty acids</th>
<th>P.S</th>
</tr>
</thead>
<tbody>
<tr>
<td>1920–1929†</td>
<td>2</td>
<td>148</td>
<td>35.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1930–1939‡</td>
<td>2</td>
<td>37</td>
<td>41.2</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>1940–1949§</td>
<td>20</td>
<td>1,178</td>
<td>37.6</td>
<td>15.3 [64]</td>
<td>10.7 [64]</td>
<td>2.5 [64]</td>
</tr>
<tr>
<td>1950–1959¶</td>
<td>46</td>
<td>20,932</td>
<td>40.5</td>
<td>16.6 [3951]</td>
<td>20.8 [3863]</td>
<td>4.3 [3951]</td>
</tr>
<tr>
<td>1970–1979**</td>
<td>53</td>
<td>73,499</td>
<td>37.8</td>
<td>13.8 [61068]</td>
<td>14.0 [54909]</td>
<td>5.1 [61134]</td>
</tr>
<tr>
<td>1980–1985††</td>
<td>20</td>
<td>1,492</td>
<td>37.5</td>
<td>11.8 [178]</td>
<td>12.4 [52]</td>
<td>5.4 [155]</td>
</tr>
</tbody>
</table>
cast a shadow over SFA was not obesity research but the growing heart disease problem. A scientific movement against animal fats began in 1913 with Anitschkow and Chalatow’s cholesterol-fed rabbit and reached a fever pitch in the 1960s with Ancel Keys’s writings on heart disease factors. The 1970 publication of the so-called Seven Countries Study laid the cause of the biggest killer in America at the feet of cholesterol and SFA [31]. Elevated blood cholesterol was believed to be the cause of atherosclerosis, and although there was confusion as to whether it was dietary cholesterol or dietary SFA that raised blood cholesterol, almost every food that contains one contains the other. The blood pressure lowering effects of vegetarian diets [32] and hypolipidemic effects of polyunsaturated fat (PUFA) [33] bolster the story of SFA being uniquely unhealthy.

What happened from the mid-1970’s on was a reduction in fat intake, SFA in particular [28], simultaneous with the largest recorded increase in obesity rates of about ten percentage points. This was so counter-intuitive to the dominant thinking on obesity that it was dubbed the American Paradox [34], a reference to the high animal fat, low obesity phenomenon known as the French Paradox. Taking a long view of the entire 20th century, consumption of high SFA foods such as beef, eggs, dairy, pork, and solid cooking fats have all decreased [35]. Based on the available data, dietary fat intake is flat whereas SFA intake is inversely correlated with rising obesity rates.
1.5 Dietary PUFA

Table 2: Major sources of calories in 1909 and 1999 [35].

<table>
<thead>
<tr>
<th>Food category</th>
<th>1909</th>
<th>1999</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soybean oil</td>
<td>0.006</td>
<td>7.38</td>
<td>123,810</td>
</tr>
<tr>
<td>Poultry</td>
<td>0.94</td>
<td>4.94</td>
<td>426</td>
</tr>
<tr>
<td>Spice</td>
<td>0.22</td>
<td>0.85</td>
<td>277</td>
</tr>
<tr>
<td>Oils</td>
<td>0.44</td>
<td>1.55</td>
<td>250</td>
</tr>
<tr>
<td>Shellfish</td>
<td>0.06</td>
<td>0.15</td>
<td>163</td>
</tr>
<tr>
<td>Shortening</td>
<td>2.17</td>
<td>5.67</td>
<td>161</td>
</tr>
<tr>
<td>Nuts</td>
<td>0.65</td>
<td>1.62</td>
<td>149</td>
</tr>
<tr>
<td>Sugars</td>
<td>10.64</td>
<td>16.95</td>
<td>59</td>
</tr>
<tr>
<td>Fruit</td>
<td>2.52</td>
<td>3.36</td>
<td>33</td>
</tr>
<tr>
<td>Game</td>
<td>0.12</td>
<td>0.12</td>
<td>0</td>
</tr>
<tr>
<td>Beef</td>
<td>5.46</td>
<td>4.98</td>
<td>-9</td>
</tr>
<tr>
<td>Eggs</td>
<td>1.56</td>
<td>1.4</td>
<td>-10</td>
</tr>
<tr>
<td>Dairy</td>
<td>15.44</td>
<td>13.54</td>
<td>-12</td>
</tr>
<tr>
<td>Finfish</td>
<td>0.47</td>
<td>0.39</td>
<td>-16</td>
</tr>
<tr>
<td>Vegetables</td>
<td>6.35</td>
<td>4.75</td>
<td>-25</td>
</tr>
<tr>
<td>Pork</td>
<td>6.18</td>
<td>4.28</td>
<td>-31</td>
</tr>
<tr>
<td>Grains</td>
<td>36.75</td>
<td>22.43</td>
<td>-39</td>
</tr>
<tr>
<td>Fats</td>
<td>8.63</td>
<td>5.22</td>
<td>-39</td>
</tr>
<tr>
<td>Legumes</td>
<td>0.98</td>
<td>0.35</td>
<td>-64</td>
</tr>
<tr>
<td>Lamb</td>
<td>0.42</td>
<td>0.07</td>
<td>-83</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>—</td>
</tr>
</tbody>
</table>

Unlike consumption of SFA, which has decreased, consumption of PUFA has increased unambiguously and dramatically. Although fatty fish and fish oil consumption has been a popular dietary trend for the past 10-15 years, it is the n-6 linoleic acid (LA), not the n-3 PUFA in fish oils, whose dietary contribution has surged. Over the course of the 20th century, percent kcal contribution from solid fats went from 8.63 to 5.22 (39% decrease) whereas non-soy liquid oils
increased from 0.44 to 1.22 (250% increase) and soybean oil from a negligible 0.006 to 7.38 (123,810% increase) [35].

Regarding solid fats, the PUFA content of pig lard has doubled from the 1950s USDA measurements still extant on their nutrition website to today; most of this change came from LA at the expense of oleic, palmitic, and stearic acids [36]. Pig adipose is a direct reflection of the fatty acid (FA) profile of their diet, even being used as a predictive tool by buyers and sellers of specialty Iberian pork [37]. Beef fat is less influenced by diet, but increased LA is measurable in the FA profile of grain fed versus grass fed cows [38]. The contemporary American is consuming 8-10% of their kcals from LA, whereas the early 20th century American consumed 1% or less. This increase was not steady, but loosely fits a dual inflection parabola.

The first inflection point is a geometric increase starting in the 1940s (2.5% kcals from PUFA) moving through the 1960s and 1970s (5-5.5% kcals from PUFA) and peaking sometime in the 1990s or early 21st century (>15% kcals from PUFA) [28], and tapering off as a limit function approaching the year 2000. It is tempting to see the similarities between this and the increase in obesity, which started in the early 20th century but experienced the greatest rates of increase from the 1970s through the 1990s, leveling off after that [39]. A related metric, vegetable oil consumption, nearly doubled between the years 1960 and 2000, from 19g to 37g per day [40] but has since slowed down in its
rate of increase. Vegetable oil is approximately 50% LA. There is a positive correlation between dietary PUFA, specifically LA, and the rate of obesity.

1.6 Experimental models of obesity

Animal models are the primary experimental tool for studying human conditions like obesity and metabolic syndrome. Spontaneous mutations such as the leptin deficient ob/ob mouse or the Zucker rat have been useful for studying obesity physiology, but since the current obesity epidemic does not have a monogenetic origin, diet-induced obesity (DIO) is a more appropriate technique. Engineered mutations, chemical treatments, and surgical manipulations are also unrepresentative of the cause of common human obesity. Several diets have been used to induce obesity in rodents. This is, in one aspect, unfortunate, that aspect being inter-experimental comparability. For example, the cafeteria diet is highly variable in composition and based on the individual researcher, but is often a fat and carbohydrate rich, energy dense, processed food diet that might be described simply as “junk food.” Some authors, especially early in obesity research, have used the terms cafeteria diet and high-fat diet (HFD) interchangeably [41].

This type of diet presents a problem for ascribing causation, as factors suspected to induce obesity are intermixed. A cafeteria diet, or early field HFD, may have any and all of the following qualities: high in fat, starch, sugar, and overall energy density, low in micronutrient content and fiber, hyper-palatability,
and so on. As the field progressed, HFD emerged as an experimental diet paradigm that, although having different iterations, attempted to standardize many factors and induce obesity primarily with an elevated fat content. It was then possible to show that, other variables held steady, an \textit{ad libitum}-fed HFD could induce obesity in rodents.

\subsection*{1.7 Fatty-acid profile of obesity-inducing experimental diets}

The next development in the field was to consider what type of fat was most effective at inducing obesity. Although it is now common for researchers to present evidence for different metabolic effects by fat type \cite{42}, early research treated fat as a non-specific component for increasing energy density. By an accident of history, the vast majority of HFDs were, and still are, lard based.

\subsubsection*{1.7.1 Change in lard use}

Consumer lard use had been decreasing since the early 20\textsuperscript{th} century, due largely to coincidental public relations events. The change in consumer perception of lard went from positive, its use as the primary cooking fat, to negative and something to be avoided, due to the portrayal of disgusting rendering conditions including men falling into vats and being rendered with pork fat in Upton Sinclair’s “The Jungle,” the introduction of “clean” and “healthier” hydrogenated seed oils by Procter and Gamble chemist E. C. Kayser, and
culminating in the characterization of all animal tissue-based fats as causing heart disease [43].

The result of this change in consumption was an excess supply looking for a use, and lard is a convenient fat for mixing and pelleting. Since the common knowledge is that animal fats are predominantly saturated, researchers began reporting their results in producing obesity, insulin resistance, hypertension, dyslipidemia and the rest of the metabolic syndrome in rodents as being caused by a high SFA diet.

1.7.2 Change in lard composition

The problem with this reporting is that it did not take into account changing animal husbandry methods in the second half of the 20th century. Specifically, commercial pig feeding shifted from so-called slop made from damaged and otherwise unsellable produce and other scraps, to grain feed supplemented with seed oils. One of the reasons for this change was price, coming from subsidization of grain and soy agriculture in the Farm Bill [44], while at the same time demand for seed oil paints, coatings, and adhesives was replaced by petroleum products [45]. Farmers and animal scientists were also keen on following health trends and experimented as early as the 1930s [46] through the 1970s [47] with increasing the PUFA content, thereby decreasing the SFA and monounsaturated fatty acid (MUFA) content, of pork fat through seed oil feeding.
This leads to a question of what exactly has been the FA profile of the average experimental HFD? At one extreme, the pigs on the south Pacific island of Tokelau, fed on breadfruit, pandanus and coconut, have body fat with a PUFA content of 3.1%, 2.0% of that being LA [48]. The USDA’s National Nutrient Database for Standard Reference lists pork fat as having a PUFA content of 11.5% [49]. This number likely represents the lard of pigs fed mixed slop and grain. Testing of lard by Research Diets in 2011 and 2012 (Table 3) show a PUFA content of ~28%, with ~24% as LA [50]. Soybean or safflower oil are customarily added for linolenic acid, resulting in a lard-based HFD in which LA is ~30% of total fat [51]. The ubiquitous designation of these HFDs, and more importantly their pathological effects, as originating from mostly SFA is a mistake whose scope and consequence in the field is hard to overstate.

1.8 Fatty-acid profile interpretation in obesity research

As researchers made more of an effort to examine different types of fats and confirm the FA profile of their HFDs, results in the field have diverged. Some studies maintain that SFAs are especially obesogenic [52]. Others contradict this, demonstrating safflower oil and even olive oil to be more obesogenic than tallow [53]. To avoid the mixed nature of fatty acids in tallow, coconut oil has been compared to vegetable oil and shown to be less [54] or even non-obesogenic [55]. One study reports a similar level of obesity from SFA and PUFA, making a distinction between the mechanism involved [56]. Meanwhile, some authors
misrepresent their dietary composition, such as reporting results in a study using a <13.8% SFA HFD as SFAs causing inflammation and subsequent obesity [57]. Arithmetic shows that a 13.8% SFA HFD is 86.2% not SFA; this kind of misrepresentation is unfortunately common in the field.

1.9 Hypothalamic targets, neuroinflammation, and obesity

As shown in lesion experiments, the hypothalamus is a controlling center for energy balance [58]. The areas that, post-lesioning, resulted in obesity highly express leptin receptor, relating the lesion experiments to the ob-/ob- mouse [59]. Seated atop a fenestrated capillary bed partially bypassing the blood-brain barrier, the Arcuate nucleus (ARC) of the hypothalamus contains two populations of neurons important for energy balance, pro-opiomelanocortin/cocaine and amphetamine related transcript (POMC/CART) producing and neuropeptide Y/Agouti-related peptide (NPY/AgRP) producing neurons [60]. The anorexigenic POMC/CART and orexigenic NPY/AgRP neurons receive neural, peptide (insulin, leptin, adiponectin etc.) and nutrient (circulating glucose and free fatty acids) borne energy status information. In addition to having access to the periphery, these nuclei crosstalk with others, innervating and being innervated by neurons of the lateral, ventromedial, paraventricular and dorsomedial nuclei of the hypothalamus [60]. The whole of these neuronal interactions with the periphery and with each other, deemed the “first order” energy sensing system, integrates energy status for relay downstream. The first order system acts agonistically or
antagonistically towards the “second order” energy sensing system of melanocortin receptors 3 and 4 (MC3R and MC4R) [61]. The stimulation or inhibition of that system can suppresses food intake and increase energy expenditure or the reverse [62–64]. Hypothalamic inflammation (HI) can influence parts of these energy-sensing complexes, and molecular evidence for it is found in rodent obesity studies whenever investigated [65–67].

HI has been suggested to have a variety of causes. Activation of pattern recognition receptors (PRR) [57] of the innate immune system and related reactive oxygen species (ROS) production [68] can initiate HI. Increased cytokine/chemokine activity impairs insulin signaling to POMC and NPY neurons through interference with the signaling cascade of insulin receptor substrate (IRS) to phosphoinositide-3-kinase (PI3K) [69]. Similarly, both insulin and leptin signaling are blocked by activation of suppressor of cytokine signaling 3 (SOCS3) and its inhibition of signal transducer and activator of transcription 3 (STAT3) [70,71]. Resistance to these signals of adiposity negative feedback progressively increase the defended body weight, consistent with the difficulty observed in maintaining weight loss achieved through diet and exercise [9,72,73]. Reversal of the HI induced obese phenotype has been shown from anti-inflammatory drug treatment [57,74] and also dietary FA manipulation [75]. Deletion of the MyD88 gene that couples PRR binding to nuclear transcription factor kappa-β (Nf-κβ) activation, a potent inflammatory signaling mechanism, protected mice against DIO and leptin resistance [76].
1.10 Hypothalamic inflammation and fatty-acids

Within the HI hypothesis of obesity, FAs feature prominently as a cause for many of the defects [66]. There is evidence of toll-like receptor 4 (TLR4) activation, a receptor previously known to respond to bacterial lipopolysaccharide (LPS), by SFA, leading to neuroinflammation [77].

Alternatively, the prostaglandins that induce inflammatory cytokine production are products of n-6, and to a lesser extent n-3, PUFA liberated via lipolysis [78]. Furthermore, lipid oxide and peroxide radicals, which stimulate inflammation, can only be produced from PUFA [79]. The theoretical potential for both SFA and n-6 PUFA to cause obesity inducing HI makes the importance of proper reporting of HFDs necessary for progress in exploring this mechanism.

1.11 Blood sugar and fatty-acids

Disturbed glucose metabolism is a common comorbidity with obesity, and seems to be directly related to FA metabolism. Originally described as the glucose-FA cycle in 1963, the competition between glucose and FAs for use as energy substrate is the most important non-hormonal factor in glucose metabolism. Put simply, free FAs in the blood restrict tissue glucose uptake and usage, and high blood glucose restricts lipolysis and shunts FAs into esterification rather than mitochondrial oxidation [80]. Insulin enhances glucose metabolism, while cortisol, glucocorticoids, and growth hormone do the opposite.
Most importantly, for a given level of circulating insulin, an increase in blood FAs will reduce its ability to modulate glucose metabolism, or cause resistance to insulin. In this scheme, glucose use is spared through an inhibition, in order of greatest to least importance, of decarboxylation, multiple steps of phosphorylation, and transport. The primary enzymes involved are pyruvate dehydrogenase complex (PDH), 6-phosphofructo-1-kinase (PFK1), and, depending on the tissue, hexokinase or glucokinase and glucose transporter type 4 (GLUT4) or glucose transporter type 2 (GLUT2). Multiple kinases inhibit PDH through reversible phosphorylation [81]; this inhibition is stimulated by products of PDH, acetyl-CoA in particular, which is also a FA product. Citrate, a product of fat and sugar energy substrates one step removed from acetyl-CoA, is a PFK1 inhibitor [82]. Liver glucokinase is directly inhibited by acyl-CoA [83].

In addition to their effect on glucose metabolism through beta-oxidation, FAs can bind to peroxisome proliferator-activated receptors (PPARs). The alpha isoform, activated by n-6 and n-3 PUFA, stimulates beta-oxidation primarily in liver, kidney, and heart tissues [84]. The gamma isoform, expressed in adipose tissue, facilitates adipocyte differentiation and FA uptake in adipose [85] and in muscle [86]. That PUFA seem to be more effective ligands than SFA for the PPARs [87] suggests a possible mechanism for a difference in glucose metabolism effects from the different types of FAs. Another mechanism by which
PUFA and SFA may alter glucose metabolism differently is the greater reliance of the former on the acyl carnitine transfer system to translocate into mitochondria and begin the process of beta-oxidation [88].

The ability to effectively use glucose, or the loss of that ability, is tied to obesity, metabolic syndrome, and many diseases. Aging alone shifts the body towards oxidizing FAs over glucose, presenting with higher insulinemia and increased fat mass [89]. That FAs affect glucose metabolism is widely accepted, but whether different types of fats have different affects is much less understood.

1.12 Sex differences and estrogen

Obesity presents with different patterns of fat distribution, which differ in their association with risk factors for disease [4,74,90] and their prevalence between sexes [91,92]. The rate of obesity in men is about 3% higher than women from the ages of 30 through 50; after that they are equal [93]. This disparity, and its loss, may be related to the steep decline in ovarian steroids. Hormones important to energy balance such as leptin are known to differ in level and activity by sex both in adolescence [94] and adulthood [95] and both obesity [96] and its health damaging comorbidities [97] appear to be sexually dimorphic. Beyond BMI measurements, adipose accumulates differently in males and females, an effect related to differences in leptin signaling in the brain [98].

The sex steroid 17beta-estradiol (E2) is important in feeding behavior and heat production, and is shown to be protective against insulin resistance [99].
These effects of E2 occur through peripheral and central mechanisms. E2, specifically through actions in the brain, is known to suppress feeding and fat accumulation and augment energy expenditure and activity. The key brain regions mediating E2’s effects on energy homeostasis are the hypothalamus and the hindbrain [100–103]. Specifically, estrogen receptor alpha is highly expressed in the same nuclei involved in HI, and silencing this receptor induces the metabolic syndrome in female mice and rats [103].

A decrease in circulating estrogens due to ovariectomy or menopause is associated with weight gain in both rodent models and humans, which is attenuated by E2 replacement [104]. Obesity researchers tend to use male animals because the cycling nature of estrogen in female animals is a confounder that must be controlled for. This produces a relative paucity of DIO experimentation data from female animals. This lack is exacerbated in explorations of more novel mechanisms, such as HI, because of the confounding sensitivity to those hypothalamic energy balance systems to changes in estrogen signaling. In order to maximize the relevance of a nutritional manipulation experiment, however, it should be performed on both sexes.

1.13 Maternal diet obesity physiology

Taking into account the nutritional status of the mother, rather than just that of the offspring, in studies of obesity was began by the Barker “fetal/infant origins of disease hypothesis.” Stated simply: a poor nutritional environment
during pregnancy, lactation and early infancy predisposes those offspring, whose nutritional environment is now more abundant, to chronic diseases later in life such as heart disease, type II diabetes mellitus, and obesity [105]. Attempts at modeling this obesity predisposition through maternal under nutrition have had mixed results. They do, however, suggest an altered development of the hypothalamic appetite regulating system attributed to a reduced postnatal leptin surge and “catch-up” weight gain [106–108]. These findings, although interesting, are largely inapplicable to the Western obesity epidemic that is characterized by nutritional abundance as opposed to restriction. This maternal environment of over nutrition is most often studied in rodent DIO models. Some studies show, similar to maternal under nutrition, lower birth weight in treated offspring than control offspring followed by a catch-up weight gain and eventual obesity and insulin resistance [109,110]. Higher birth and adult weights in offspring of DIO dams compared to control offspring has also been observed [111].

There is a considerable body of research on maternal HFD influencing offspring obesity and gene expression, but few studies have looked specifically at different types of HFD’s or at markers of HI. Type of FAs in circulation during gestation do affect gestation length and birth weight and length [112], and indeed maternal LA intake was the only factor negatively correlating with fetal growth in a prospective, longitudinal study of maternal FA status [113]. Maternal intake is the primary driver of fetal FA profile, but throughout pregnancy the efficiency of transfer of some FAs through the placenta changes [114]. During lactation,
dietary FAs are well represented in milk [115]. Maternal diet FA profile is largely delivered to the offspring during gestation and lactation, and appears to exert effects in addition to use as fuel.

Expression of PPARs, likely a control point for lipid metabolism and adipocyte differentiation through FA, and specifically PUFA, signaling, is altered through maternal HFD [116]. Hypothalamic IKKbeta activation, a powerful inflammatory switch, is modulated by maternal HFD [117]. The particular reflection of fetal brain FA profile to maternal diet [118] makes plausible the possibility of effects on the hypothalamic energy balance nuclei. Studies showing clear adult obesity effects [109] and even hypothalamic leptin resistance [119] from maternal high-fat feeding suffer from the aforementioned weakness of not reporting FA profile or using standardized HFD. There is evidence of a link between specifically LA in maternal diet to changes in E2 signaling and metabolism in offspring [120]. In female offspring, E2 related cancer risk is elevated from high maternal dietary LA [121]. Thus there is copious evidence that maternal HFD affects offspring metabolism and can cause obesity, and that specific FAs like LA exert distinct effect from maternal exposure. What is missing is the study of adult obesity from maternal exposure to HFDs of specific FA profiles.

1.14 Summary and objectives
This dissertation aims to determine whether LA or SFA is more obesogenic in the three experimental contexts described in the objectives below. Assays assessing general metabolic health, and the mechanistic involvement of HI, will also be used.

**Objective 1: Determine how HFDs with different FA profiles (SFA versus LA) differentially produce obesity through modulation of hypothalamic inflammation.** We will test the hypothesis that a high LA diet will more strongly induce the altered central and peripheral states associated with DIO than a high SFA diet in intact male mice. Isocaloric and isolipidic diets will be designed with a high LA or SFA content. We will measure body weight and composition, food intake, glucose homeostasis, indirect calorimetry, plasma metabolic peptides and mRNA associated with inflammatory cytokines and metabolism related neuropeptides in the hypothalamus. The results of this aim will help explain the mechanism of DIO produced by HFD and describe the relative potency FA species in causing chronic positive energy balance.

**Objective 2: Determine how E2 has differential effects on the obesogenesis of high SFA or high LA diet in females.** We will test the hypothesis that females will respond to DIO from our high SFA or high LA diets, and the effects of hypothalamic inflammation and the dysregulation of energy homeostasis will be observed in an LA and an E2-dependent manner. We will
measure hypothalamic levels of inflammatory cytokines and regulatory neuropeptide mRNA as well as body weight and composition, food intake, glucose homeostasis and indirect calorimetry in female mice following ovariectomy with or without E2 treatment and fed high SFA or high n-6 PUFA diets. The results of this aim will contribute to our understanding of the influence of sex steroids on the differences in the hypothalamic control of energy homeostasis under the physiological challenge of DIO from different FAs.

**Objective 3: Determine the metabolic effects on offspring of dams fed high SFA or high LA HFDs.** We will test the hypothesis that an HFD high in LA fed maternally will cause more metabolic disturbance and obesity in control and HFD fed offspring than an HFD high in SFA. We will measure body weight and composition, food intake, glucose homeostasis, metabolic rate, and inflammatory cytokine serum levels. The results of this aim will increase the understanding of maternal influence on obesity and metabolism by focusing on FA type, a hitherto ignored nutritional variable in transgenerational obesity research.

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CHAPTER 2: LINOLEIC ACID CAUSES GREATER WEIGHT GAIN THAN SATURATED FAT WITHOUT HYPOTHALAMIC INFLAMMATION IN THE MALE MOUSE
2. Linoleic acid causes greater weight gain than saturated fat without hypothalamic inflammation in the male mouse

2.1 Abstract

A significant change in the Western diet, concurrent with the obesity epidemic, was a substitution of saturated fatty acids with polyunsaturated, specifically linoleic acid (LA). Despite increasing investigation on type as well as amount of fat, it is unclear which fatty acids are most obesogenic. The objective of this study was to determine the obesogenic potency of LA vs. saturated fatty acids, and the involvement of hypothalamic inflammation. Forty-eight mice were divided into 4 groups: low-fat or 3 high fat diets (HFD, 45% kcals from fat) with LA comprising 1%, 15%, and 22.5% of kilocalories, the balance being saturated fatty acids. Over 12 weeks, bodyweight and composition, food intake, calorimetry, and glycemia assays were performed. Arcuate nucleus and blood were collected for mRNA and protein analysis. All HFD-fed mice were heavier and less glucose tolerant than control. 22.5% LA caused greater bodyweight gain, decreased activity and insulin resistance compared to control and 1% LA. All HFDs elevated leptin and decreased ghrelin in plasma. Neuropeptides gene expression was higher in 22.5% HFD. The inflammatory gene Ikk was suppressed in 1% and 22.5% LA. No consistent pattern of inflammatory gene expression was observed, with suppression and augmentation of genes by one or all of the HFDs relative to control. These data indicate that in male mice, LA induces obesity and insulin
resistance, and reduces activity, more than saturated fat, supporting the hypothesis that increased LA intake may be a contributor to the obesity epidemic.

2.2 Introduction

Obesity in the United States is increasing in prevalence, and shows association with many serious, non-communicable diseases (1). This has motivated an extensive effort to discover causes of obesity and develop methods for prevention and treatment. The high-fat diet (HFD)-fed mouse is a widely used model for diet-induced obesity (DIO) (2). Compared to the “cafeteria diet,” it provides nutrient consistency, and high sucrose diets, unless also high in fat (3,4), produce a lean mouse (5,6). Although recognized as the best DIO model, a better understanding of the influence of fatty acid (FA) profile would aid in discovery of mechanisms and treatments.

Both saturated (7,8) and polyunsaturated (9,10) fatty acids (SFA and PUFA) have been identified as obesogenic compared to other FA. Incorrect FA profile reporting may be causing these divergent results. The FA profile of experimental diets is rarely confirmed with gas chromatography, but instead reported from a nutrient database. Recent testing by Research Diets (Research Diets, Inc., New Brunswick, NJ) showed that lard, commonly used in HFDs, contains twice the amount of PUFA, mostly as linoleic acid (LA), reported on the USDA National Nutrient Database for Standard Reference Release (11). Similarly, the FAs consumed by the relevant human population should factor into
DIO research. Total fat intake was not greatly changed during the 20\textsuperscript{th} century, and any change in total fat was dwarfed by the increase in PUFA (primarily LA) at the expense of SFA and oleic acid (12). Accurate reporting of FA profile and integrating epidemiological data are necessary prerequisites to theorizing on mechanisms of obesity.

One candidate mechanism for DIO is hypothalamic inflammation (HI), in which fatty acids play a large part (13–17). In short, chronic low-grade inflammation impairs neuronal sensing of energy status, resulting in a melanocortin system that acts as if adipose storage was insufficient on downstream neuronal circuits controlling feeding behavior and energy expenditure. One proposed HI pathway is activation of Toll-like receptor 4 (TLR4) by SFA (7,18). Indeed, knockout of TLR4 abrogated DIO from an SFA-rich HFD but, interestingly, not from an LA-rich HFD, which produced greater obesity regardless of TLR4 status (19). Although important to innate immunity through the recognition of lipopolysaccharide, TLR4 is not the entirety of the inflammatory system. Central inflammation (20) and metabolic derangement due to HI (21) can be a response to reactive oxidative species. PUFA are the only fatty acids prone to non-enzymatic oxidation at mammalian body temperatures. Acyl chain carbon-hydrogen bonds surrounded by carbon-carbon double bonds are susceptible to nucleophilic attack (22). Thus, inflammatory mechanisms exist for SFA and PUFA.
Due to the contradictory data on FAs in rodent DIO research, the Western diet being modeled, and the involvement of FA in HI, we hypothesized that LA will cause more weight gain, metabolic derangements, and greater expression of HI markers than SFA. We fed male WT C57BL6/J mice one of three HFDs with equal fat content but different FA profiles and measured weight, food intake, glucose metabolism, indirect calorimetry, and activity. Upon sacrifice, we measured plasma metabolic peptides and biomarker mRNA of HI and metabolism in the arcuate nucleus of the hypothalamus.

2.3 Material and methods

2.3.1 Animal care

All animal treatments were in accordance with institutional guidelines based on National Institutes of Health standards and performed with Institutional Animal Care and Use Committee approval at Rutgers University. Male WT C57BL6/J mice were selectively bred in-house, maintained under controlled temperature (23°C) and photoperiod conditions (12/12 h light/dark cycle), and given access to food and water ad libitum. Mice were weaned and ear-tagged at post-natal day 21 and housed in groups until start of experiment.

2.3.2 Experimental diets

All experimental diets were prepared as pellets by Research Diets (New Brunswick, NJ). FA profile was assured through in-house gas
chromatography/mass spectroscopy. Our control diet (CON) was Research Diets D12450B (10% kcals from fat). This was to control for environmental effects, no diet can truly control for fat amount or type, as macronutrient exclusion requires either compensatory nutrient inclusion or relative caloric deficit. Our 3 HFDs were isocaloric and isolipidic to Research Diets D12451 (45% kcals from fat) and named for the amount of calories derived from LA; 1% = mostly coconut oil with some seed oils, 15% = a more even mixture of coconut oil and seed oils, 22.5% = mostly safflower and sunflower seed oils (see Table 1 for FA profile). We chose to maintain a constant n-3 content rather than a constant n-6/n-3 ratio, as this is more similar to the Western dietary change we are modeling. Coconut oil was used for SFA because all other food sources, such as lard, butter, or tallow, have significant amounts of n-3 and n-6 PUFA, and comparing synthetic- with food-derived FAs would also confound the interpretation. Although the chain length is probably a factor in obesity, palmitate from tallow has been shown to be less obesogenic than safflower oil (23). All diets had identical protein, fiber, and micronutrient contents.

2.3.3 Experimental design

Experimental feeding began at 12 weeks of age. See Table 2 for timeline. Mice were housed 3 per cage and given ad libitum access to food and water. Body weight and food intake (per cage food intake) were recorded weekly for 12 weeks followed by body composition measurements using an EchoMRI 3-in-1
Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) and calorimetric and activity measurements (48 h run) via Columbus Instruments’ Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Inc., Columbus, OH, USA). A glucose tolerance test (GTT), following an overnight fast, was administered via intraperitoneal (ip) injection of 2g/kg glucose in 0.9% saline solution. Blood glucose (BG) from tail blood was measured with an AlphaTrak 2 Blood Glucose Monitoring System (Abbott Laboratories, Abbott Park, IL, USA) pre-injection and 15, 30, 60, 90, 120 and 180 minutes post-injection. An insulin tolerance test (ITT) following a 5 h fast involved an injection of 0.5 U/kg insulin (Humulin R, Lilly, Indianapolis, IN, USA) in 0.9% saline solution and followed the same BG measurement scheme as GTT. Mice were given 4 days of rest each between CLAMS, GTT, and ITT.

2.3.4 Tissue collection

At completion of physiological assays, mice were given another 4 days of rest while remaining on the same diet and then killed by decapitation after ketamine sedation (100 μl of 100 mg/ml, ip). Trunk blood was collected and prepared for plasma analysis of peptide hormones and cytokines by Luminex Magpix multiplex (EMD Millipore, Billerica, Massachusetts, USA). Plasma was prepared by adding a protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mg/mL, Sigma-Aldrich) to each K⁺ EDTA collection tube. Samples were maintained on ice until centrifugation at 3,000 rpm
for 10 min at 4°C. Supernatant was then collected and stored at −80°C until analysis. In preparation for RNA extraction and measurement, the basal hypothalamus was cut using a brain slicer matrix (Ted Pella, Inc., Redding, CA, USA), into one mm thick coronal rostral and caudal slices corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates (24). The slices were transferred to RNAlater (Life Technologies, Inc., Grand Island, NE, USA) and stored overnight at 4°C. The rostral and caudal parts of the arcuate nucleus were dissected using a dissection microscope. The combined arcuate tissue was stored at −80°C. Total RNA was extracted from combined nuclei (rostral and caudal arcuate) using Ambion RNAqueous-Micro Kits (Life Technologies, Inc.) according to the manufacturer’s protocol. Total RNA was DNase I-treated, using the extraction kits, at 37°C for 30 min to minimize genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA, USA). Samples with an RNA Integrity Number below 6 were not used.

2.3.5 Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 μl 5× Buffer, 25 mM MgCl₂, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng
random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/µl Rnasin (Promega) and 100 mM DTT in DEPC-treated water (GeneMate, Bioexpress, Inc., Kaysville, UT, USA) to a total volume of 20 µl. Reverse transcription was conducted using the following protocol: 5 min at 25°C, 60 min at 50°C, and 15 min at 70°C. The cDNA was diluted 1:20 with Nuclease-free water (GeneMate, Bioexpress) for a final cDNA concentration of 0.5 ng/µl and stored at −20°C. All primers were designed to span exon–exon junctions and synthesized by Life Technologies, Inc., using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 2 for a list of all primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 µl of cDNA template (an equivalent of 2 ng total RNA) was amplified using either PowerSYBR Green master mix (Life Technologies) or Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in triplicate to determine the efficiency \( E = 10^{-\frac{1}{m}} - 1, \ m = \text{slope} \) of each primer pair. All efficiencies expressed as percent efficiency were approximately equal (one doubling per cycle, 90–110%). The relative mRNA expression data was analyzed using the \( \Delta \Delta C_T \) method (25,26). Amplification protocol for all the genes was as follows: initial denaturing at 95°C for 10 min (PowerSYBR) or 3 min (Sso Advanced) followed by 40 cycles of amplification at 94°C for 10 s (denaturing), 60°C for 45 s (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 s, 65°C to 95°C (in increments of
0.5°C) for 5 s and 95°C for 5 s. The Cq geomean of reference genes, ActB and Hprt, were used to calculate fold change. Quantification values were generated only from samples showing a single product at the expected melting point.

2.3.6 Data analysis

All data are expressed as means ± SEM. All data from the weekly body weight measurements, GTT, ITT, and all CLAMS data were analyzed using a repeated measures two-way ANOVA followed by a post hoc Bonferroni-Dunn multiple comparisons test. All data from the food intake, body composition, plasma protein, and arcuate qPCR experiments were analyzed using a one-way ANOVA followed by a post hoc Bonferroni-Dunn multiple comparisons test (unpaired). All data analyses were performed on GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) and in all cases, effects were considered significant at p<0.05.

2.4 Results

2.4.1 Body weight is affected primarily by HFD, secondarily by LA

To determine the potential of LA vs. SFA to cause obesity, we fed male mice one of three HFDs with a range of LA concentrations (1%, 15%, 22.5%), or a low fat control diet (CON) for 12 weeks. All 3 HFD groups gained more than twice the amount of weight as CON (F (3, 44) = 33.58, p < 0.0001). Weight gain of 22.5% became greater than CON at week 1, which persisted for the remaining
11 weeks; 15% and 1% diverged from CON at weeks 2 and 3, respectively (Figure 1A). At week 6, weight gain of 22.5% became greater than 1% (p < 0.05), and remained so through week 12. Weight gain of 15% was higher than 1% at weeks 10 and 12 (p < 0.05). Food intake (F (3, 12) = 4.448, p = 0.0254) and feeding efficiency (F (3, 40) = 4.148, p < 0.0119) trended higher for all HFDs; food intake of 22.5% (p<0.05, Figure 1B) and feeding efficiency of 15% (p < 0.05, Figure 1C) were different than CON. All 3 HFD (p < 0.0001) groups had a similar body fat percentage, which was higher than CON (Figure 1D; F (1, 88) = 743.1, p < 0.0001).

2.4.2 Glucose metabolism altered by HFD and LA

The effects of LA and SFA on glucose metabolism were measured through glucose and insulin tolerance tests administered post 12-week experimental feeding. Fasting glucose was elevated in all HFD groups (mean BG = 165.4 ± 6.0 mg/dl, 171.6 ± 7.8 mg/dL, and 180.3 ± 12.6 mg/dL for 1%, 15%, and 22.5%, respectively) compared to CON (mean = 112.8 ± 6.7 mg/dL; F (3, 44) = 148.0, p < 0.0001). Glucose disposal during GTT was similarly impaired in all 3 HFD groups compared to CON from the 60 through 180-min time points (Figure 2A; F (3, 43) = 12.98, p < 0.0001). GTT AUC values for the HFD groups (p < 0.0001) were higher than CON (Figure 2B; F (3, 43) = 14.59, p < 0.0001), suggesting fat content was the primary factor influencing glucose tolerance.
Insulin suppressed BG equally in CON, 1% and 15%; 22.5% had higher BG during most time points, including the endpoint (Figure 2C; p < 0.0001), of the 2-hour challenge (F (3, 41) = 6.056, p < 0.0016). ITT AUC was higher in 22.5% than CON (p < 0.05) and also higher than 1% and 15% (Figure 2D; p < 0.01), indicating insulin resistance from LA (F (3, 41) = 6.065, p < 0.0016).

2.4.3 CO2 production reduced by HFD, O2 consumption mildly reduced by SFA

To characterize the effect of LA and SFA on metabolism, we measured O2 consumption and CO2 production by mice singly housed in Columbus Instruments CLAMS cages. Data from light (day) and dark (night) periods from the last 24 h were analyzed separately. Nighttime O2 consumption was higher than daytime for all diets (F (1, 80) = 31.76, p < 0.0001). Within nighttime hours, O2 consumption was lower in 1% (p < 0.05) and 15% (p < 0.05) than CON (Figure 3A; diet: F (3, 40) = 3.989, p < 0.05; time: F (1, 40) = 252.3, p < 0.0001), while daytime O2 consumption was only lower in 1% (p < 0.05, Figure 3A). This suggests suppressed respiration by SFA or increased extra-respiratory consumption of O2 by PUFA (22). All 3 HFD groups had lower day and night CO2 production than CON (Figure 3B; p < 0.0001 except daytime CON vs. 22.5% p < 0.01). As expected from greater availability of fat for use as energy substrate, there were effects of diet (F (3, 40) = 24.26, p < 0.0001), time (F (1, 40) = 136.8, p < 0.0001); and time*diet (F (3, 40) = 5.998, p < 0.01). Respiratory exchange
ratio (RER), or $\frac{\text{VCO}_2}{\text{VO}_2}$, followed the VCO$_2$ pattern; all 3 HFD groups had lower RER than CON during both day and night ($p < 0.0001$, Figure 3C; diet: F (3, 40) = 69.8, $p < 0.0001$; time*diet: F (3, 40) = 5.811, $p < 0.01$). Interestingly, daytime vs. nighttime RER was only different for CON ($p < 0.001$) and was not different within HFD groups. Heat, an indirect measurement of metabolism using the product of RER and $\text{VO}_2$, was higher in 15% ($p < 0.01$) and 22.5% ($p < 0.0001$) than CON (Figure 3D; diet: F (3, 40) = 9.135, $p < 0.0001$; time: F (1, 40) = 216.9, $p < 0.0001$). Daytime heat was higher in 22.5% than 1% ($p < 0.05$).

### 2.4.4 Nighttime activity reduced by LA

Beam breaks in the X and Z-axes were counted to determine differences in spontaneous activity. Daytime activity was similar across all groups. Activity was different between the daytime and nighttime in all diets except for the 22.5% diet in both X total and X ambulatory (Figure 4A-B). Elevated LA content in the HFD reduced X total and X ambulatory nighttime activity. Nighttime total X plane activity was lower in the 22.5% than both the 1% ($p < 0.001$) and 15% ($p < 0.01$; time: F (1, 40) = 179.1, $p < 0.0001$; time*diet: F (3, 40) = 6.897, $p < 0.001$). Nighttime ambulatory X plane activity was also lower in the 22.5% diet than both the 1% ($p < 0.0001$) and 15% ($p < 0.05$; diet: F (3, 40) = 2.875, $p < 0.05$; time: F (1, 40) = 128.7, $p < 0.0001$; time*diet: F (3, 40) = 6.67, $p < 0.001$). Nighttime Z plane activity was reduced by 22.5% LA compared to 1% ($p < 0.05$; time: F (1, 40) = 116.0, $p < 0.00001$; time*diet: F (3, 40) = 4.439, $p < 0.01$).
2.4.5 Plasma ghrelin and leptin affected by HFD

Plasma concentrations of the cytokines IL-6, MCP-1, and TNF-α and the peptide hormones ghrelin, insulin, and leptin were measured to indicate systemic inflammation and metabolic syndrome from LA and SFA feeding. There were no differences in plasma levels of IL-6, insulin, MCP-1, or TNF-α (Figure 5B, C, E, F). Plasma ghrelin was higher in CON than 1% (p < 0.01), 15% (p < 0.001) and 22.5% (p < 0.01), indicating a suppression of ghrelin by dietary fat (Figure 5A; F (3, 23) = 9.630, p < 0.001). Plasma leptin had an opposite pattern to ghrelin, being lower in CON than 1% (p < 0.01), 15% (p < 0.01), and 22.5% (p < 0.05; Figure 5D; F (3, 43) = 5.795, p < 0.01). All HFD groups had similar leptin levels, consistent with the correlation between fat mass and circulating leptin.

2.4.6 Hypothalamic neuropeptide expression affected by LA

The expression of orexigenic and anorexigenic genes from the arcuate nucleus was measured to identify differences in energy balance related neuronal activity between groups. mRNA of all neuropeptides trended higher in 22.5%. Expression of Pomc was higher in 22.5% than 15% (p<0.05; Figure 6A; F (3, 37) = 3.300, p < 0.05), and that of Cart was higher in 22.5% than CON (p<0.05; Figure 6B; F (3, 37) = 3.259, p < 0.05). Agrp expression was greater in 22.5% than both 1% and 15% (p<0.05; Figure 7D; F (3, 36) = 4.431, p < 0.01)).
2.4.7 Ikk expression suppressed by LA and SFA in arcuate nucleus

In order to further characterize a central obesogenic profile, expression of hormone receptors and down-stream transducers of hormone and cytokine signaling was measured. Despite higher serum leptin in the HFD groups, arcuate \textit{Lepr} expression was not different between diets (Figure 7A). \textit{Insr}, \textit{Stat3}, \textit{Socs3}, \textit{Irak1} and \textit{Traf6} likewise were not different between groups. \textit{Tlr4} trended higher in 1% but exhibited large variability (Figure 7C; F (3, 30) = 2.293; p < 0.0981). The inflammatory switch \textit{Ikk} was slightly lower in 22.5% than 1% (p<0.0001; Figure 7F; F (3, 31) = 52.44, p < 0.0001), but both 1% and 22.5% had greatly reduced \textit{Ikk} expression compared to CON and 15% (p<0.0001).

2.4.8 CX3CL1 suppressed by LA, IL-6 by LA and SFA, other cytokines unchanged

Expression of inflammation-related cytokine and chemo-attractant genes was measured in the arcuate nucleus as an indication of HI. The cytokines \textit{Il-1} and \textit{Il15} (Figures 8A, C) were not changed by diet. The serine protease inhibitor \textit{Pai1} (Figure 8D) was also not different between groups. \textit{Il-6} expression was suppressed in 1% and 22.5% relative to CON (p<0.01; Figure 8B; F (3, 30) = 6.473, p < 0.01), similar to \textit{Ikk}. Expression of the adhesion molecule \textit{Icam1} was similar between 1% and 15% but was lower in 22.5% than 1% (p<0.05) and lower in CON than both 1% and 15% (p<0.001; Figure 8E; F (3, 26) = 13.22, p < 0.0001) The chemokine \textit{Cx3cl1} had similar expression in 1% and 15% and CON
expression was lower than 1% (p<0.05) and 22.5% was less than CON (p<0.01) and both 1% and 15% (p<0.0001; Figure 8F; F (3, 29) = 20.08, p < 0.0001).

2.5 Discussion

There is a body of working showing LA to be more obesogenic than SFA. In the current study, we showed that LA-rich HFD caused greater weight gain than SFA, although the effect is not as large as the results obtained previously by other groups. In those studies, mice or rats were fed SFA from coconut oil, which resulted in no difference in weight gain and adiposity compared to CON (10) or greater weight gain than CON but much less than the LA group (9). In another study, rats were fed HFDs made with either tallow, olive oil, or sunflower seed oil; the tallow HFD caused the least weight gain (23). Although total dietary fat was the most important metabolic factor in our study, 22.5% LA HFD did decrease activity and induce insulin resistance compared to 1%, and altered arcuate melanocortin gene expression compared to the other groups. Arcuate expression of inflammatory genes was variably suppressed, depending on the gene, by both SFA and LA.

Alternatively, there are studies showing SFA to be more obesogenic than PUFA, but the majority of these studies used lard as the SFA source. Lard is not a suitable fat for comparing weight gain from different fatty acids since most lard now contains an FA profile approximately balanced between SFA, PUFA, and MUFA (11). When demonstrating lard to be more obesogenic than soy and
cottonseed oil (27) or flaxseed and olive oil (28), the comparison is not between SFA and PUFA in general but between palmitic and oleic acids, or stearic and linolenic acids, depending on the study. In both cases, LA is not an experimental variable, maintaining a steady content in those experimental HFDs. The dietary change that occurred in Western countries, however, was a large increase in LA at the expense of other FAs. The effects of LA compared to other FAs should be a focus of DIO research.

The maintenance of glucose homeostasis was affected by HFD and LA. In response to a glucose challenge, all 3 HFD groups responded with similarly impaired glucose disposal relative to CON. This is in agreement with the Randle Cycle (29), whereby the oxidation of fatty acids in mitochondria inhibits that of glucose and by extension cellular glucose uptake. The amount of insulin needed to drive the observed clearance of glucose is unknown because we did not measure blood insulin during the GTT. When a dose of insulin based on body weight was given, however, it had a similar effect on blood glucose for CON, 1%, and 15%, but elicited a reduced effect in 22.5%. These results suggest that the 22.5% group would require more insulin in order to achieve a rate of glucose disposal equal to CON, 1% and 15%. Previously, impairment of glucose disposal has been shown during both GTT and hyperinsulinemic-euglycemic clamp proportional to dietary n-6 PUFA (30). This suggests fatty acids are not equal in their participation in the Randle Cycle. Indeed, medium chain saturated fatty
acids are not dependent on nor do they affect the carnitine acyltransferase system as do the longer, unsaturated fatty acids (31,32).

All HFD groups had lower RER than CON, a result of their depressed VCO₂. This is consistent with the ability of RER to predict substrate intake (33) as mitochondrial oxidation of FA produces less CO₂ per molecule ATP than does carbohydrate oxidation. VO₂ between the 3 HFD groups was not different, but compared to CON both 1% and 15% nighttime and 1% daytime VO₂ was slightly lower, indicating a trend of VO₂ suppression by SFA or increase by LA. Presumably, this effect is not due to an increased metabolic rate by LA as 22.5% was the heaviest group and had similar feeding efficiency as 1% and 15%. In indirect calorimetry, VO₂ is measured at the whole animal rather than cellular/organelle level, hence the quotient of VCO₂/VO₂ reported as RER and not respiratory quotient. This is because non-respiratory O₂ consumption is counted the same as respiratory consumption. An example of non-respiratory O₂ consumption is lipid peroxidation, such as oxidation of PUFA-rich LDL (34). As previously mentioned, only PUFA oxidize non-enzymatically at physiological temperatures, with greater chain length and number of double bonds increasing oxidative risk.

There was an effect of LA in reducing activity, particularly during nighttime, as shown in X and Z plane counts for 22.5% vs. 1%. In our study, calorimetry was performed after the 12-week experimental feeding, at which time all HFD mice were obese. Therefore, reduced activity may have been causal of or caused
by DIO. Previous studies have alternately concluded activity does (35) and does not (36) contribute to DIO in mice. Our study suggests that LA may contribute more to reduced activity than SFA. Previously, substitution of n-3 with n-6 PUFA causes anxiety-like behavior in mice such as reduced spontaneous activity (37), but it is unclear whether it is the presence of n-6 or absence of n-3 PUFA that is responsible. The nature of removing nutrients, such as FA, in experimental diets cannot be isolated from the presence of its substitute, obfuscating causation.

We found that plasma ghrelin levels were lower and leptin levels higher in all HFD groups compared to CON. Ghrelin is an orexigen, and a ligand for the growth hormone secretagogue receptor. It has been shown in obese humans (38) and HFD-fed mice (39) that ghrelin levels are lower as an adaptation to positive energy balance. Our results with plasma leptin suggest that fatty acid type does not strongly affect leptin secretion and confirms its correspondence to adiposity.

Food intake was fairly consistent across groups, with only an increase in 22.5% compared to CON; this could be due to higher Agrp expression. A high SFA-containing HFD has been shown to suppress Npy and Agrp expression (40); the higher SFA content in the 1% and 15% groups may explain why they did not have increased expression of either gene compared to CON, while maintaining high food intake during positive energy balance. The pattern of neuropeptide expression did not fully correlate with observed food intake and body weight gain. Although leptin, a stimulator of hypothalamic Pomp production, was higher in the
plasma of all HFD groups, only 22.5% had increased Pomc expression. The co-expressed neuropeptide Cart, also stimulated by leptin, showed a similar expression pattern. Npy and Agrp, neuropeptides associated with orexigenic behavior, were largely unchanged between groups except for 22.5% having increased Agrp.

Arcuate mRNA levels of hormone receptors, cytokine receptors, and their ligands did not point to any group having significantly elevated HI or blunted receptor signaling. Interestingly, arcuate Lepr expression was not different between groups, despite higher plasma leptin in the HFD groups. Expression of Tlr4 trended higher in 1% compared to the other groups, consistent with previous studies showing SFA activation of Tlr4 (7,18,19), supposedly through resemblance to the putative ligand, lipopolysaccharide. Production of TLR4 transcription targets, however, was mostly suppressed by 1% and 22.5%, especially Ikk, Il-1 and Il-6. Furthermore, it is unclear whether increased expression of Tlr4 indicates receptor activation or, if it has negative feedback, absence of activation. Among TLR4’s many proposed ligands, pathways, and targets, some hundreds of positive and negative feedback loops occur (41); little is known about what controls receptor abundance. TLR4 activation by SFA could have caused the slightly increased expression in 1% of CX3CL1, a chemokine that is produced by dendritic cells under lipopolysaccharide stimulation (42), and Icam1.
Furthermore, the hypothesis that SFA induces TLR4 activation may be artifactual or, at least, confounded by the unforeseen effects of experimental reagents. Indeed, the research showing this is primarily treatment of immortalized cell lines with FA in bovine serum albumin (BSA); treatment of BSA with polymyxin-B to rid it of LPS does not remove other immune-activating contaminants such as di and tri-acyl lipopeptides and flagellin (43). While showing BSAs ability to activate TLR4 by itself, Erridge and Samani also showed that SFA, whether in decontaminated BSA or delivered purely, is unable to do so. TLR4 activation by dietary SFA in vivo has not been investigated to the same extent as in vitro, and how the two phenomena are related is unknown.

In conclusion, SFA- and LA-rich HFDs induced obesity and disturbed metabolic profiles in the mouse compared to a low-fat diet. However, LA produced greater body weight gain and insulin resistance, and suppressed activity more than the SFA. The dissimilar results between this study and many others (9,10,23) describing the effects of HFD differing in FA composition illustrates the need for carefully designing and reporting experimental diets and viewing results within that context. Although it seems obvious that fat intake is involved in the obesity epidemic, at the molecular level it is unclear which fats mediate DIO or the particular mechanism. It may be that different FAs induce obesity through different mechanisms, for example SFA through TLR signaling and LA through inhibiting glucose oxidation via the Randle Cycle. Future experiments should characterize the effects of these FA on hypothalamic
neuronal activity (POMC and NPY/AgRP) and on other measurements and pathways activated during hypothalamic inflammation from DIO. Other elements in experimental diets and overall experimental design may potentiate or contribute to these mechanisms. Improved attention to these details of experimental design and observation will be necessary to fully elucidate mechanisms of DIO in humans and in animal models.

2.6 Acknowledgements

The authors thank Dr. Sara Campbell for the use of the EMD Millipore MAGPIX® Multiplex® System and Dr. Judith Storch for the use of the Comprehensive Lab Animal Monitoring System and EchoMRI Body Composition Analyzer. This research was supported by funds from USDA-NIFA NJ06107 and from National Institutes of Health R00DK083457, R00DK083457-S1, and P30ES005022.
References


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### Tables

#### Table 1. Diet Fat Compositions

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Figures

**Figure 1.** Body weight gain, composition, and food intake in mice fed low vs. high LA HFD. (A) Three month old male mice fed either CON, 1%, 15% or 22.5% were weighed weekly for 12 weeks. Asterisks at weeks 7 and 8 denote comparison between 1% and 22.5% and at weeks 9-12 between 1% and both 15% and 22.5%. (B) Weekly food weight (g) was summed and multiplied by caloric content (kcals/g) to calculate cumulative intake in kcals. (C) Feeding efficiency was calculated by dividing weight gained (g) by food consumed (kcals) over 12 weeks. (D) Body composition was measured after 12 weeks by EchoMRI; body fat was divided by body weight. Data (n=12) were analyzed by a repeated measures, two-way ANOVA (A) or one-way ANOVA (B, C, D) with Bonferroni-Dunn multiple comparisons test. (* p<0.05; ** p<0.01; **** p<0.0001).

**Figure 2.** Glucose metabolism is impaired by HFD and LA. (A) Blood glucose was measured for 3 hours post IP glucose injection into fasted mice (n=11). (B) Area under the curve integrated from glucose challenge time point measurements. (C) Blood glucose was measured for 2 hours post IP insulin injection into fasted mice (n=9). (D) Area under the curve integrated from insulin challenge time point measurements. Data were analyzed by a repeated measures, two-way (A, C) or one-way (B, D) ANOVA with Bonferroni-Dunn multiple comparisons test. (* p<0.05; ** p<0.01; **** p<0.0001).
Figure 3. Twenty-four hour indirect calorimetry of mice fed low vs. high LA HFD. (A) Nighttime (black bars) and daytime (grey bars) average $O_2$ consumption (mL/min) was divided by bodyweight (kg). (B) Nighttime and daytime average $CO_2$ production (mL/min) was divided by bodyweight (kg). (C) $VCO_2/VO_2$. (D) Heat is the product of “calorific value” (3.815 + 1.232 * RER) and $VO_2$. Data (n=10) were analyzed by a repeated measures, two-way ANOVA with Bonferroni-Dunn multiple comparisons test. Asterisks denote comparisons between diets and letters denote comparisons within diets (a,* p<0.05; b,** p<0.01; c,***p<0.001; d,**** p<0.0001).

Figure 4. Nighttime movement was reduced by LA. (A) Average nighttime (black bars) and daytime (grey bars) beam breaks in the X plane. (B) Average nighttime and daytime novel beam breaks, indicating movement (ambulation) in the X plane. (C) Average nighttime and daytime beam breaks in the Z plane. Data (n=10) were analyzed by a repeated measures, two-way ANOVA with Bonferroni-Dunn multiple comparisons test. Asterisks denote comparisons between diets and letters denote comparisons within diets (a,* p<0.05; b,** p<0.01; c,***p<0.001; d,**** p<0.0001).

Figure 5. Serum ghrelin and leptin affected by HFD. Average serum protein levels (ng/mL) of (A) ghrelin (n=6), (B) interleukin-6 (n=11), (C) insulin (n=12), (D) leptin (n=12), (E) MCP-1 (n=8), and (F) TNF-α (n=8). Data were analyzed by a
one-way ANOVA with Bonferroni-Dunn multiple comparisons test (* p<0.05; ** p<0.01; *** p<0.001).

**Figure 6.** Neuropeptide mRNA levels of mice fed low vs. high LA HFD. Average relative expression of (A) *Pomc*, (B) *Cart*, (C) *Npy*, and (D) *Agrp* neuropeptides in arcuate nucleus. Data (n=9-11) were analyzed by a one-way ANOVA with Bonferroni-Dunn multiple comparisons test (* p<0.05).

**Figure 7.** Receptor and signal transducer mRNA. Average relative expression of (A) *Lepr*, (B) *Stat3*, (C) *Tlr4*, (D) *Irak1*, (E) *Insr*, (F) *Socs3*, (G) *Traf6*, and (H) *Ikk* mRNA in arcuate nucleus. Data (n=7-11) were analyzed by a one-way ANOVA with Bonferroni-Dunn multiple comparisons test (**** p<0.0001).

**Figure 8.** Inflammatory cytokine and chemoattractant mRNA. Average relative expression of (A) *Il-1*, (B) *Il-6*, (C) *Il-15*, (D) *Pai1*, (E) *Icam1*, and (F) *Cx3cl1* mRNA in arcuate nucleus. Data (n=7-10) were analyzed by a one-way ANOVA with Bonferroni-Dunn multiple comparisons test (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).
Figure 1

A. Weekly Bodyweight

B. Food Intake

C. Feeding Efficiency

D. Body Fat

---

* CON
* 1%
* 15%
* 22.5%

---

% of Initial

Week

kcal

CON 1% 15% 22.5%

---

g/kcal

CON 1% 15% 22.5%

---

(% BW)

CON 1% 15% 22.5%

---

* p < 0.05

**** p < 0.0001
**Figure 2**

**A** Glucose Tolerance

- Blood Glucose (mg/dL)
- Time (min)
- Control, 1%, 15%, 22.5%

**B** AUC (md/dL/min)

- CON, 1%, 15%, 22.5%

**C** Insulin Tolerance

- Blood Glucose (mg/dL)
- Time (min)
- Control, 1%, 15%, 22.5%

**D** AUC (md/dL/min)

- CON, 1%, 15%, 22.5%
Figure 4

A. X Total

B. X Ambulatory

C. Z Total
Figure 5
Figure 6

- **Pomc**:
  - CON: 1.5
  - 1%: 2.0
  - 15%: 2.5
  - 22.5%: 3.0

- **Cart**: (Data not presented on this page)

- **Npy**: (Data not presented on this page)

- **Agrp**: (Data not presented on this page)
Figure 7

![Bar charts showing fold change for various genes across different conditions: Lepr, Insr, Stat3, Socs3, Tlr4, Ikk, Irak1, and Traf6. The fold change is presented for CON, 1%, 15%, and 22.5% conditions. The error bars indicate the standard error of the mean.](image-url)
Figure 8

**II-1**

**II-6**

**II-15**

**Pai1**

**Icam1**

**Cx3cl1**
CHAPTER 3: THE INTERACTIONS OF 17β-ESTRADIOL AND THE FATTY ACID TYPE OF HIGH-FAT DIETS ON ENERGY AND GLUCOSE HOMEOSTASIS IN FEMALE MICE
3. The interactions of 17b-estradiol and the fatty acid type of high-fat diets on energy and glucose homeostasis in female mice

3.1 Abstract

Women are partially protected from obesity and comorbidities compared to men until after menopause, likely due to the loss of circulating 17β-estradiol (E2). The modern obesity epidemic, as understood experimentally, is associated with inflammation from dietary fatty acids. It is unclear whether saturated fat, or n-6 polyunsaturated fat, is the primary driver of high-fat diet induced obesity. It is also unknown how metabolic inflammation from different fatty acids interacts with E2, since the majority of obesity studies are performed on male animals. In particular hypothalamic inflammation as a mechanism for obesity has not been studied extensively in females, even though E2 is known to affect the relevant neuronal populations. Our objective is to determine the effects of high fat diets with varying levels of linoleic acid (LA) and saturated fat on the energy and glucose homeostasis in female mice with and without E2. Female C57BL/6J mice were fed either a control diet or a 45% kilocalories from fat diet with varying levels of LA (1%, 15%, or 22.5% kilocalories from LA) with or without E2 (300 mg/kg/day orally). After 8 weeks, the oil-treated high fat groups gained more weight than control regardless of fat type. E2 reduced body weight fat accumulation in all high fat groups. Glucose clearance from glucose challenge was impaired by LA. Nighttime O₂ consumption was increased by E2, regardless of diet and
independent of activity. Neuropeptides and inflammatory genes were not affected by LA or SFA content. These data show that fatty acid type does not affect body weight, but does affect glucose metabolism in females, and this effect is not due to hypothalamic inflammation.

3.2 Introduction

The growing health concern of obesity almost certainly derives a good deal of causation from diet and lifestyle (1), but also has an endocrinological aspect that is different between the sexes. Cardiovascular disease, type II diabetes, and metabolic syndrome, the generally recognized comorbidities of obesity, are substantially more common in postmenopausal than premenopausal women, an effect seen in tandem with a reduction in circulating 17b-estradiol (E2) (2–5). Food intake, for example, tracks the menstrual cycle in humans, forming a peak during the luteal phase and a nadir in the peri-ovulatory (6–9). Unsurprisingly, the post-menopausal fall in circulating E2 is associated with weight gain (10). Hormone replacement therapy has been shown to reduce some of these effects (11). A dietary, as well as pharmacological, treatment for obesity and its comorbidities in women requires a greater understanding of the physiology of obesity in females.

Originally considered a behavioral problem, the etiology of obesity is now believed to be primary physiological, especially after demonstrating it via
hypothalamic lesion (12) and the discovery of the so-called obese, or ob, gene leptin (13). In both humans and rodents, hypothalamic lesion and leptin deficiency cause severe obesity. Case studies of obesity from brain damage and monogenic mutation are rare, but are evidence for a central mechanism for obesity that is physiological rather than psychological. Hypothalamic inflammation (HI), a molecular proxy to lesion damage, can impair the energy balance regulatory system (14,15), including sensitivity to leptin. Whether HI causes or is caused by obesity is an ongoing question (16), but it is suggestive that HI is characterized by dysregulation of the same hormone and signaling pathways that are involved in genetic or lesion-induced obesity. HI can be induced by diet, specifically by fatty acids (FA) (17), which could serve as the connection between the molecular physiology of obesity and its presence at greater rates in the population.

Nutritionally, the idea of obesity being caused by non-specific overnutrition has transitioned to a focus on individual nutrients possessing obesogenic, endocrine-like qualities. Increased caloric density from dietary fat, for example, could not explain the current obesity epidemic, since over the relevant time period calories from fat have not increased (18–20). FA profile, however, has greatly changed; monounsaturated and saturated fats (MUFA and SFA) have been replaced by polyunsaturated fats (PUFA), in particular linoleic acid (LA) (21). Although conflicted on which FAs cause obesity and its comorbidities, it is now accepted that the metabolic effects of FAs are not the
same (22). Sugar consumption also increased during the obesity epidemic (23), but obesity induced by sugar alone has not been shown in animals (24) or humans (25). High-fat diet (HFD) without sugar, however, can induce obesity (26).

Although MUFA induced obesity has been studied (27), the majority of HFD research targets obesity caused by SFA (28) or obesity caused by LA (29). Determining FA-specific effects is difficult because foods do not contain individual FAs, but a mixture that is determined by the source organisms (plant vs. animal) and its nutritional sources. The most common source of HFD fat is lard, which has the most variable FA profile of any source commercially available. Pig adipose directly reflects the FA profile of its diet (30). The FA profile of beef fat is less diet dependent than lard, but is still influenced by feeding (31). Coconut oil has the highest and most consistent amount of SFA and has been used to compare other FAs to SFA in studies of obesity (29) and heart disease (32). Other than isolated FAs and/or triacylglycerols, which have different olfactory and taste properties than whole foods (33), coconut oil is the best representative of pure SFA.

Compared to the advances in basic obesity research in the areas of mechanism and nutrition, little has been done to address sex differences. Even though sex differences have been shown in body weight and metabolic effects of HFDs from different fat sources (34), more research into the molecular mechanisms of obesity is performed on male animals because of the added complication of
cycling levels of E2. E2 is likely part of the reason for the observed differences in obesity (35) and obesity-related diseases (36) between the sexes. Within the context of HI and energy balance, E2 is directly involved in these pathways (37), compounding the case for greater experimental consideration. The anorectic effect of intracerebroventricular (icv) leptin and insulin treatment are different between males and females, whereas that of melanocortin agonist is not, suggesting the sex difference is in nuclei upstream of the melanocortin receptors (arcuate POMC) (38). These are likely the same leptin receptor expressing neurons damaged in lesion experiments and inactive in ob -/- mutants.

The question we are addressing is whether obesity and metabolic damage from HFDs high in SFA or LA can be linked to HI gene expression patterns in E2-deficient and E2-treated ovariectomized (ovx) female mice. We hypothesize that mice fed the higher LA HFDs will gain more weight, show decreased glucose clearance, and exhibit higher expression of HI gene biomarkers compared to mice fed high SFA HFD. We expect these effects to be more pronounced in the oil treated than in E2 treated mice. To address this objective, ovx females were fed 3 different HFDs over 8 weeks with weekly measurements of body weight and food intake followed by body composition assessment, indirect calorimetry, and glucose and insulin tolerance tests (GTT and ITT).

3.3 Materials and Methods

3.3.1 Animals
All animal treatments were in accordance with guidelines based on National Institutes of Health standards and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Female wild-type (WT C57BL/6J) mice were selectively bred in-house, and maintained under controlled temperature (23°C) and photoperiod conditions (12/12 h light/dark cycle) with food and water ad libitum.

3.3.2 Drugs and Diets

Estradiol benzoate (EB) and 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mg/mL) were purchased from Sigma-Aldrich. Ketamine, Marcaine®, and Rimadyl® were purchased from Henry Schein Animal Health (Columbus, OH, USA). EB was dissolved in 100% ethanol (Sigma) prior to dissolving in coconut oil (Nature’s Way). Diets were purchased from Research Diets (New Brunswick, NJ): CON (10% kcal fat; D12450B) and 3 HFDs based on Research Diets 45% kcal fat diet D12451, with either 1%, 15% or 22.5% of kcals from LA, the remainder consisting of coconut oil SFAs. See Table 1 for FA profile of experimental diets.

3.3.3 Experimental Design

Adult females (10 weeks old) were ovariectomized (ovx) via a single ventral incision under isoflurane anesthesia (2% in O₂:N₂O (2:1)) delivered by face mask with a local injection of Marcaine® (2 mg/kg) followed by 48 h of pain
management using an injection of Rimadyl® (4 mg/kg, every 24 h; Henry Schein). Females fed ad libitum CON; (n = 24) or one of 3 high-fat diets (1%, 15% or 22.5%; n = 24 for each diet) for 8 weeks. Half of each diet group was administered oil and the other half EB (300 mg/kg) daily perorally via a peanut butter carrier (all groups: n = 12 – CON-oil, CON-E2, 1%-oil, 1%-E2, 15%-oil, 15%-E2, 22.5%-oil, 22.5%-E2). We chose peroral E2 dosage to reduce the stress-inducing effects of repeated injection and to maintain a constant systemic level of E2, similar to hormone replacement therapy in postmenopausal women (39). Females were housed in group-matched pairs and body weight and food intake were measured weekly. At the end of 8 weeks, body composition was measured using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA). Calorimetric and activity measurements (48 h run) were performed using a Columbus Instruments’ Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Inc., Columbus, OH, USA). After suitable recovery, a glucose tolerance test (GTT) was performed on each female. Females were fasted overnight (1700h-900h) in a new cage. At the start of the test and 30 min after application of a local anesthetic (Lidocaine®; Henry Schein) to the tail, mice were placed in Plexiglass restrainers and tails were nicked to collect a baseline (time=0) glucose reading using a glucometer (AlphaTRAK2, Carlsbad, CA, USA). Immediately after baseline, females were given an intraperitoneal (i.p.) glucose injection (2.0 g/kg body weight) and placed back individually into clean cages. Tail blood samples were collected at 15, 30,
60, 90, 120, and 180 min post-injection. After 180 min, all mice were returned to their home cages with ad libitum access to water and food. After sufficient recovery (~3-4 d), an insulin tolerance test (ITT) was performed after a 5 h fast in a similar manner as the GTT, with an i.p. injection of insulin (0.75 units/kg). Blood samples were collected from the tail in individual cages at 15, 30, 60, 90, and 120 min post-injection.

3.3.4 Brain and Body Dissections

After ~1 week recovery from ITT, females were dosed at 0900h and decapitated after sedation with ketamine (100 µl of 100 mg/ml, i.p.) at 1000h. Food was removed at the last dosing. Trunk blood was collected in a K+ EDTA collection tube. Plasma was prepared for peptide analysis by adding a protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mg/mL) to each collection tube. Samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at 4°C. Plasma was stored at –80°C until analysis. Leptin and interleukin-6 (II-6) were determined by multiplex assay (#MMHMAG-44K; EMD Millipore, Billerica, MA, USA). Total plasma E2 levels were measured using Mouse/Rat Estradiol ELISA kit (ES180S-100; Calbiotech, Spring Valley, CA, USA).

The brain was immediately extracted from the skull and rinsed in ice-cold Sorensen’s buffer for 30 sec, then cut using a brain matrix (Ted Pella, Redding, CA, USA) into one mm thick coronal rostral and caudal blocks corresponding to
Plates 42 to 47 and Plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates (Paxinos & Franklin 2008, 3rd Edition) (40). Blocks of the basal hypothalamus (BH) were transferred to RNAlater (Life Technologies) and stored overnight at 4°C. The rostral and caudal parts of the arcuate nucleus were dissected from slices using a dissecting microscope. Dissected tissue was stored at −80°C. Total RNA was extracted from the combined nucleus (rostral and caudal arcuate) using Ambion RNAqueous-Micro Kits (Life Technologies) according to the manufacturer’s protocol. Total RNA was also DNase I-treated, using the extraction kits, at 37°C for 30 min. to minimize any genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Santa Clara, CA, USA). Only samples with RNA Integrity Number > 8 were used in the analysis of gene expression.

3.3.5 Quantitative Real-Time PCR

Complementary DNA (cDNA) was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies), 4 µl 5x Buffer, 25 mM MgCl2, 10 mM dNTP (Clontech Laboratories, Mountain View, CA, USA), 100 ng random hexamer primers (Promega, Madison, WI, USA), 40 U/µl Rnasin (Promega) and 100 mM DTT in DEPC-treated water (Bioexpress, Kaysville, UT, USA) in total volume of 20 µl. Reverse transcription was conducted using the
following protocol: 5 min at 25°C, 60 min at 50°C, 15 min at 70°C. The cDNA was diluted to 1:20 with nuclease-free water (Bioexpress) for a final cDNA concentration 0.5 ng/µl and stored at −20°C. Negative control (no reverse transcriptase) was processed simultaneously with the experimental samples.

All primers were designed to span exon-exon junctions and synthesized by Life Technologies, using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 2 for a listing of all the primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 µl cDNA template was amplified using either PowerSYBR Green master mix (Life Technologies) or Sso Advanced SYBR Green (BioRad, Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in triplicate to determine the efficiency \( E=10^{\left(-\frac{1}{m}\right)} - 1 \), \( m=\text{slope} \) of each primer pair. All efficiencies expressed as percent efficiency were approximately equal (one doubling per cycle, 90-110%). The relative mRNA expression was calculated using the \( \Delta\Delta C_T \) method utilizing a calibrator of diluted (1:20) cDNA from BH of an untreated male. The amplification protocol for all the genes was as follows: initial denaturing – 95°C for 10 min (PowerSYBR) or 3 min (SsoAdvanced) followed by 40 cycles of amplification at 94°C for 10 sec (denaturing), 60°C for 45 sec (annealing), and completed with a dissociation step for melting point analysis with 60 cycles of 95°C for 10 sec, 65 °C to 95°C (in increments of 0.5°C) for 5 sec and 95°C for 5 sec. The geometric mean of the reference genes \textit{Actb}, \textit{Hprt}, and \textit{Gapdh} was used to calculate \( \delta C_q \) values.
Quantification values were generated only from samples showing a single product at the expected melting point. All gene expression data were expressed as an $n$-fold difference relative to the calibrator.

### 3.3.6 Statistical Analysis

All data were expressed as mean ± SEM, and analyzed by a multi-factorial (steroid and diet) ANOVA followed by a post-hoc Bonferroni test using Graphpad Prism software (GraphPad Software, Inc., La Jolla, CA 92037 USA). Body weight, GTT, and ITT data were analyzed using repeated-measures, two-way ANOVA with a post-hoc Bonferroni test. All gene expression data were analyzed using two-way ANOVA with a post-hoc Bonferroni test within each genotype because expression was normalized to the CON-oil samples of each genotype. In all experiments, effects were considered significant at $a \leq 0.05$.

### 3.4 Results

#### 3.4.1 E2 reduces weight gain from LA and SFA

We fed one of three HFDs with a range of LA concentrations (1%, 15%, 22.5%), or a low fat control diet (CON) to ovx female mice with and without E2 treatment for 8 weeks and measured weight gain. By the second week, weight gain for 1% and 22.5% oil became greater than CON; weight gain for 15% oil became greater than CON by week 3 (Figure 1A-C). Weight gain from week 2 onwards was also greater for oil vs. E2 treated mice within all three HFDs. At
weeks 6-8, weight gain of 1% and 22.5% E2 became greater than CON. The final body weights of oil treated 1%, 15%, and 22.5% were greater than CON (Figure 1D). Diet (F (3, 88) = 14.64; p < 0.0001), steroid (F (1, 88) = 39.79; p < 0.0001), and their interaction (F (3, 88) = 2.845; p < 0.05) affected body weights. Within diets, E2 treatment reduced body weights in 15% and 22.5%, but not 1% or CON.

3.4.2 HFD effects on intake and body composition opposed by E2

We determined weekly energy intake (Figure 2A) by multiplying food intake in grams by the caloric density of diets. Steroid (F (1, 56) = 11.9; p < 0.01) and diet (F (3, 56) = 11.19; p < 0.0001) affected food intake, but there was only a trend towards an effect of E2 within any diet. E2-treated 15% (p<0.001) and 22.5% (p<0.0001) consumed more food than E2-treated CON; all oil-treated HFD groups consumed more food than oil-treated CON. Feeding efficiency (Figure 2B) was determined by dividing weekly weight gained in grams by food consumed that week in kcals. The effects of E2 (F (1, 39) = 11.07; p < 0.01) resulted in a greater FE in 22.5% oil than their E2-treated counterparts (p<0.0001). The effects of diet on FE (F (3, 39) = 5.079; p < 0.01) were seen in all HFD oils being more efficient than E2-treated.

Lean mass (Figure 2C) and fat mass (Figure 2D), determined by MRI and reported as percentage of body weight, were, respectively, affected by diet (F (3, 87) = 14.21; p < 0.0001, F (3, 87) = 17.17; p < 0.0001) and by steroid (F (1, 87) =
95.56; p < 0.0001, F (1, 87) = 112.6; p < 0.0001). Fat mass (F (3, 87) = 3.786; p < 0.05) but not lean mass (F (3, 87) = 1.117; p = 0.3468) showed significant variability from their interaction. Lean mass was reduced in 1% and 22.5% both oil and E2 compared to CON, but only 15% oil had a reduction, in 15% E2 was more protective. Fat mass was higher in all oil treated HFDs than CON, but also higher in 1% E2 compared to CON. In 15% and 22.5% E2 was again protective. Fat mass in E2-treated 1% was also higher than E2-treated 15%; showing that E2 is more protective from the dystrophic effects of HFD for a FA profile balanced between LA and SFA.

3.4.3 SFA boosts triglycerides, LA slows glucose disposal

Triglycerides (Figure 3A) and BG (Figure 3B) were measured from tail blood following a fast. Triglycerides were, as is usually seen, increased by SFA feeding, but only in the E2-treated 1% group. Fasting BG was affected by steroid (F (1, 86) = 9.146; p < 0.01), but due to variability from diets did not produce any differences; CON oil vs. 22.5% oil were nearly so (p = 0.0672). Differences in glucose metabolism were, however, uncovered during GTT and ITT.

GTT and ITT were performed after 8 weeks of feeding CON, 1%, 15% and 22.5% diets to determine the effects on glucose metabolism in oil- and E2-treated female mice. At several time points of the GTT, for both oil and E2 treated animals, 22.5% had higher BG than control and other HFDs (Figure 4A-B). Time (F (6, 264) = 152.9; p < 0.0001) and the interaction of time and diet (F (18, 264) =
4.502; p < 0.0001) were significant sources of variation within oil treated animals, whereas time (F (6, 240) = 90.46; p < 0.0001), diet (F (3, 40) = 4.582; p < 0.01), and their interaction (F (18, 240) = 1.986; p < 0.05) were sources for E2 treated animals. At 90 min post-injection 22.5% oil had higher BG than 1% oil, and at 120 minutes 22.5% oil had higher BG than 1% oil and CON oil. All oil treated HFDs had higher BG than CON at 180 minutes. E2 accelerated glucose disposal in all diet except 22.5%, which had higher BG than CON at 30 through 120 min post-injection. At 120 min, 22.5% BG was also higher than 1%, and 15% BG was higher than CON. The area under the curve (AUC) analysis of GTT BG measurements was higher for oil compared to E2 groups within each diet, although the difference between oil and E2 groups was smaller in the high LA HFD. Between diets, 22.5% E2 and oil, and 15% E2, had elevated AUC (Figure 4E). Diet and E2 (F (3, 84) = 8.143; p < 0.0001, F (1, 84) = 63.05; p < 0.0001), but not their interaction, were significant sources of variation in GTT AUC data. The trends and differences in GTT data suggest a dose-response of LA for glucose intolerance.

BG suppression by insulin injection was similar across diets (Figure 4C-D). For both oil and E2 treated mice, only time (F (5, 210) = 207.2; p < 0.0001, F (5, 195) = 124.9; p < 0.0001) significantly affected inter-group variability. Within oil treated mice, diet trended towards being significant (F (3, 42) = 2.325; p = 0.0886) and 22.5% trended towards higher BG in the later time points. At 120 min post-injection, 1% E2 had a greater recovery and was closer to its initial BG,
being higher than 15% E2 and CON E2, but not 22.5% E2. The higher trending BG over the course of ITT in 22.5% oil resulted in a higher AUC (Figure 4F). The glucose metabolism impairment of LA, as a response to insulin challenge, was more tempered than during glucose challenge.

3.4.4 CO2 production, but not O2 consumption, reduced by HFD

O2 consumption was not altered by diet (Figure 5A). The day/night cycle (F(1, 170) = 33.89; p < 0.0001) and steroid (F(7, 170) = 17.65; p < 0.0001) both affected variability; during the night E2 treated mice had higher O2 consumption than oil treated within all diets, whereas only 15% showed a higher O2 consumption from E2 during the day. Although trending higher in all diets, E2 treatment induced more CO2 production only in 15% at night (Figure 5B). Day/night (F(1, 170) = 22.37; p < 0.0001) and diet/E2 (F(7, 170) = 22.37; p < 0.0001) were both significant sources of variability. CO2 production was suppressed by all HFDs during both the day and night, except for 15% E2 during the day, which was the same as CON.

The respiratory exchange ratio (RER), the ratio of CO2 produced to O2 consumed, followed the CO2 pattern of being lower in all HFD fed mice regardless of the day/night cycle or steroid treatment (Figure 5C). Daytime RER was lower within CON diet for E2 treated animals due to a higher trend of daytime O2 consumption compared to CO2 production. Time (F(1, 170) = 4.764; p < 0.05) and treatment (F(7, 170) = 78.14; p < 0.0001) caused variability. Heat
production, a product of O2 consumption and the estimated caloric value of the diet, was not different between diets or time of day (Figure 5D). Although both treatment ($F (7, 170) = 44.59; p < 0.0001$) and time ($F (7, 170) = 2.279; p < 0.05$) affected variability, their interaction did not ($F (7, 170) = 0.5128; p = 0.8241$), producing little difference between groups.

Spontaneous activity as measured by movement in the X and Z-axes during calorimetry was not affected by diet or steroid (Figure 6A-C). The day/night cycle was the sole source of variation in X total ($F (1, 170) = 76.99; p < 0.0001$), X ambulatory ($F (1, 170) = 45.57; p < 0.0001$), and Z total ($F (1, 170) = 77.3; p < 0.0001$) counts; all mice, regardless of diet or E2, had increased nighttime activity.

3.4.5 SFA suppresses leptin and IL6 compared to LA in E2 deficient mice

Serum E2 was higher in all E2 treated mice compared to oil treatment (Figure 7A). Diet did not affect circulating E2 ($F (3, 72) = 0.5621; p = 0.6418$). All HFD mice, regardless of E2 treatment, had higher circulating leptin than CON, which is consistent with their higher fat mass (Figure 7B). Diet was the primary driver of variation ($F (3, 56) = 79.74; p < 0.0001$). 1% oil, but not 1% E2, had lower circulating leptin than their 15% and 22.5% counterparts, despite having similar fat masses. Circulating IL6 levels followed a similar pattern as leptin (Figure 7C). Nearly all HFD groups had higher IL6 than CON (Figure 6B). Diet ($F (3, 48) = 110.5; p < 0.0001$), E2 ($F (1, 48) = 8.547; p < 0.01$), and their interaction
(F (3, 48) = 12.57; p < 0.0001) were all significant variables. 1% oil had lower IL6 than 15% and 22.5% oil, being suppressed all the way to the CON level. IL6 was also suppressed in 1% E2 compared to 15% and 22.5% E2, but not to the same degree as in oil treatment.

3.4.6 Arcuate neuropeptide and inflammatory gene expression

The expression of anorexigenic neuropeptides Pomc and Cart was not affected by diet (Figure 8A-B). CON and 22.5% Pomc expression was suppressed by E2 (F (1, 64) = 17.62; p < 0.0001. None of the experimental variables significantly affected Npy expression (Figure 8C). Diet was the more important variable for Agrp expression (F (3, 63) = 2.93; p < 0.05), which was suppressed by 22.5% in E2 treated mice (Figure 8D); a decreasing trend is apparent in the HFD-fed groups.

Tlr4 expression trended towards suppression in the CON oil group compared to CON E2 (p = 0.0631) but high variability within groups prevented significant inter-group variability from either diet (F (3, 63) = 0.9228; p = 0.4351) or E2 (F (1, 63) = 1.37; p = 0.2462) (Figure 9A). IL1 expression was suppressed by E2 (F (1, 61) = 17.6; p < 0.0001) but was only lower between CON oil and CON E2 (Figure 9B). IL15 was mostly affected by diet (F (3, 63) = 3.219; p = 0.05) and suppressed by HFDs, and by E2 in 22.5% HFD (Figure 9C). Although circulating leptin was higher in HFD fed animals, and highest from LA, arcuate nucleus expression of its receptor Lepr was not different between groups (data
not shown). The expression of Insr, Stat3, Socs3, Irap1, Traf6, and Ikk were not different between any groups (data not shown).

3.5 Discussion

3.5.1 Summary

The contemporary obesity trend affects the sexes differently, but perhaps not unequally. Although young women have some protection from obesity-associated comorbidities, menopause is a powerful physiological leveler that induces rapid catch-up in many of those conditions. The unpredictable nature of findings when studying a phenomenon as general as diet-induced obesity (DIO) suggests that an artificial exclusion on research subjects, such as sex, limits that potential. Any great discoveries on the cause or prevention of DIO will eventually find its way to application through science communication and the advice of physicians and dieticians, and a discovery found through basic research conducted on solely male animals may prove ineffective for women. Even some men, those with extreme obesity and metabolic syndrome, can have an atypical steroid profile with quite elevated E2 and suppressed testosterone, and may benefit from a different obesity treatment than that effective for men with a typical steroid profile. This concept should translate into research not simply studying the effects of estrogen on weight gain, but the application of the current leading theories on obesity to females and in the context of female steroids. Therefore,
we looked at the effects of FA profile of HFD on DIO, and the potential involvement of HI, in female mice in the context of E2 deficiency vs. sufficiency.

Here we showed that E2 suppressed weight gain from high SFA and high LA HFD, in a seemingly FA-independent manner. Glucose metabolism, however, was affected in an FA-dependent manner. During both GTT and ITT, 22.5% diet impaired glucose disposal in E2 deficient females, while E2 treatment failed to rescue high-LA consuming females to levels commensurate of the other groups.

The other substantial difference seen in different FA profile was the slight potentiation of leptin secretion, and the more substantial potentiation of IL-6 secretion, by LA, especially when comparing E2 deficient females. These peptides did not cause a difference in weight gain, but may be involved in the differences in glucose metabolism.

3.5.2 Weight gain

Previously, E2 has been shown to decrease food intake, acting through estrogen receptor-α (ERα) and cholecystokinin (41) and reduce the post-ovariectomy body weight gain (42). In our study, the HFD fed, E2 treated mice did not eat less food than those treated with oil. This difference is likely due to the administration route of E2; the anorectic effects of E2 are more pronounced in direct hindbrain compared to oral administration (41) or the crude measurements of food intake in paired-housed females. Hypothalamic E2 administration also inhibits food intake, but only acutely (43). Although no exogenous administration
of E2 can mimic identically ovarian secretion, oral delivery and subcutaneous injection are more similar to it than is delivery through central cannulation.

Furthermore, pair feeding of ovx to intact mice resulted in 25% greater weight gain in ovx mice (44), a similar gain as we are reporting herein, indicating that hyperphagia is not necessary for E2 deficiency-induced weight gain. The observed anorectic effects of E2 may be of an acute, secondary nature relative to chronic metabolic effects, analogous to epinephrine’s acute control of heart rate (45), whereas long term heart rate is correlated primarily with oxygen consumption(46). Within E2 treated animals, 1% and 22.5% began gaining weight faster than 15% and CON at the later stages of the experiment. This was not sufficient to result in a difference in cumulative weight gain at 8 weeks, although if feeding had continued longer, the weight gain may have continued to diverge. In a 24 week HFD feeding experiment, weight gain was ongoing in female, intact mice consuming Research Diets D12492 (47), which is similar to the 15% diet in our study. Greater weight gain in females fed either high LA or high SFA HFD, compared to an even (15% LA) mixture, is noteworthy in the context of proposed obesogenic mechanisms for those different FAs.

3.5.3 Glucose metabolism

We previously showed in male mice that LA disrupted glucose metabolism more than SFA (48). In that study, all HFD mice had impaired glucose disposal from glucose challenge compared to CON, but only 22.5% had impaired glucose
disposal from insulin challenge. In the present study in female mice, glucose disposal during glucose challenge, and not insulin challenge, was impaired by LA. LA is a known determinant of hyperglycemia (22), through a mechanism related to participation in the carnitine palmitoyl transferase (CPT1) system. CPT1 expression is higher in liver cells of rats fed high n-6 PUFA fats compared to high SFA fats (49), and inhibition of CPT1 by methyl palnoxirate treatment reduces rats’ ability to oxidize corn oil triglycerides but not medium chain saturated triglycerides [50]. The short and medium chain SFAs in coconut oil are also protective of beta cell toxicity from alcohol [51] and alloxan [52].

Another difference between SFA and PUFA is the effect on PPARs, that being generally a much higher potency of activation by PUFA [53]. PPAR signaling is considered adaptive; shunting FAs to oxidation or other beneficial uses when there are perceived in the body, but the type of FA activating these receptors is a factor in the response. LA and other n-6 PUFA can induce adipogenesis through PPAR activation where n-3 PUFA do not [54]. The resulting inflammation from this adipogenic environment, as well as the downstream eicosanoids of n-6 PUFA [55], would have a negative effect on blood glucose clearance. The estrogen receptor system acts on, and is acted on, PPAR gamma [56], a potential reason why E2 opposes the glucose metabolism impairing effects of HFD.

Likewise, activation of ERa by E2 increases the rate of fat oxidation and decreases tissue accumulation of diacylglycerol [57], resulting in less long chain,
polyunsaturated FAs. The accelerated clearance of FAs from circulation and tissues by E2 may be the primary, although indirect, mechanism of improving glucose metabolism. By chronically relieving cells of FAs inhibitory to glucose metabolism, the glucose oxidation rate, and consequently the rate of glucose drawn from blood, is increased. Because of this estrogen mediated boosting of fat clearance, FA profile might be a more important health concern for men and post-menopausal women than for cycling women.

3.5.3 Calorimetry

O$_2$ consumption trended lower in HFD oil groups than CON, and this trend was stronger during the night. During the night, E2 caused an increase in O$_2$ consumption, especially in HFD fed mice. A limitation of using whole animal O$_2$ consumption as a proxy for metabolic rate is the inability to differentiate between O$_2$ used in cellular respiration and O$_2$ used in non-respiratory oxidation.

Higher tissue and circulating FAs, specifically polyunsaturated, can take up O$_2$ to produce peroxyls and peroxides. It is likely that the trend towards lower O$_2$ consumption in HFD-fed oil-treated mice would be a significant difference if non-respiratory O2 use could be isolated and subtracted. An equation, similar to those developed by Bauer [58] and others for estimation of tissue n-3 and n-6 PUFA content from intake, that estimates FA peroxidation from factors such as PUFA intake, metal catalyst concentration, and O2/CO2 ratio, would be a useful addition to indirect calorimetrics. An open container of highly oxidizable material
would register as “respiring” according to the current method of measuring VO2. Without such an equation to quantify this phenomenon, however, all that can be said is that VO2 is likely overestimated to some extent in animals based on fat intake and especially PUFA intake.

The presence of higher circulating and tissue FAs in HFD-fed mice is supported by their reduced CO₂ production. A reduction in CO₂ production, particularly in the presence of a relatively stable O₂ consumption, indicates an increased ratio of FAs as energy substrate. This increased presence of FAs, specifically long chained unsaturated FAs, explains glucose intolerance in 22.5% through CPT1 and the Randle Effect [59]. Respiratory quotient, which had O2 as its denominator and therefore depends on physiological O2 fate for accuracy, has also been suggested to be an independent predictor of body mass changes [60].

There is evidence from the basic chemistry of the Bohr effect on hemoglobin [61], as well as the hypercapnic activation of vitamin K2 dependent enzymes [62], that CO2 per se supports the metabolic rate. There has been much conjecture on why Boulder and other high altitude Colorado cities [63], and even exotic locales famed for longevity and leanness such as the Hunza Valley in Pakistan, have substantially lower rates of obesity and associated metabolic disease. Since a low-fat diet induced glucose-based metabolism has the same effect on capnia as altitude, this seems like a more obvious place to start in explaining low obesity rates common in high altitude populations than their idiosyncratic diets and cultures.
3.5.4 Blood peptides

Circulating leptin was correlated with fat mass. Since leptin is mostly produced by white adipose tissue [64], circulating levels are controlled at the level of secretion by body fatness. In this study, we show that although all HFD mice have higher serum leptin than CON, 1% oil was an intermediate between CON and the higher LA HFD oil mice. The lower leptin of 1% oil compared to 15% and 22.5% did not cause a difference in weight gain or arcuate leptin receptor mRNA, which was not different between groups. An even greater difference was observed in circulating IL-6, where 1% oil showed suppression equal to CON. Another study measuring E2’s effects on cytokines showed an increase in IL-6 from E2 while feeding an HFD made from corn oil and lard [65], both good sources of LA.

This is consistent with our results, where the oil-treated, 1% LA HFD-fed mice had low levels of IL-6 compared to the elevated IL-6 levels in E2-treated, HFD-fed mice. Leptin and IL-6 both being reduced in the serum of E2 and LA deficient mice may have something to do with their molecular similarity. They are both cytokines with receptors from the same family, and are induced similarly by agents such as LPS, other cytokines, and even E2 [66]. Higher circulating cytokines likely play a part in the glucose intolerance seen in 22.5%; elevated peripheral IL-6 is commonly seen in insulin resistance [67], and blocks the suppression of hepatic gluconeogenesis by insulin [68].
3.5.5 Arcuate gene expression

This difference in circulating IL-6, like the difference in leptin, did not influence arcuate gene expression. Arcuate cytokine mRNA, including Il-6, was not different between groups. Hormone and cytokine receptors, and energy balance neuropeptides, reflect this as well. The moderate suppression by 22.5%, of arcuate Pomc and Agrp did not co food intake, which was unaffected by treatment.

It is reasonable to conclude that peripheral hormone and inflammatory activity is more reflective of dietary influence, such as type and amount of FAs, than is central. The hypothalamus is not protected by a complete blood brain barrier, but is still less available to circulating molecules than is the periphery. It may be that a greater change in hypothalamic gene expression may require a longer dietary intervention. It has been shown previously that approximately 5 weeks post-ovx, expression of energy balance related genes in the arcuate nucleus is similar to that of intact females, despite an enduring effect on body weight [69]. The normalization of neuropeptide levels at a higher plateau of obesity is reminiscent of the set point observations on leptin during weight loss, where after the body reaches a level of adiposity it defends it in the same way body weight is defended in a normal weight individual.

3.5.6 Conclusion
There is a significant challenge in experimentally capturing both obesity and pre-obesity events causative of weight gain. Inflammation measured in obese animals can be interpreted as a symptom or a cause of the obesity. Early onset inflammation, before an animal becomes obese, relies on the assumption of future obesity and consistent production. IL-6 can stimulate the pancreas to release insulin and control glycemia [70], but over a different course of time can blunt insulin’s ability to suppress hepatic glucose production [68]. In this study we chose to measure hypothalamic gene expression and serum protein at time of sacrifice, when oil treated HFD mice were obese, and found that circulating IL-6 and leptin were related to glucose intolerance but not obesity or hypothalamic inflammation.

Overall, our results show that E2 makes female mice more resistant to HFD induced obesity from different types of FAs, but did not protect against impaired glucose disposal from LA. These data suggest that young, cycling women who present with symptoms of pre-diabetes may be able to halt or reverse diabetic progression by reducing dietary LA. For post-menopausal women, E2 treatment may combat obesity, but they may also benefit from a low LA diet for preserving proper glucose metabolism. Our finding that E2 increased circulating IL-6 in HFD-fed mice, especially higher LA HFD, is not evidence in favor of E2 as a risk free obesity treatment. Estrogen replacement in post-menopausal women has long been controversial because of the increased risk of some cancers. Endometrial cancer risk is greatly increased by estrogen
replacement, and further in obesity, but is reduced by progesterone [71]. Indeed, progesterone in combination with E2 is required to induce higher leptin secretion in women [72], and may play a role in the resistance to obesity related disease in cycling women. Reducing dietary fat is a safe strategy to prevent obesity for women regardless of menopausal status, and targeting LA for reduction may specifically benefit glucose metabolism in those same women.

Acknowledgements

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References


[10] Sullivan EL, Shearin J, Koegler FH, Cameron JL. Selective estrogen receptor modulator promotes weight loss in ovariectomized female rhesus monkeys (Macaca mulatta) by decreasing food intake and increasing


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### Table 1. Diet Fat Compositions

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### Table 2. Primer sequences

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Figures

Figure 1: Body weight gain

A. Weekly weight gain of CON vs. 1% fed oil and E2 treated mice. Two-way ANOVA: interaction time*trt: F (24, 352) = 16.11; p < 0.0001. B. Weekly weight gain of CON vs. 15% fed oil and E2 treated mice. Two-way ANOVA: interaction time*trt: F (24, 352) = 17.23; p < 0.0001. C. Weekly weight gain of CON vs. 22.5% fed oil and E2 treated mice. Two-way ANOVA: interaction time*trt: F (24, 352) = 16; p < 0.0001. D. Cumulative weight gain over 8 weeks. Two-way ANOVA: interaction: diet*steroid: F (3, 88) = 2.845; p < 0.05. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two-way ANOVA (repeated measures or standard) with post-hoc Bonferroni multiple pairwise comparisons test. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*)& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

Figure 2: Feeding and body composition

A. Weekly average food intake. Two-way ANOVA: diet: F (3, 56) = 11.19; p < 0.0001, steroid: F (1, 56) = 11.9; p < 0.01. B. Weekly average feeding efficiency. Two-way ANOVA: diet: F (5, 39) = 5.079; p < 0.01, steroid: F (1, 39) = 11.07; p < 0.01. C. Lean mass as a percent of body weight. Two-way ANOVA: diet: F (3, 87)
= 14.21; p < 0.0001, steroid: F (1, 87) = 95.56; p < 0.0001. D. Fat mass as a percent of body weight. Two-way ANOVA: diet: F (3, 87) = 17.17; p < 0.0001, steroid: F (1, 87) = 112.6; p < 0.0001. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

Figure 3: Blood chemistry

A. Fasting blood triglycerides. Two-way ANOVA: diet: F (3, 84) = 4.798; p < 0.01.

B. Fasting blood glucose. Two-way ANOVA: steroid: F (1, 84) = 5.926; p < 0.01.

Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

Figure 4: Glucose metabolism
A. 180 minute glucose tolerance test between oil-treated females. Two-way ANOVA: interaction time*diet: F (18, 264) = 4.02; p < 0.0001. B. 180 minute glucose tolerance test between E2-treated females. Two-way ANOVA: interaction time*diet: F (18, 240) = 1.986; p < 0.05. C. 120 minute insulin tolerance test between oil-treated females. Two-way ANOVA: time: F (5, 210) = 207.2; p < 0.0001. D. 120 minute insulin tolerance test between E2-treated females. Two-way ANOVA: time: F (5, 195) = 124.9; p < 0.0001. E. Area under the curve of blood glucose during glucose tolerance test. Two-way ANOVA: diet: F (3, 88) = 9.86; p < 0.0001, steroid: F (1, 88) = 64.69; p < 0.0001. F. Area under the curve of blood glucose during insulin tolerance test. Two-way ANOVA: steroid: F (1, 82) = 3.687; p = 0.0583. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*)& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

Figure 5: Calorimetry

A. Volume of oxygen consumption during the night and day. Two-way ANOVA: trt: F (7, 170) = 17.65; p < 0.0001, time: F (1, 170) = 33.89; p < 0.0001. B. Volume of carbon dioxide production during the night and day. Two-way ANOVA:
trt: F (7, 170) = 22.33; p < 0.0001, time: F (1, 170) = 32.28; p < 0.0001. C. Ratio of carbon dioxide production to volume of oxygen consumption during the night and day. Two-way ANOVA: trt: F (7, 170) = 78.14; p < 0.0001, time: F (1, 170) = 4.764; p < 0.05. D. Heat production during the night and day. Two-way ANOVA: trt: F (7, 170) = 2.279; p < 0.05, time: F (1, 170) = 44.59; p < 0.0001. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

**Figure 6: Spontaneous activity**

A. Total beam breaks in the x-axis during the night and day. Two-way ANOVA: time: F (1, 170) = 76.99; p < 0.0001. B. Novel beam breaks in the x-axis during the night and day. Two-way ANOVA: time: F (1, 170) = 47.57; p < 0.0001. C. Total beam breaks in the z-axis during the night and day. Two-way ANOVA: time: F (1, 170) = 77.3; p < 0.0001. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within
steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

**Figure 7: Plasma E2 and peptides**

A. Plasma E2 (pg/mL). Two-way ANOVA: steroid: F (1, 72) = 48.03; p < 0.0001.
B. Plasma leptin (pg/mL). Two-way ANOVA: diet: F (3, 56) = 79.74; p < 0.0001.
C. Plasma IL-6 (pg/mL). Two-way ANOVA: interaction diet*steroid: F (3, 48) = 12.57; p < 0.0001. Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

**Figure 8: Expression of arcuate nucleus neuropeptides**

A. Expression of *Pomc* relative to reference genes. Two-way ANOVA: steroid: F (1, 64) = 17.62; p < 0.0001. B. Expression of *Cart* relative to reference genes. Two-way ANOVA: steroid: F (1, 64) = 6.689; p < 0.05. C. Expression of *Npy* relative to reference genes. No significant effect of diet or steroid. D. Expression of *Agrp* relative to reference genes. Two-way ANOVA: diet: F (3, 63) = 2.93; p <
Data are represented as means ± SEM. Sample size for each group was 12. Data were analyzed by two-way ANOVA (repeated measures or standard) with post-hoc Bonferroni multiple pairwise comparisons test. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).

Figure 9: Expression of arcuate nucleus inflammatory genes

A. Expression of Tlr4 relative to reference genes. No significant effect of diet or steroid. B. Expression of Il-1 relative to reference genes. Two-way ANOVA: steroid: F (1, 61) = 17.6; p < 0.0001. C. Expression of Il-15 relative to reference genes. Two-way ANOVA: diet: F (3, 63) = 3.219; p < 0.05. Data are represented as mean ± SEM. Sample size for each group was 12. Data were analyzed by two-way ANOVA with post-hoc Bonferroni multiple pairwise comparison tests. Letters denote comparisons within diet between steroid treatments; asterisks denote comparisons within steroid treatment between HFD and CON. Comparisons within steroid, between HFDs are denoted with asterisks over capped lines. (*& a = p<0.5; ** & b = p<0.01; *** & c = p<0.001; **** & d = p<0.0001).
Figure 1

A. CON vs. 1%
- 1% OIL
- 1% E2
- CON OIL
- CON E2

B. CON vs. 15%
- 15% OIL
- 15% E2
- CON OIL
- CON E2

C. CON vs. 22.5%
- 22.5% OIL
- 22.5% E2
- CON OIL
- CON E2

D. Final bodyweight
- OIL
- E2
- CON
- 1%
- 15%
- 22.5%
Figure 2

A. Cumulative Food Intake

B. Cumulative Feeding Efficiency

C. Lean

D. Fat

% Body weight
Figure 3

**Fasting Triglycerides**

mg/dL

**Fasting Glucose**

mg/dL

CON  1%  15%  22.5%

OIL  E2
Figure 6

A 1000

Hourly Counts

X Total

B 600

Hourly Counts

X Ambulatory

C 250

Hourly Counts

Z Total

[Graphs showing data for Hourly Counts across different conditions.]
Figure 7

(A) Estradiol levels measured in pg/mL across different concentrations (CON, 1%, 15%, 22.5%).

(B) Leptin levels measured in ng/mL across different concentrations (CON, 1%, 15%, 22.5%). Asterisks indicate significant differences, with **** indicating a p-value < 0.0001 and ** indicating a p-value < 0.01.

(C) Interleukin 6 levels measured in ng/mL across different concentrations (CON, 1%, 15%, 22.5%). Asterisks indicate significant differences, with **** indicating a p-value < 0.0001 and * indicating a p-value < 0.05.
Figure 8

A) Pomc

B) Cart

C) Npy

D) Agrp

Relative Expression

CON 1% 15% 22.5%

Relative Expression

CON 1% 15% 22.5%

Relative Expression

CON 1% 15% 22.5%

Relative Expression

CON 1% 15% 22.5%

**
Figure 9

**Tlr4**

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* Significant difference
CHAPTER 4: THE EFFECTS ON OFFSPRING METABOLISM OF MATERNAL HIGH-FAT DIET COMPRISED PRIMARILY OF SATURATED FATTY ACIDS OR LINOLEIC ACID
4. The effects on offspring metabolism of maternal high-fat diet comprised of saturated fatty acids or linoleic acid

4.1 Abstract

The early-life origins of disease hypothesis has become part of the obesity research field. Using the established high-fat diet feeding technique to induce obesity in dams, the effects on offspring metabolism can be studied. Since the modern obesity epidemic is associated with a change in type of fat, rather than amount, the manipulation of fatty acid profile of experimental diets can be used to imitate this condition. Since fatty acids are now known to interact with cellular receptors, they are a likely category of nutrient to have complex endocrinological effects that could persist intergenerationally. We fed wild-type C57BL6/J mating pairs diets high in either saturated fat from coconut oil or linoleic acid from vegetable oil from pregnancy through lactation and followed the offspring. Body weight and food intake were measured weekly for 25 weeks. Assays for glucose metabolism, body composition and indirect calorimetry were performed at 25 weeks, and plasma metabolic peptides were measured at sacrifice. Maternal diet affected male offspring body weights but not female. Linoleic acid caused greater weight gain in males weaned onto high-fat diet, and impaired glucose disposal. Females of dams fed linoleic acid were insulin resistant when weaned onto high-fat diet. Plasma leptin was increased in obese males from maternal linoleic acid feeding, but was proportional to adiposity in females. These data indicate that
The fatty acid profile of maternal diet is more causative of obesity in male offspring than female, as they are likely protected by the metabolic effects of estrogen. Maternal linoleic acid feeding was shown to alter glucose metabolism, as it does when fed to adult animals. Our results suggest that fatty acid profile can have intergenerational metabolic effects and should be studied as a potential epigenetic modifier.

4.2 Introduction

In the early 21st century, obesity in the United States occurs in approximately 1 out of 3 American adults and 17% of children (1). In addition to the widely discussed lifestyle changes of increased sedentarism and a greater amount of meals coming from processed or fast foods, the question of epigenetic or early-life programming contributing to child obesity is being investigated. The early-life programming, or Barker, hypothesis began from evidence in Europe of strong correlations between low birth weight and adult cardiovascular disease (2), and experiments have investigated hypocaloric undernutrition (3) and protein restriction (4) as models to mimic low birth weight in the context of disturbing the energy balance system, causing obesity, and producing biomarkers of metabolic disease in adult offspring. Since the modern obesity epidemic is characterized by food abundance rather than restriction, a more relevant experimental question is how maternal overnutrition effects offspring energy homeostasis as adults.
Indeed, maternal diet-induced obesity (DIO) (5), even if restricted to high-fat diet (HFD) feeding during the pregnancy and lactation periods alone (6), causes obesity in male offspring seemingly in disregard to their diet. In addition to obesity, glucose metabolism is disturbed in these offspring (7). There is also evidence of early hypothalamic, but not peripheral, inflammation, which mechanistically ties the early-life programming hypothesis to the hypothalamic inflammation hypothesis of obesity (8). Female offspring may be partially protected from the obesogenic effects of maternal obesity through the role of 17β-estradiol in the control of hypothalamic and peripheral energy homeostasis (9).

Although the current obesity epidemic is more characterized by food abundance, as opposed to food restriction as described in the Barker hypothesis, perhaps an even greater distinction should be made regarding food quality. Feeding dams HFD to induce obesity in themselves and their offspring, while closer to the modern Western lifestyle, is not wholly accurate. Fat intake, even during the obesity epidemic, did not substantially increase (10). What did increase during that time was the intake of oils with a high linoleic acid (LA) content (11). LA content in breast milk has increased along with availability, and infant formulas use vegetable oil as their primary fat source (12). The DIO field has pivoted towards targeting specific fatty acids (FA) in their HFDs (13). High LA oils have been shown to be obesogenic compared to saturated fat (SFA) (14), while other studies show SFA (15) and even monounsaturated fat (16) causative
of obesity. These conflicting results may be due to inconsistencies in FA profile reporting, especially when using the variable fat source lard without confirming its FA profile with gas chromatography-mass spectrometry (17,18). Our group recently showed that both SFA and LA were obesogenic in mice, but that LA was more so (19). Since the change in modern dietary FA profile is so dramatic, and the integration of this information has firmly taken hold in the DIO field, early-life programming studies should shift focus towards this factor in maternal HFD feeding as well.

There is already reason to believe that FA profile, in addition to total fat content, has physiological consequences for offspring. High LA intake during pregnancy increases hypothalamic estrogen receptor abundance in female offspring, as well as voluntary alcohol intake (20). Focusing on the production of arachidonic acid derived prostaglandins, and their activation of molecular pathways inducing the maturation of preadipocytes to adipocytes, maternal high LA feeding produced obesity and greater adipocyte maturation and size in offspring, an effect that was blunted by including greater alpha-linoleic acid (ALA) as an enzymatic competitor (21). The stimulatory effect of LA/ALA ratio on early-life adipogenesis has been shown in guinea pigs (22), a particularly good model for human adipose tissue, as well as other animals (23). Although these studies look at the effects on offspring obesity of maternal intake of LA per se, comparing a high LA/ALA ratio to a balanced one, rather than to HFD high in SFA, ignores findings in the traditional DIO field. Increasing intake of LA without a
commensurate increase in ALA causes a greater LA/ALA ratio, but the characteristic pre-obesity epidemic FA profile was not one of both high LA and ALA intake, but a ratio brought into balance by a low LA intake, with a much higher SFA intake. Comparing the effects of feeding dams LA or SFA on offspring obesity and metabolism more directly addresses modern human DIO.

We hypothesize that offspring of dams fed high LA HFD will develop greater obesity and metabolic impairments than offspring of dams fed high SFA HFD. To address this we fed female, wild type C57BL6/J mice one of 2 HFDs, either primarily vegetable oil or primarily coconut oil, or a standard low-fat control (CON), during breeding, pregnancy and lactation. Male and female offspring were weaned onto either CON or HFD, and body weight and food intake were measured weekly followed by measurements of body composition, indirect calorimetry, and glucose and insulin tolerance tests after 25 weeks.

4.3 Materials and Methods

4.3.1 Animal Care

All animal treatments were in accordance with institutional guidelines based on National Institutes of Health standards and performed with Institutional Animal Care and Use Committee approval at Rutgers University. Female and male WT C57BL6/J mice were selectively bred in-house, maintained under controlled temperature (23°C) and photoperiod conditions (12/12 h light/dark
cycle), and given access to food and water *ad libitum*. Mice were weaned and ear-tagged at post-natal day 21 and housed in groups until start of experiment.

**4.3.2 Breeding**

At ~7 weeks of age female dams (n=6-8/diet) were acclimated for 4 week to their experimental diet, then paired with males for mating. Males were removed at first sign of pregnancy, either copulation plug or rapid body weight gain in breeders. Dams were maintained on their experimental diet through pregnancy and lactation, and offspring were weaned, tagged, and housed in groups by sex at postnatal day 21 (PND 21). Litters were culled to 6-8 to remove potential litter size effects. Mating males were reintroduced for a second breeding where they continued to be maintained on their experimental diet. All females were limited to 2 matings to reduce the impact of multiparity on offspring energy homeostasis (24).

**4.3.3 Experimental Diets**

All experimental diets were prepared as pellets by Research Diets (New Brunswick, NJ). FA profile was assured through in-house gas chromatography - mass spectroscopy. Research Diets D12450B (10% kcals from fat) was used as the control diet (CON). We used 2 HFDs that were isocaloric and isolipidic to Research Diets D12451 (45% kcals from fat) and named for the amount of calories derived from LA; 1% = mostly coconut oil with some seed oils, 22.5% =
mostly safflower and sunflower seed oils (see Table 1 for FA profile). A constant n-3 ratio content, which led to a rising n-6/n-3 ratio in 22.5%, was used to model the changes in Western dietary FA profile during the 20th century. Coconut oil was chosen as the source of SFA because it is 98% saturated and does not contain the long chain PUFA that animal sources of SFA contain, making these diets a direct comparison of SFA and LA. All diets had identical protein, fiber, and micronutrient contents.

4.3.4 Offspring Experimental Design

Experimental feeding of offspring began at weaning on PND 21. Mice were housed by sex, 3-6 per cage depending on litter size, and given ad libitum access to food and water. Offspring diet was either CON or Research Diets D12451 (HFD). Body weight and food intake (per cage food intake) were recorded weekly for 25 weeks followed by body composition measurements using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) and calorimetric and activity measurements (48 h run) via Columbus Instruments’ Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Inc., Columbus, OH, USA). A glucose tolerance test (GTT), following a 5 h fast, was administered via intraperitoneal (ip) injection of 2g/kg glucose in 0.9% saline solution. Blood glucose (BG) from tail blood was measured with an AlphaTrak 2 Blood Glucose Monitoring System (Abbott Laboratories, Abbott Park, IL, USA) pre-injection and 15, 30, 60, 90, 120 and 180
minutes post-injection. An insulin tolerance test (ITT) following a 5 h fast involved an injection of 0.5 U/kg insulin (Humulin R, Lilly, Indianapolis, IN, USA) in 0.9% saline solution and followed the same BG measurement scheme as GTT. Mice were given 4 days of rest each between CLAMS, GTT, and ITT.

4.3.5 Tissue Collection

At completion of physiological assays, mice were given another 4 days of rest while remaining on the same diet and then killed by decapitation after ketamine sedation (100 μl of 100 mg/ml, ip). Trunk blood was collected and prepared for plasma analysis of the peptide hormones insulin and leptin, and the cytokines interleukin-6 (IL6), and monocyte chemoattractant protein-1 (MCP1) by Luminex Magpix multiplex (EMD Millipore, Billerica, Massachusetts, USA). Plasma was prepared by adding a protease inhibitor, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF, 1 mg/mL, Sigma-Aldrich) to each K+ EDTA collection tube. Samples were maintained on ice until centrifugation at 3,000 rpm for 10 min at 4°C. Supernatant was then collected and stored at –80°C until analysis.

4.3.6 Data analysis

All data are expressed as means ± SEM. All data from the weekly body weight and food intake measurements, GTT, and ITT were analyzed using a repeated measures two-way ANOVA followed by a post hoc Bonferroni-Dunn
multiple comparisons test. CLAMS calorimetry analysis used a regular two-way ANOVA with Bonferroni-Dunn post hoc multiple comparisons test. Body composition, CLAMS activity, and plasma protein were analyzed using a one-way ANOVA followed by a post hoc Bonferroni-Dunn multiple comparisons test (unpaired). All data analyses were performed on GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA) and in all cases, effects were considered significant at p<0.05.

4.4 Results

4.4.1 Body Weight, Composition, and Food Intake

Male and female weaning weights at PND 21 were generally similar, male: (CON: 10.1 ± 0.5 g (n = 5); 1%: 11.3 ± 0.5 g (n = 7); 22.5%: 10.5 ± 0.8 g (n = 5)), female: (CON: 9.6 ± 0.4 g (n = 7); 1%: 10.8 ± 0.5 g (n = 4); 22.5%: 9.9 ± 0.9 g (n = 5). Litters of 1% fed dams had higher average weaning weights in both sexes, but not significantly so. This may be related to the lower body weights in male offspring of 1% fed dams.

In male offspring, both the maternal diet (F (2, 69) = 4.35; p = 0.05) and offspring diet (F (1, 69) = 27.26; p < 0.0001) affected final bodyweights (Figure 1A). Male offspring of CON-fed dams had similar final body weights regardless of the diet they were weaned onto. Male offspring of dams fed 1% or 22.5% gained more weight when weaned onto HFD than onto CON. Within CON-fed offspring, 1% maternal feeding resulted in lower body weights than maternal CON feeding.
Within HFD-fed offspring, 22.5% maternal feeding resulted in higher body weights than both 1% (p < 0.05) and CON (p < 0.01). On a weekly basis, within CON-fed males, offspring of dams fed 22.5% gained less weight than offspring of dams fed CON from weeks 15 (p < 0.05) to 25 (p < 0.01) (Figure 1B). CON-fed male offspring of dams fed 1% gained less weight from weeks 20 (p < 0.05) to 25 (p < 0.001). Within HFD-fed males, offspring of dams fed 22.5% gained more weight than those of CON-fed dams at weeks 10 (p < 0.01) to 20 (p < 0.0001), and offspring of dams fed 1% gained more at than those of CON-fed dams at weeks 15 (p < 0.05) to 20 (p < 0.01) (Figure 1C).

In female offspring, only offspring diet (F (1, 57) = 87.42; p < 0.0001) and not maternal diet affected final body weights (Figure 1D). HFD-fed female offspring gained more weight over 25 weeks than CON-fed of dams fed CON (p < 0.0001), 1% (p < 0.0001), or 22.5% (p < 0.001). Within CON-fed females, offspring of dams fed 1% began experimental feeding heavier than offspring of dams fed 22.5% (p < 0.001), and weighed more at weeks 15 (p < 0.01) to 25 (p < 0.001) (Figure 1E). CON-fed female offspring of dams fed 1% weighed more at week 25 than offspring of dams fed CON (p < 0.05). HFD-fed female offspring of dams fed 1% or 22.5% weighed more than those of dams fed CON at weeks 15 (p < 0.05) to 25 (p < 0.01) (Figure 1F).

Lean tissue mass in males was affected by offspring diet (F (1, 68) = 23.72; p < 0.001) but not maternal diet (Figure 2A). Percent lean mass was higher in CON-fed compared to HFD-fed offspring of 1% (p < 0.0001) and 22.5%
(p < 0.01) fed dams. Male fat mass was also affected by offspring diet (F (1, 68) = 37.91; p < 0.0001) and not maternal diet (Figure 2B). Body fat percentage was higher in HFD-fed compared to CON-fed offspring of 1% (p < 0.0001) and 22.5% (p < 0.0001) fed dams. Female lean tissue mass was affected by both maternal (F (2, 60) = 5.638; p < 0.01) and offspring (F (1, 60) = 102.8; p < 0.0001) diets (Figure 2C). Percent lean mass was lower in HFD-fed female offspring of 1% (p < 0.01) and 22.5% (p < 0.05) fed dams. Offspring (F (1, 60) = 132.4; p < 0.0001) and maternal (F (2, 60) = 3.911; p < 0.05) diet affected body fat mass (Figure 2D). HFD increased body fat in all maternal diets (p < 0.0001). HFD-fed female from 1%-fed dams exhibited higher body fat mass compared to HFD-fed female offspring from CON-fed dams (p < 0.05).

Average weekly food intake in males was primarily affected by offspring diet (F (1, 12) = 19.82; p < 0.001), leading to an increased intake in HFD-fed compared to CON-fed offspring of 1%-fed (p < 0.05) and 22.5%-fed (p < 0.05) dams, but not Con-fed dams (Figure 3A). There was a trend for of variation in food intake coming from maternal diet (F (2, 12) = 2.589; p = 0.1163), causing a higher weekly intake in HFD-fed offspring of 22.5% fed dams compared to HFD-fed offspring of CON-fed dams (p < 0.05). Female average weekly food intake was also mostly determined by offspring diet (F (1, 10) = 55.1; p < 0.0001) (Figure 3B). HFD-fed female offspring of CON- (p < 0.05), 1%- (p < 0.01), and 22.5%- (p < 0.01) fed dams consumed more food than CON-fed females of the same maternal diet. Feeding efficiency in males was affected by offspring diet (F
(1, 12) = 11.74; p < 0.01), but was not different between groups within maternal or offspring diets (Figure 3C). Feeding efficiency in females was also affected by offspring diet (F (1, 10) = 25.44; p < 0.001) (Figure 3D). Female offspring of 1% fed dams had higher efficiency on HFD than on CON (p < 0.05).

4.4.2 Glucose Metabolism

Offspring HFD was the primary driver of higher fasting blood glucose in males (F (1, 66) = 8.094; p < 0.01) (Figure 4A). While adult HFD in males from Con-fed dams did not alter fasting glucose, adult HFD increased fasting glucose in males from both 1%- and 22.5%-fed dams. Fasting glucose was higher than CON-fed offspring of CON and 1% fed dams (p < 0.01). During GTT, time (F (6, 396) = 83.22; p < 0.0001) and an interaction of maternal and offspring diet (F (5, 66) = 6.434; p < 0.0001) were both responsible for changes in blood glucose (BG). Total area under the curve (AUC) of GTT BG was controlled by offspring diet (F (1, 66) = 24.24; p < 0.0001), with HFD-fed offspring of CON (p < 0.05) and 22.5% (p < 0.01) fed dams being higher than their CON-fed counterparts (Figure 4B). Within CON-fed males, offspring of 1% fed dams had higher BG than offspring of CON fed dams at 60 min. (p < 0.05) and higher BG than offspring of CON and 22.5% fed dams at 90 min. (p < 0.05) (Figure 4C). HFD-fed males had a higher BG spike and slower disposal, with average readings breaking and maintaining over 600 mg/dL, than CON fed, but were not different from each other from maternal diet (Figure 4D).
During ITT, time (F (5, 325) = 46.85; p < 0.0001) and an interaction of maternal and offspring diet (F (5, 65) = 5.866; p < 0.001) affected BG. Total AUC of BG was different between groups through an interaction of maternal and offspring diet (F (2, 65) = 7.502; p < 0.01) (Figure 5A). CON-fed offspring of CON-fed dams, and HFD-fed offspring of 22.5% fed dams, were similar, both being higher than other CON-fed offspring (p < 0.05). In males from CON-fed dams, HFD increased insulin-stimulated glucose clearance (p < 0.05). Conversely, in males from 22.5%-fed dams, HFD reduced insulin-stimulated glucose clearance (p < 0.05), while no difference was found in males from 1%-fed dams. Comparing CON-fed male offspring over time, maternal diet influenced BG (F (2, 43) = 9.96; p < 0.001), as offspring of both 1%- and 22.5%-fed dams had lower BG than offspring of CON-fed dams from 30 minutes on (Figure 5B). Maternal diet also affected on ITT BG in HFD-fed male offspring (F (2, 22) = 3.686; p < 0.05) (Figure 5C). Throughout the ITT, BG trended lowest in offspring of CON-fed dams, followed by 1% fed, and highest in offspring of 22.5% fed dams, but due to large individual subject variability BG in offspring of 22.5% fed dams early in the test at 15 minutes (p < 0.01).

In females, offspring diet rather than maternal diet was more important in glucose metabolism compared to male offspring. Variability in fasting glucose was driven by offspring diet (F (1, 60) = 27.24; p < 0.0001), but only offspring of 22.5% fed dams showed a significant increase in fasting BG from HFD compared to CON diet (p < 0.0001) (Figure 6A). CON-fed offspring of 22.5% fed dams,
however, had a suppressed BG compared to CON-fed offspring of CON fed dams (p < 0.05). GTT AUC was similar in all female offspring fed the same diet, but all maternal diet group’s offspring had lower AUC on CON compared to HFD (F (1, 60) = 59.54; p < 0.0001) (Figure 6B). Maternal diet did not affect BG during GTT in CON-fed offspring (Figure 6C) or HFD-fed offspring (Figure 6D); within CON-fed females offspring of 22.5% fed dams had higher BG at the 60 min. time point, but returned to being similar for the rest of the GTT. Similar to the males, BG peak during GTT was 100-200 md/dL higher in HFD-fed than CON-fed females.

Maternal diet has a greater effect on BG during ITT than on GTT in females. Total BG AUC variability was driven mostly by offspring diet (F (1, 50) = 34.16; p < 0.0001). HFD-fed offspring from CON-fed (p < 0.05) and 22.5%-fed (p < 0.0001) dams both had higher BG AUC than CON-fed counterparts (Figure 7A). HFD-fed offspring of 1%-fed dams, however, had suppressed BG AUC compared to those of 22.5% fed dams (p < 0.05), and were not different from CON-fed offspring from the same maternal diet. Comparing within offspring diet over time, maternal diet affected CON-fed (F (2, 27) = 4.265; p < 0.05) and HFD-fed offspring BG (F (2, 23) = 3.72; p < 0.05). BG during ITT of CON-fed females was similarly repressed until the last time point, 120 min, when offspring of 22.5% fed dams maintained a lower BG than offspring of 1% (p < 0.0001) and of CON-fed dams (p < 0.05) (Figure 7B). In HFD-fed females, however, offspring of 1% fed dams had lower BG at 15 min compared to offspring of CON-fed (p <
Offspring of 22.5%-fed dams expressed higher BG at 60 min (p < 0.05) and maintained higher BG through 120 min (p < 0.001).

4.4.3 Indirect Calorimetry and Activity

O2 consumption was generally higher in males during the night F (1, 60) = 381; p < 0.0001). Within CON-fed males, the effects of nighttime on O2 consumption were blunted in offspring of CON-fed and 22.5% fed dams (Figure 8A). The effect of maternal diet (F (2, 39) = 4.404; p < 0.05) was seen in higher nighttime O2 consumption in offspring of 1% fed dams (p < 0.05) and higher daytime O2 consumption in offspring of 22.5% fed dams (p < 0.05) compared to offspring of CON-fed dams. Within HFD-fed males, the effect of maternal diet (F (2, 21) = 4.267; p < 0.05) was seen as greater night and day O2 consumption in offspring of 1% fed dams than those of 22.5% fed dams (p < 0.05) (Figure 8B). Compared within maternal diets, HFD feeding suppressed O2 consumption in offspring of 1% fed dams during the night (p < 0.05) and in offspring of 22.5% dams during both day and night (p < 0.0001).

CO2 production was also lower during the day than during the night in CON-fed males (F (1, 39) = 132.1; p < 0.0001) (Figure 8C). Maternal diet (F (2, 39) = 6.217; p < 0.01) caused an increase in CO2 production in offspring of 1% and 22.5% fed dams compared to offspring of CON-fed dams during the day (p < 0.05), but only for offspring of 1% fed dams during the night (p < 0.001). CO2
production in HFD-fed males was higher during the night (F (1, 21) = 262.4; p < 0.0001), but the effects of maternal diet were blunted (F (2, 21) = 2.898; p = 0.0774) (Figure 8D). Compared within maternal diets, HFD-fed offspring of 1% (p < 0.001) and 22.5% (p < 0.0001) fed dams had higher day and night CO2 production than CON-fed offspring; this effect was not seen in male offspring of CON-fed dams. The ratio of CO2 produced to O2 consumed, respiratory exchange ratio (RER), was unaffected by time of day (F (1, 39) = 3.335; p = 0.0755) but was affected by maternal diet (F (2, 39) = 8.85; p < 0.001) in CON-fed males (Figure 8E). Nighttime RER was higher in male CON-fed offspring of 1% (p < 0.0001) and 22.5% (p < 0.05) fed dams compared to those of CON-fed dams; daytime RER was higher in offspring of 1% fed dams than of CON-fed dams (p < 0.01). HFD-fed males also showed no effects of time of day on RER (Figure 8F). Daytime RER was suppressed in offspring of 1% fed dams compared to offspring of CON-fed dams (p < 0.01) and to offspring of 22.5% fed dams (p < 0.05); effects of maternal diet (F (2, 21) = 5.381; p < 0.05).

Nighttime O2 consumption was higher in CON-fed (F (1, 36) = 145; p < 0.0001) (Figure 9A) and HFD-fed (F (1, 24) = 116.6; p < 0.0001) (Figure 9B) females. Maternal diet did not affect O2 consumption of CON-fed females but did for HFD-fed (F (2, 24) = 7.022; p < 0.01). HFD-fed offspring of 22.5% fed dams had reduced O2 consumption during the night (p < 0.01) and day (p < 0.001). The same pattern was observed in CO2 production, with both CON (F (1, 36) = 135.4; p < 0.0001) and HFD-fed (F (1, 24) = 109.8; p < 0.0001) females having
higher nighttime VCO2 than during the day (Figure 8C-D). Again, maternal diet only affected HFD-fed females ($F (2, 24) = 6.996; p < 0.01$), with offspring of 22.5% fed dams having suppressed night and day CO2 production ($p < 0.01$). RER was not affected by time of day or maternal diet in CON or HFD-fed females.

Spontaneous activity in males and females was not affected by maternal diet. Both total ($F (1, 39) = 76.28; p < 0.0001$) and ambulatory ($F (1, 39) = 52.24; p < 0.0001$) activity in the X-axis in males was elevated during the night than the day, as was movement in the Z-axis ($F (1, 39) = 71.67; p < 0.0001$) (Figure 10A-F). Females reacted similarly with X-axis total ($F (1, 36) = 53.35; p < 0.0001$), X ambulatory ($F (1, 36) = 44.71; p < 0.0001$) and Z-axis ($F (1, 36) = 26.84; p < 0.0001$) movement elevated during the night than during the day (Figure 11A-F).

### 4.4.5 Plasma Proteins

Circulating insulin in males was largely unaffected by offspring ($F (1, 40) = 0.9816; p = 0.3277$) or maternal ($F (2, 40) = 1,597; p = 0.2151$) diet (Figure 12A). HFD-fed offspring of 22.5% fed dams, however, had higher insulin than their CON-fed counterparts and the HFD-fed offspring of 1% fed dams ($p < 0.05$). Leptin was affected by both offspring ($F (1, 40) = 28.25; p < 0.0001$) and maternal ($F (2, 40) = 6.357; p < 0.01$) diet in males. HFD feeding resulted in higher plasma leptin in offspring of 1%-fed ($p < 0.0001$) and 22.5%-fed ($p < 0.05$) dams compared to CON feeding (Figure 12B). HFD-fed offspring of 1%-fed dams
had higher leptin than HFD-fed offspring of 22.5% dams (p < 0.01). Circulating IL-6 was highest in CON-fed offspring of CON-fed dams, being suppressed by offspring HFD (F (1, 39) = 8.015; p < 0.01) and an interaction of maternal and offspring diet in the other groups (F (2, 39) = 4.44; p < 0.05) (Figure 12C). Plasma MCP-1 expression was suppressed by HFD feeding in offspring of CON-fed dams (p < 0.05), and by maternal 22.5% HFD in CON-fed males being (p < 0.05; maternal and adult diet interaction: F (2, 37) = 5.354; p < 0.01; Figure 12D).

Circulating insulin tended to be suppressed by offspring HFD or maternal HFD through an effect of maternal diet (F (2, 44) = 4.485; p < 0.05) and its interaction with offspring diet (F (2, 44) = 4.01; p < 0.05) (Figure 13A). Within offspring of CON-fed dams, HFD feeding lowered plasma insulin (p < 0.05). The other maternal groups were not different based on offspring diet, but CON-fed offspring of 1% fed dams (p < 0.05) and 22.5% fed dams (p < 0.05) also had lower plasma insulin than offspring of CON-fed dams. Plasma leptin was highest in HFD-fed offspring of 1% fed and 22.5% fed dams with an effect of maternal (F (2, 45) = 5.002; p < 0.05) and offspring (F (1, 45) = 46.05; p < 0.0001) diets (Figure 13B). There were no effects of maternal or adult diet on circulating IL-6 in females (Figure 13C) or MCP-1 (Figure 13D).

4.5 Discussion

It has been shown previously that male offspring are susceptible to maternal overnutrition-induced obesity and metabolic impairment (5,6), and that
female offspring, when studied, are at least partially protected from these effects at the gross anatomy and at the molecular level (25). In our study, we included male and female offspring in the analysis and observed sex-specific results. Previous findings of protection from intergenerational diet effects in females may not apply equally across all diet manipulations, and the comparison of different FAs in the context of DIO is not necessarily equivalent to simple overnutrition.

Proof of concept of specific effects of LA on female offspring was already present (20), and the interaction with estrogen receptor-alpha abundance suggests interaction with the steroid system proposed to be protective in females. Matching the Barker hypothesis with a dietary change that occurred during the relevant time course of the obesity epidemic is the most likely experimental design to yield useful insights for application. The substantial increase in LA intake (11) is given little consideration in obesity research relative to other changes such as increases in sugar consumption or use of processed foods, especially when comparing these changes on a percentage basis of diet. The evidence for an endocrinological, in addition to nutritive, action of FAs through cell surface (26) and nuclear (27) receptors makes dietary fat an even more attractive candidate for intergenerational changes at the cellular and molecular levels. We found that FA profile did have an effect on obesity and some parameters of metabolism in male and female offspring.

In CON-fed males, maternal high-fat feeding did not cause greater weight gain. Specifically, low LA HFD suppressed weight gain compared to the low-fat
control. HFD maternal feeding also led to a suppression of IL-6 in CON-fed males, which may have a causal or effect relationship with suppressed body weights. One factor to make sure of is that the essential FA (EFA) content of the 1% LA diet we used was sufficient for growth. The fourth revised addition of Nutrient Requirements of Laboratory Animals recommends 0.68% of energy from LA as necessary to prevent symptoms of EFA deficiency such as growth retardation and acrodynia (28). The necessity of even this requirement is unclear as evidence has been available since the 1940s that so-called EFA deficiency may be a B vitamin, specifically B5 and B6, deficiency, demonstrated by Schneider et al., Quackenbush et al. and others (29,30).

Regardless, the LA content of our 1% diet obviates this potential problem. The reduced weight in CON-fed offspring of 1% fed dams was also not pathologically underweight, but closer to a normal weight. At 25 weeks those males reached a weight approximately 300% greater than their weight at 21 days of age, whereas offspring of CON-fed dams, and HFD-fed offspring of dams fed either HFD, reached body weights 400% and above their 21 day weight. No scaling of thickening of the trunk or tail skin, a more conspicuous sign of EFA deficiency than lower growth rates, was seen (31). Additionally, lean mass was higher and fat mass lower in CON-fed offspring of dams fed either HFD, making underdevelopment unlikely.

HFD-feeding in dams of HFD-fed males, however, caused greater weight gain. HFD-fed offspring of 22.5% fed dams, specifically, had higher body weights
than HFD-fed offspring of 1% or CON-fed dams. The cause of early-life induced obesity from LA may be different than the causes, such as endocannabinoid tone, suggested for obesity produced in mature animals by n-6 PUFA (32). The receptors purported to control differentiation and maturation of preadipocytes, the peroxisome proliferator activated receptor (PPAR) family, are thought to show binding affinity to PUFA and their downstream eicosanoid metabolites more than other FAs (33). This is seen in the ability of PUFA, but not SFA or MUFA, to reduce circulating LDL via PPAR action (34,35). PPAR activation, in particular by n-6 PUFA early in life, produces a pro-adipoblastic signaling environment in preadipocytes through elevated cyclic AMP (36). The resulting higher number of mature adipocytes would tend to accumulate FAs more readily from the circulation (37), and, when induced, produce more obesogenic cytokines (38).

Several activators of PPARs, especially PPAR gamma, are known epigenetic actors (39) as well as controlling the fate of cellular development in the current generation. FA receptors, such as the PPARs, would make an effective tool for monitoring environmental conditions and programming that information into future generations, since they interact with the highest energy nutrient.

In addition to greater susceptibility to DIO in adolescence, the likely long-term outcome of a greater number of maturing adipocytes is resistance to weight loss in adulthood. Even after the recent advances in understanding molecular mechanisms of obesity, the primary focus of research and of physicians is the amount of calories consumed and burned. If specific nutrients can, early in life,
induce a greater cellular development of adipose tissue that will follow that individual throughout life as a higher propensity to store energy as fat and alter normal metabolism, preventing that would be extremely helpful in combating obesity.

Plasma insulin, interestingly, did not strictly follow adiposity as is usually seen (40), but is reduced in the heaviest group, HFD-fed offspring of 22.5% fed dams, compared to HFD-fed offspring of 1% fed dams. Reduced secretion of insulin could contribute to that group’s greater weight gain through reduced satiety; HFD-fed offspring of 22.5% fed dams also had the greatest weekly food intake. HFD-fed male offspring of 22.5% fed dams also had higher circulating leptin than the other groups, and this may have been interactive with reduced insulin. Higher body weight likely contributed to higher leptin levels, but the shared downstream signaling cascade between insulin and leptin (41) is also a potential reason for elevated leptin in tandem with suppressed insulin.

As expected, females showed some protection from maternal overnutrition. Body weight gain followed offspring diet, being higher in HFD-fed than CON-fed females regardless of maternal diet. This finding, in the context of maternal LA intake, suggests that estrogen may be involved in PPAR-induced adipogenic differentiation. This involvement of estrogen in maturation of preadipocytes (42) and of mesenchymal stem cells into adipocytes (43) in differentiation medium has been studied in vitro but not in vivo. The recognition of FAs, specifically PUFA, as potent signaling molecules within this differentiation
milieu, and the involvement of sex steroids, would integrate real environmental changes for humans within early-life adipogenesis research. An interesting future direction would be to ovariectomize female offspring, or use a transgenic or Cre-LoxP knockdown model of estrogen, to compare the effects of maternal overnutrition on intact and steroid deficient female offspring. One effect of maternal diet was seen in body composition of HFD-fed females born to HFD-fed dams, where lean mass was reduced 7-10% compared to HFD-fed offspring of CON-fed dams. Plasma leptin was higher in HFD-fed females, but was potentiated in offspring of 1% fed females, although this had no effect on food intake.

Fasting BG in males was highest in the heaviest group, HFD-fed offspring of 22.5% fed dams, but this translated only a small impairment in glucose disposal during GTT. This group did, however, have higher BG during ITT, suggesting insulin resistance. Insulin resistance has been previously demonstrated from dietary LA (13), through the mechanism of increasing stimulation of the CPT1 system (44) and subsequent inhibition of mitochondrial glucose oxidation. Our results suggest that this effect can be produced through perinatal LA delivery, or delivery during lactation. Females were protected from effects of obesity on glucose metabolism, except for offspring of 22.5% fed dams, which had suppressed fasting glucose when CON-fed but elevated when HFD-fed. The effect was not seen in GTT, but during ITT HFD-fed offspring of 22.5% fed dams had impaired glucose disposal. Surprisingly, their plasma insulin was
not elevated as it was in males of that group. Normally, the pancreatic islets of female mice would be potentiated by endogenous estrogen to secrete insulin in the face of high BG (45). Plasma leptin in females was mostly proportional to body weight and fat mass. The highest plasma leptin was seen in HFD-fed female offspring of 1% fed dams, which also had somewhat lower insulin, potentially following the same relationship of insulin and leptin seen in the males. O2 consumption and CO2 production were higher in CON-fed male offspring of 1% fed dams. In addition to a similar food intake between offspring of CON-fed and 1% fed dams, this suggests that the reduced body weights in that group are due to a higher metabolic rate. HFD-fed female offspring of 22.5% fed dams had suppressed O2 consumption and CO2 production, likely caused by suppressed glucose metabolism.

Because our measurement of spontaneous activity did not show any effects of maternal or offspring diet, but only classic effects of night and day, the differences in weight gain and body composition are likely due to metabolic rate. This is supported by the reduced O2 consumption in obese groups compared to the lighter and leaner ones. O2 consumption from cellular respiration is likely overestimated in animals with high concentrations of PUFA in their tissues, due to oxygen being added to reactive lipids to form peroxides (46). The difference in O2 used in cellular respiration is, therefore, likely larger than that measured through indirect calorimetry.
In summary, that a high LA HFD potentiated weight gain in male offspring fed a high-fat diet, but not offspring fed low-fat. Female offspring were resistant to these maternal diet effects when fed HFD or CON, and gained body weight according to the diet they were weaned on to. It is proposed that obesity induced by early-life LA intake is due to the pro-adipogenic molecular effects of n-6 PUFA on pre-adipocytes through binding of PPARs, and that estrogen receptor alpha signaling in female offspring is a source of protection from these effects. A maternal diet high in LA also impaired glucose metabolism in HFD-fed male and female offspring, due to the accumulation of LA in tissues and its antagonistic effects to glucose metabolism within mitochondria. If a dietary aspect as simple as FA profile can alter the propensity of offspring to become obese and glucose intolerant, addressing this in the food supply would be a more efficient strategy than trying to treat children and adults already presenting with stubborn obesity. Future experiments will determine the effect of these maternal diets on gene expression of energy balance and inflammation related genes in the arcuate nucleus of the hypothalamus and in the liver of male and female offspring.

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Figures

Figure 1: Body weight
A. Male cumulative weight gain 25 weeks post-weaning. B. CON-fed weekly male weight gain at 5-week intervals. C. HFD-fed weekly male weight gain at 5-week intervals. D. Female cumulative weight gain 25 weeks post-weaning. E. CON-fed weekly female weight gain at 5-week intervals. F. HFD-fed weekly female weight gain at 5-week intervals. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* = p < 0.05, ** = p < 0.01, *** or c = p < 0.001, **** or d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

Figure 2: Body composition
A. Male lean mass as a percentage of body weight. B. Male fat mass as a percentage of body weight. C. Female lean mass as a percentage of body weight. D. Female fat mass as a percentage of body weight. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* = p < 0.05, ** or b = p < 0.01, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.
**Figure 3: Food intake**

A. Average weekly food intake in males. B. Average weekly food intake in females. C. Average weekly feeding efficiency (grams body weight gained/kcals consumed) in males. D. Average weekly feeding efficiency in females. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, b = p < 0.01). Asterisks over columns denote comparison to maternal CON. Letters denote comparisons within maternal diet.

**Figure 4: Male glucose tolerance**

A. Fasting glucose. B. Area under the curve of blood glucose throughout GTT. C. Post-glucose challenge blood glucose over 180 minutes in CON-fed. D. Post-glucose challenge blood glucose over 180 minutes in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, ** or b = p < 0.01). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 5: Male insulin tolerance**

A. Area under the curve of blood glucose throughout ITT. C. Post-insulin challenge blood glucose over 120 minutes in CON-fed. D. Post-insulin challenge blood glucose over 120 minutes in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001). Asterisks over columns denote
comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 6: Female glucose tolerance**

A. Fasting glucose. B. Area under the curve of blood glucose throughout GTT. C. Post-glucose challenge blood glucose over 180 minutes in CON-fed. D. Post-glucose challenge blood glucose over 180 minutes in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* = p < 0.05, b = p < 0.01, c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 7: Female insulin tolerance**

A. Area under the curve of blood glucose throughout ITT. C. Post-insulin challenge blood glucose over 120 minutes in CON-fed. D. Post-insulin challenge blood glucose over 120 minutes in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** or d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted
with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 8: Male indirect calorimetry during night and day**

A. Volume of oxygen consumption in CON-fed. B. Volume of oxygen consumption in HFD-fed. C. Volume of carbon dioxide production in CON-fed. D. Volume of carbon dioxide production in HFD-fed. E. Ratio of VCO2/VO2 in CON-fed. F. Ratio of VCO2/VO2 in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* = p < 0.05, ** or b = p < 0.01, *** = p < 0.001, **** or d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 9: Female indirect calorimetry during night and day**

A. Volume of oxygen consumption in CON-fed. B. Volume of oxygen consumption in HFD-fed. C. Volume of carbon dioxide production in CON-fed. D. Volume of carbon dioxide production in HFD-fed. E. Ratio of VCO2/VO2 in CON-fed. F. Ratio of VCO2/VO2 in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (a = p < 0.05, ** or b = p < 0.01, *** or c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted
with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 10: Male spontaneous activity during night and day**

A. Beam breaks in the x-axis in CON-fed. B. Beam breaks in the x-axis in HFD-fed. C. Novel beam breaks in the x-axis in CON-fed. D. Novel beam breaks in the x-axis in HFD-fed. E. Beam breaks in the z-axis in CON-fed. F. Beam breaks in the z-axis in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (a = p < 0.05, b = p < 0.01, c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

**Figure 11: Female spontaneous activity during night and day**

A. Beam breaks in the x-axis in CON-fed. B. Beam breaks in the x-axis in HFD-fed. C. Novel beam breaks in the x-axis in CON-fed. D. Novel beam breaks in the x-axis in HFD-fed. E. Beam breaks in the z-axis in CON-fed. F. Beam breaks in the z-axis in HFD-fed. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (a = p < 0.05, b = p < 0.01, c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.
Figure 12: Male plasma peptides

A. Plasma insulin. B. Leptin. C. IL-6. D. MCP-1. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, ** or b = p < 0.01, *** or c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.

Figure 13: Male plasma peptides

A. Plasma insulin. B. Leptin. C. IL-6. D. MCP-1. All data were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni’s analysis (* or a = p < 0.05, ** = p < 0.01, c = p < 0.001, d = p < 0.0001). Asterisks over columns denote comparison to maternal CON, comparisons between maternal HFDs denoted with asterisks over capped lines. Letters denote comparisons within maternal diet.
Figure 1

A. Male Cumulative Weight Gain

B. Weekly CON Male Weight Gain

C. Weekly HFD Male Weight Gain

D. Female Cumulative Weight Gain

E. Weekly CON Female Weight Gain

F. Weekly HFD Female Weight Gain
Figure 2

**A. Male Lean Mass**

- CON: Black bars
- HFD: Grey bars

**B. Male Fat Mass**

**C. Female Lean Mass**

**D. Female Fat Mass**

- CON: Black bars
- 1%: Grey bars
- 22.5%: Black bars

Key:
- d
- b
- **d**
- **d**
- **d**
- **d**
- **d**
- **d**

Significance levels are indicated by symbols above the bars.
Figure 3

A. Male Food Intake

B. Female Food Intake

C. Male Feeding Efficiency

D. Female Feeding Efficiency
Figure 4

A. Male Fasting Glucose

B. Male GTT AUC

C. Male CON GTT

D. Male HFD GTT
Figure 5

A. Male ITT AUC

B. Male CON ITT

C. Male HFD ITT
Figure 6

A) Female Fasting BG

B) Female GTT AUC

C) Female CON GTT

D) Female HFD GTT
Figure 8

A. Male CON VO2

B. Male HFD VO2

C. Male CON VCO2

D. Male HFD VCO2

E. Male CON RER

F. Male HFD RER
Figure 9

A. Female CON VO2

B. Female HFD VO2

C. Female CON VCO2

D. Female HFD VCO2

E. Female CON RER

F. Female HFD RER
Figure 10

A  Male CON X Total

B  Male HFD X Total

C  Male CON X Ambulatory

D  Male HFD X Ambulatory

E  Male CON Z Total

F  Male HFD Z Total
Figure 11

A  Female CON X Total

B  Female HFD X Total

C  Female CON X Ambulatory

D  Female HFD X Ambulatory

E  Female CON Z Total

F  Female HFD Z Total
Figure 12

A. Male Plasma Insulin

B. Male Plasma Leptin

C. Male Plasma IL-6

D. Male Plasma MCP-1

**A**

**B**

**C**

**D**
Figure 13

A. Female Plasma Insulin

B. Female Plasma Leptin

C. Female Plasma IL-6

D. Female Plasma MCP-1
CHAPTER 5: SUMMARY AND FUTURE DIRECTIONS
5. Summary and future directions

5.1 Chapter 2 summary (male experiment)

The first experimental chapter investigated the effects of high-fat diet (HFD) feeding in male mice, comparing linoleic acid (LA) to saturated fat (SFA) as the primary dietary fat. The involvement of hypothalamic inflammation (HI) as a mechanism was determined by measuring gene expression of energy balance and inflammatory genes in the arcuate nucleus. The predictions were that high-LA HFD would cause greater weight gain, markers of metabolic syndrome, and HI than SFA. We found that a 45% of kilocalories (kcals) from fat HFD, whether comprised of primarily LA, SFA, or a mixture, produced an obese mouse with glucose intolerance and higher fat to lean body composition ratio compared to a low-fat control (CON) diet. There was no difference in calorie content between HFD, regardless of LA content. However, the CON diet had a lower calorie content than all HFD, 3.85 kcals/gram compared to 4.73 kcals/gram. A minor increase in body weight as well as insulin resistance during insulin challenge was seen in the higher LA group.

The hypothesis was partially supported by the data, with the caveat that high-SFA HFD-fed mice, despite some metabolic protections compared to the highest LA fed mice, were closer to the other two HFD groups than they were to CON in terms of obesity and metabolism. HI was not an apparent mechanism of obesity. Interleukin-6 (IL-6) and inhibitor of IκB kinase (IKK) were suppressed by SFA and LA, neuropeptides were upregulated by LA, and other differences were
measured, but they were not consistently correlated to obesity or any metabolic parameter.

5.2 Chapter 3 summary (female experiment)

The second experimental chapter repeated application of the same 3 HFDs on female ovariectomized (ovx) mice with and without 17β-estradiol (E2) treatment. The hypothesis tested was again that LA, in the context of HFD, would result in more obesity and markers of metabolic syndrome than SFA. The results were similar to those in males in that HFD, rather than LA or SFA, was the primary driver of obesity and related metabolic changes. E2 was protective of obesity from all HFDs, although the highest LA and SFA HFDs caused a slightly higher fat to lean body composition ratio. LA impaired glucose disposal during glucose challenge in both E2 treated and deficient females, and impaired glucose disposal during insulin challenge in E2 deficient females.

The prediction of greater body weight from LA was not borne out. Only impaired glucose homeostasis, and not greater body weight, was produced in females from LA compared to SFA. Plasma leptin was upregulated by LA as well as body adiposity; IL-6 was upregulated by LA and E2. Gene markers of metabolism and HI did not follow a pattern that could be causally related to differences in obesity, glucose metabolism, or energy expenditure between groups. One important caveat to this chapter is that all females were ovariectomized, and none intact. Potentially, the loss of the cyclical nature of
steroid concentrations found in intact females could reduce the effects of HFD on energy and glucose homeostasis and HI similar to what is found in intact males from Chapter 2.

5.3 Chapter 4 summary (maternal experiment)

The third experimental chapter applied feeding of 2 of the HFDs from previous chapters, the highest in SFA and the highest in LA, to female mice before and during pregnancy and lactation. The body weight and metabolic parameters and assays used in the previous chapters were used on the offspring. Gene expression of metabolic and HI related genes was not performed. Offspring were weaned onto a CON diet or a standard HFD, Research Diets D12451 (~14% kcals from LA). The prediction continued to be that LA feeding, this time in maternal diet, would result in greater weight gain and aspects of metabolic syndrome in offspring than SFA.

We further predicted that the effect would be greater in male offspring, since there is evidence that females have protection from maternal over and under-nutrition, and that the effects would be greater in HFD-fed rather than CON-fed offspring. Like the previous chapters’ results, increased calorie intake from the HFD was the primary driver of obesity. Maternal LA caused greater obesity in HFD-fed male offspring, whereas females were only affected by their diet. Glucose clearance from an insulin tolerance test, but not glucose tolerance test, was impaired in male and female HFD-fed offspring of LA-fed dams. Plasma
leptin was upregulated by maternal LA in HFD-fed males, and to a lesser extent by maternal SFA in HFD-fed females. Similar to previous chapters, the most striking results were in impairments to glucose metabolism by LA, whereas body weight differences were statistically significant but slight in magnitude.

5.4 Overall summary

Taken together, our results suggest that in mice LA is as or slightly more obesogenic than SFA in HFD adult and maternal feeding. Specifically, male mice fed LA gained approximately 25% and male offspring of dams fed LA gained approximately 50% more weight than their SFA fed counterparts. Irrespective of body weight or fatness, LA induced an impairment of glucose metabolism, as measured by slowed glucose disposal during glucose or insulin challenge, in male and female mice and in offspring of dams fed LA-rich HFD. Specifically, adult fed males were insulin resistant when fed LA, adult fed females were glucose intolerant when fed LA, and maternally exposed males and females were insulin resistant when their mothers were fed LA. Greater weight gain was associated with a slight increase in food intake, and a slight decrease in oxygen consumption compared to lean animals. A clear pattern of evidence for HI being a causative mechanism for the results, as measured by expression of arcuate nucleus genes, was not found.

5.5 Glucose Metabolism
The most significant effects observed from the different HFDs were on glucose metabolism. Each experimental model resulted in a difference in glucose metabolism from the type of FAs consumed. LA induced either glucose or insulin resistance in the highest percentage group. This effect did not coincide with a pattern of HI biomarkers, so a different mechanism is likely at work.

5.5.1 Fatty acid chain length

The rationale for using coconut oil as the source of SFAs was that it is only whole food fat source with a nearly entirely saturated FA profile. Using coconut oil introduced another variable, that being FA chain length. Approximately 89% of the SFAs in coconut oil have a chain length of 14 and below [1], while safflower oil, the main source of LA used in our experiments, counts more than 95% of its FAs as 16 carbons and longer [2]. For context, the percentage of SFAs below 16 carbons in length in beef tallow, perhaps a more representative dietary fat for Americans, is between 6 and 13 depending on the animals diet [3]. Chain length is an independent variable affecting many aspects of physiology. In the endocrine system, the secretion of insulin [4], cholecystokinin [5], and ghrelin, peptide yy and glucagon-like-peptide-2 [6] is enhanced or suppressed by chain length of dietary FAs. Another effect influenced by chain length, relevant to glucose metabolism, is FFA induced beta-cell cytotoxicity. Although the choice of coconut oil was justified, it was limitation for interpreting our findings.
5.5.2 Lipotoxicity

High concentrations of circulating FFAs can be toxic to beta cells, inducing apoptosis and potentially causing diabetes [7,8]. The difference between short and long chain SFAs could be significant in this effect, as long chain SFAs are considered more potent toxins [9] through the production of ceramide [10]. Lipotoxicity has also been shown from protein kinase C activation by diacylglycerol [11,12], which results in greater reactive oxidative species (ROS) and cytokine production. Interestingly, although fasting glucose was different in some of the experiments, fasting insulin was not. The effect on insulin secretion from type of FA may only be during glucose stimulation, as has been shown [13], and inability to sustain low fasting levels of insulin may be a late effect of beta cell death. Shorter chain SFAs do not have this cytotoxic effect, and may even protect cells from toxicity. Medium chain SFAs have been shown to protect liver from steatosis [14], and longer chain SFAs but not PUFA are protective of ethanol-induced fatty liver [15]; paradoxically, there is evidence long chain SFAs induce non-alcoholic fatty liver [16].

Most relevant to diabetes, in work related to his 1947 Nobel Prize winning discovery of the role of pituitary hormones in glucose metabolism, Bernardo Houssay showed that coconut oil, unlike other fats, conferred nearly complete protection from pancreatic beta cell destruction by the toxic glucose analogue alloxan [17]. He later showed that all SFA, even hydrogenated sunflower oil, were protective from alloxan-induced diabetes, and that all PUFA enhanced toxicity
[18]. If lipotoxicity was involved in the LA-induced impairment of glucose metabolism, the chain length of coconut oil SFAs may have played a protective role for beta cells.

### 5.5.3 The glucose-fatty acid cycle

Another way that FA chain length, and degree of unsaturation, can affect glucose metabolism is through differential participation in the Randle Effect. Classically, FFAs compete with glucose as fuel and induce, in excess, sparing of the other energy substrate [19]. There is evidence, in ex vivo liver CPT1 activity and ability of Malonyl-CoA to suppress it, that different FAs affect and are affected by Randle Effect switches differently [20]; specifically, dietary coconut oil induces less CPT1 expression than longer chained and more unsaturated FAs. Altering the interaction of Malonyl-CoA with CPT1, control points of glucose versus FA beta-oxidation, could change the glucose-sparring potential of FFAs and increase glycemia. Additionally, mitochondrial n-6 content alters the sensitivity of pyruvate dehydrogenase complex [21], the most important enzyme in determining the fate of intracellular glucose.

### 5.6 Hypothalamic inflammation

The case for FA-induced HI being causative of obesity and metabolic syndrome made in the introductory chapter, in short, centers on publications beginning in 2005 that describe a dysregulation of the first order melanocortin
energy balance system caused by FAs [22]. This dysregulation leads to chronic positive energy balance, as the body incorrectly perceives a low adipose reserve. It has been suggested that SFAs initiate this inflammation by activating TLR-4 [23,24]. Alternatively, n-6 PUFA constitute the substrate for the inflammatory eicosanoids [25], as well as for the ROS lipid products that can only be formed from PUFA. The question being asked was whether HI could be measured in mice fed a diet containing either SFAs or n-6 PUFA and how that would modulate weight gain and metabolism. Towards that end, we measured gene expression of inflammatory markers in the arcuate nucleus of the hypothalamus of male and female mice.

5.6.1 Males

Four genes, IκB Kinase (Ilk), Interleukin-6 (Il-6), Intercellular Adhesion Molecule-1 (Icam1), and Fractalkine/Chemokine (C-X3-C motif) Ligand 1 (Cx3cl1) were effected by adult diet in the male mice. Ilk and Il-6 had the same expression pattern, being suppressed by 1% and 22.5% LA-containing diets, and Icam1 and Cx3cl1 had the same pattern, expression being the highest in 1% and 15% and lower in 22.5% LA-containing diets and CON. Other than Il-6, inflammatory cytokines were not different between groups despite the difference in Ilk. Activation of the IKK kinase, and the phosphorylation and release of IκB from NF-κB, are not necessarily the same as Ilk gene expression, although the protein and mRNA tend to increase together [26]. The activation of many of these proteins is foremost a phosphorylation event, and secondarily a change in target
gene expression, and often the protein auto-targets and induces its own mRNA production. \( \text{Icam1} \), for example, is a target downstream of IKK [27], yet in our study \( \text{Ikk} \) was suppressed while \( \text{Icam1} \) was enhanced in 1% LA-containing diets. Also, despite differences in arcuate \( \text{Il-6} \), circulating IL-6 was not different between groups, but trended towards suppression in 1% LA-containing diets.

A more general problem is the notoriously inconsistent correlation between mRNA and protein abundance [28], as low as 40% agreement between change in mRNA and subsequent change in protein in some systems [29]. We chose to measure only mRNA because the protein abundance in the arcuate nucleus would require several multiples of pooling to measure, potentially quadrupling our target sample size. Another option was to use whole hypothalami for protein extraction. This was a poor option because of the disparate nuclei types that would be grouped together in the analysis. Differences that occurred in the arcuate, or paraventricular, nuclei, for example, may have been obfuscated by opposite changes in their neighbors. Because of these inherent limitations, changes in gene expression in the hypothalamus, or individual nuclei, should be considered more of a sign of endocrinological signaling rather than a definitive mechanistic action leading to proportional physiological effects. In the male mice, the changes in inflammatory gene expression we found were not correlated to the obesity caused by HFD or the insulin resistance caused by 22.5% LA-containing diet.
5.6.2 Females

There was less HI variation between groups in the female mice than in the males. *Toll-like receptor 4* trended lower in CON oils, *Interleukin-1* was suppressed by E2 in CON, and *Interleukin-15* was suppressed by 22.5% LA-containing diet in E2 treated females. These results also do not correlate with weight gain or with circulating IL-6 or leptin protein, similar to the male HI markers. In addition to the problems interpreting gene expression alone as inflammatory action, the females have the added variable of estrogen signaling. For the most part, estrogen treatment overwhelmed HFD for control of metabolic phenotype.

Relating to the hypothalamus, this may have been because the arcuate nuclei that we were analyzing for metabolic receptors and inflammatory cytokines also highly express estrogen receptors [30,31]. Although qPCR was not performed on maternal samples, female offspring were more protected than male offspring from obesity due to maternal HFD exposure; this may be a similar phenomenon as the female adults obesity and HI, whereby estrogen signaling overwhelms the dysregulatory influence of increased FAs. Estrogen receptor activity may be a more important factor in metabolic syndrome than direct dietary influence [32]. The question of sufficiency of mRNA measurements to show HI or the lack thereof is present in the female data as it was in the male data. Molecular techniques measuring protein levels and phosphorylation may be more important in females where the question of estrogen receptor activity, with its
chain of protein to protein interactions in addition it nuclear binding [33], is more variable than in males.

5.7 An alternative mechanism of inflammation

There is an alternative explanation for why SFA, centrally or peripherally, may be pro-inflammatory, and PUFA anti-inflammatory. Rather than directly binding to receptors, SFAs may enhance, and PUFAs inhibit, the binding of well-known inflammatory ligands. Erridge and Samani claimed that the in vitro studies showing TLR-4 activation from SFA may be artefactual of bacterial lipopolysaccharide (LSP), lipopeptide, and flagellum contamination [34]. The bovine serum albumin (BSA) used as a vehicle in these standard molecular experiments was shown to be contaminated with some or all of the aforementioned bacterial products, and to be insufficiently decontaminated with the standard polymixin-B treatment.

A response publication by Huang et al. countered, showing that lauric acid (C12:0) could activate TLRs with no BSA vehicle, as well as palmitic acid (C16:0) with or without BSA. The authors mention that through the limulus amoebocyte lysate (LAL) test, the BSA and even FAs used did show trace contamination, but that this amount was likely irrelevant. Even more interesting is the mechanism proposed, which did not involve direct activation of the receptors by SFA but stabilization of lipid rafts upon which TLRs “float” and complex with ligands and downstream affecters.
Since their data show that SFA has only a fraction of the potency of LPS to activate TLRs, even at triple or more the concentration, it is possible that the trace LPS seen in their reagents is simply facilitated by the lipid raft stabilizing properties of SFA. Huang et al. also found that lower albumin concentration vehicle worked more effectively to activate TLRs, and that this was through ROS production, which is a known agent of inflammation signaling. Another point not addressed by the authors was if human free fatty acid (FFA) serum concentrations of >500 uM, similar to the concentration used to mimic LPS, are easily inducible by fasting [35], why are fasted or otherwise high circulating FFA individuals not experiencing symptoms of endotoxemia? Not all of the circulating FFAs will be SFA, but enough would be to achieve levels equivalent or greater than some of the TLR-4 signaling experiments, especially since humans preferentially produce palmitate in de novo lipogenesis. This expands into the big picture question, never directly addressed, of why the mammalian physiology would produce mostly SFAs if those FAs were inflammatory and damaging to so many systems.

5.8 Inflammation and fatty acids, direct or indirect action

The lipid raft stabilization theory for SFA facilitating activation of TLRs may provide a link to another sub-field of obesity research, gut microbiome, and provide an explanation for why some studies find SFA to be inflammatory while others do not. The gut microbiome field has produced dramatic results on obesity
through fecal transplants that change the floral population [36]. Much of that field focuses on the types or ratios of bacteria, at the phylum down to the genus level, that are associated with a particular disease or obesity. The presence of a certain type of bacteria, the absence of other types, or a particular ratio of types usually correlates with the tendency towards obesity or leanness. The focus is on the types of bacteria present as the causation of metabolic problems or health.

A link between gut flora and obesity/metabolic syndrome is the association between serum LPS, inflammation, and diabetes [37]. It has been shown that, through lipid rafting in the bile and micelle driven lipid absorption process, SFA but not PUFA shuttles LPS in the gut into enterocytes [38]. From there, LPS is complexed with chylomicrons secreted into circulation where, in the case of strong immune and liver function, it is at least partially excreted into bile [39]. Referring back to Huang et al., this phenomenon hearkens to their demonstration of in vitro TLR signaling being facilitated by SFA, both phenomena suggesting the mechanism of lipid raft stabilization. Interestingly, Huang et al. also found that TLR signaling by SFA or LPS could be inhibited by PUFA. PUFAs destabilize lipid rafts, at least in comparison to SFA [40], because of decreased steric compatibility with cholesterol [41]. The concept of SFA as a delivery mechanism for LPS helps to explain the observations both in vitro and in vivo of inflammation, obesity, and metabolic syndrome in some circumstances, and the lack of this observation in others.
5.9 PUFA and enzyme inhibition

Even in a study suggesting the mechanism of TLR-4 activation for SFA-induced obesity, the PUFA control group was as obese as the experimental SFA group [42]. This begs the question of how a high PUFA diet can cause obesity. In addition to the potential to form inflammatory eicosanoids and reactive lipid peroxides, virgin PUFA exhibit an enzyme inhibition function. Proteolytic enzymes are inhibited in their function by PUFA [43,44], as well as detoxification enzymes in the liver [45,46]. Esterified PUFA inhibit chymase activity in a dose dependent manner [47], similar to pH. In plants, seed oils inhibit germination enzymes until environmental signals from heat and moisture stimulate H2O2 production and rapid consumption of seed lipid [48]. The metabolic implications of this effect are seen in its presence in the thyroid production, secretion and signaling systems [49–51].

The anti-inflammatory effect of PUFA, such as that seen in fish oil supplementation, is likely due to this inhibitory effect acting on immune cell enzymatic systems. Indeed, although PUFA are substrate for eicosanoid production, seed oil FAs inhibit the enzymes involved [52]. PUFA, outside of injury situations that liberate large amounts of FFAs for eicosanoid production, are likely not pro-inflammatory. The potential obesogenic effects of PUFA come from their ability to inhibit metabolism-related enzymatic activity.

5.10 Future directions
Taking this information on the possible divergent mechanisms of SFA and PUFA into account, further hypotheses suggest themselves. SFA-induced obesity and inflammation may be dependent on a gut floral population high in gram-negative, LPS producing bacteria. The differences in weight gain, inflammation, and sequenced DNA of gut flora between mice fed SFA versus PUFA HFDs would investigate the possible mechanistic link between HI and microbiome research. A distinction should be made between colonic and small intestinal populations, as these two areas have different physiological functions [53]. Colonic bacteria should be identified through fecal samples, small intestinal bacteria through endoscopic aspirant.

Further molecular assays should be used to differentiate the effects of SFA and LA. HI may not be fully captured through gene expression changes, since molecules like IKK and NF-kB are activated allosterically and do not necessarily result in a change in mRNA content. Measuring activated versus inactivated protein state, or mRNA of downstream molecules such as MyD88, are more direct indicators of inflammation. TLRs likely behave similarly, maintaining mRNA amount while being allosterically altered. A thiobarbituric acid reactive substances (TBARS) assay, measuring reactive lipid peroxides and non-lipid ROS from propagation, can be used to investigate the hypothesized mechanism of PUFA-induced inflammation through non-respiratory oxidation.

Continuing to explore the mechanisms studied herein, and explaining the observed metabolic effects of experimental diets by integrating new information,
is foundationally important to the obesity field. Rationality and contradiction cannot cohabitate in the same pursuit, and the current situation, in which a number of studies show SFA is obesogenic compared to PUFA while a number of other studies show the opposite, is untenable.

References


[49] Chopra IJ, Huang TS, Beredo A, Solomon DH, Chua Teco GN, Mead JF.


CHAPTER 6: APPENDIX – ESTROGEN RESPONSE ELEMENT-

INDEPENDENT SIGNALING PARTIALLY RESTORES POST-OVARIECTOMY

BODY WEIGHT GAIN BUT IS NOT SUFFICIENT FOR 17β-ESTRADIOL’S

CONTROL OF ENERGY HOMEOSTASIS
6. Appendix: Estrogen response element-independent signaling partially restores post-ovariectomy body weight gain but is not sufficient for 17β-estradiol's control of energy homeostasis

6.1 Abstract

The steroid 17β-estradiol (E2) modulates energy homeostasis by reducing feeding behavior and increasing energy expenditure primarily through estrogen receptor α (ERα)-mediated mechanisms. Intact ERαKO female mice develop obesity as adults exhibiting decreased energy expenditure and increased fat deposition. However, intact transgenic female mice expressing a DNA-binding-deficient ERα (KIKO) are not obese and have similar energy expenditure, activity and fat deposition to wild type (WT) females, suggesting that non-Estrogen Response Element (ERE)-mediated signaling is important in E2 regulation of energy homeostasis. However, initial reports did not examine the effects of ovariectomy on energy homeostasis or E2's attenuation of post-ovariectomy body weight gain. Therefore, we sought to determine if low physiological doses of E2 (250 ng QOD) known to suppress post-ovariectomy body weight gain in WT females, would suppress body weight gain in ovariectomized KIKO females. We observed that the post-ovariectomy increase in body weight was significantly greater in WT females than in KIKO females. Furthermore, E2 did not significantly attenuate the body weight gain in KIKO females as it did in WT females. E2 replacement suppressed food intake and fat accumulation while
increasing nighttime oxygen consumption and activity only in WT females. E2 replacement also increased arcuate POMC gene expression in WT females only. These data suggest that in the intact female, ERE-independent mechanisms are sufficient to maintain normal energy homeostasis and to partially restore the normal response to ovariectomy. However, they are not sufficient for E2’s suppression of post-ovariectomy body weight gain and attenuation of decreases in metabolism and activity.

6.2 Introduction

The reproductive steroid, 17β-estradiol (E2), regulates various aspects of energy homeostasis through both peripheral actions and central mechanisms. E2 suppresses feeding and fat accumulation and augments energy expenditure and activity through actions in the brain. The key brain regions mediating E2’s effects on energy homeostasis are the hypothalamus and the hindbrain (1–4). A decrease in circulating estrogens due to ovariectomy or menopause is associated with positive weight gain in both rodent models and humans, which is attenuated by E2 replacement (5–7). Furthermore, food intake varies during the menstrual cycle in humans and in intact animals with a luteal phase peak and a peri-ovulatory nadir in consumption illustrating E2’s effects (8–10).

E2 uses two classical nuclear steroid receptors, ERα and ERβ to control physiological and cellular functions and to regulate gene expression. ERα is highly expressed in the hypothalamus including the Arcuate nucleus and ERα-
mediated actions are the primary mechanisms utilized by E2 to control energy homeostasis (11). Furthermore, ERα knockouts (ERKO) exhibit an obesity phenotype with increased visceral adiposity and decreased energy expenditure (12,13). ERα is also necessary for the attenuation of weight gain and food intake and is involved in the extrahypothalamic (NTS) control of food intake by cholecystokinin (3,13). In the ventromedial hypothalamus, ERα knockdown by RNAi induces a phenotype defined by obesity, hyperphagia, glucose intolerance and reduces activity (energy expenditure), which is resistant to E2’s effect on activity (4).

Arcuate POMC and NPY neurons are involved in feeding and both express ERα. In POMC neurons, ERα is highly expressed (~75%) (14), with NPY neuronal ERα expression approximately 20% (15). Indeed, specific deletion of ERα in POMC neurons significantly increased body weight, food intake and activity, while specific deletion of ERα in SF1 neurons of the ventromedial hypothalamus decreased energy expenditure and increased fat accumulation (16). Furthermore, POMC and NPY neurons express a Gq-coupled membrane estrogen receptor that mediates E2’s effects on feeding, core body temperature and gene expression (17–21).

Both the classical ERs (ERα and ERβ) bind to DNA at estrogen response elements (ERE) to control gene expression. However, both receptors can control gene expression through ERE- independent signaling via protein-protein interactions with other transcription factors (Sp-1, Fos-Jun (AP-1) pCREB,
STATs, and NFκB) and binding to other promoter sites (22). ERs also activate membrane-initiated signaling cascades (MAPK, PLC, PI3K) that modulate cell physiology and control gene expression (18–20,23–25). Recently, the restoration of ERE-independent signaling was reported to normalize energy balance in ERKO females (26). These mice express an ERα (ERAA) that lacks the ability to bind to ERE due to mutations in the DNA-binding domain (27). Females that only express the ERAA, also called ERα KIKO (KIKO), do not become obese like their ERKO counterparts.

In wild type females, ovariectomy induces weight gain that is attenuated by E2 replacement while in the ERKO female ovariectomy does not induce significant body weight gain. Ovariectomized ERKO females also do not respond to E2 replacement (13). The lack of normal response to ovariectomy in the ERKO females is interpreted as an indicator of ERα’s role in mediating E2’s effect on energy homeostasis. Therefore, we sought to determine if the presence of ERE-independent signaling in KIKO females restores the post-ovariectomy body weight gain in females and if this positive state of energy balance is attenuated by E2 replacement. We assessed the effects of ovariectomy with or without E2 replacement in wild type, ERKO and KIKO littermate females by measuring body weight and food intake for four weeks followed by measurements of oxygen consumption, activity and body composition.

6.3 Materials and Methods
6.3.1 Animal Care

All animal treatments are in accordance with institutional guidelines based on National Institutes of Health standards, and were performed with Institutional Animal Care and Use Committee approval at Rutgers University. Female wild-type (WT), ERα KO (ERKO) and ERα KIKO (KIKO) transgenic mice (provided by Dr. Ken Korach, NIEHS) (28–30) were selectively bred in-house, and maintained under controlled temperature (25 °C) and photoperiod conditions (12/12 hr light/dark cycle) with food and water ad libitum. ERKO females were generated by breeding WT/KO heterozygotic males and females. KIKO females were generated by crossing the non-classical ER knock-in (NERKI) heterozygous males (WT/KI) with WT/KO heterozygotic females. Wild-type females were generated from both colonies and used with their KIKO and ERKO littermates. At weaning, females were tagged and ear clipped for genotyping. Genotype was determined by PCR of extracted DNA using previously published protocols (28–30).

6.3.2 Drugs

17β-estradiol benzoate (E2) was purchased from Steraloids (Newport, RI, USA) and dissolved in ethanol prior to dissolution in sesame oil (Sigma-Aldrich). Ketamine was purchased from Henry Schein (Melville, NY, USA) and used for sedation prior to killing.
6.3.3 Experimental Design

At eight weeks of age, females were ovariectomized under isoflurane anesthesia using no touch sterile techniques and allowed to recover for 5 days prior to measurements of body composition using an EchoMRI 3-in-1 Body Composition Analyzer (Echo Medical Systems, Houston, TX, USA) followed by monitoring in a Comprehensive Lab Animal Monitoring System (CLAMS) (Columbus Instruments, Inc., Columbus, OH, USA) for 48 hrs. After monitoring, females were housed alone and allowed to recover for one day prior to injection of either sesame oil or 17β-estradiol benzoate (250 ng/dose) at which time body weight and food weight were measured. Females were injected and weighed every other day (QOD) for 4 weeks. At the end of four weeks, females were analyzed for body composition using the EchoMRI and metabolic parameters and activity using the CLAMS. Monitoring was followed by another day of recovery and an injection of oil or E2 24-hr prior to killing and brain and body dissections. See Figure 1 for a timeline of the experiment. A subset of females from each genotype were not ovariectomized and subjected to the same experimental paradigm.

6.3.4 Tissue Dissections

At the end of the experiments, females were decapitated after sedation with ketamine (100 µl of 100 mg/ml, i.p.) 24 h after the final treatment injection. Trunk blood was collected and analyzed for triglyceride and glucose levels using
a CardioChek (Polymer Technology Systems, Inc., Indianapolis, IN, USA) and prepared for serum analysis of 17β-estradiol levels using Mouse Calbiotech ELISA conducted by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core (31). The E2 assay had an intra-assay variability of 3.1% and a sensitivity of 0.3–300 pg/ml. Hypothalamic nuclei were micro-dissected for RNA extraction and gene expression analysis. The basal hypothalamus (BH) was cut using a brain slicer (Ted Pella, Inc., Redding, CA, USA), into one mm thick coronal rostral and caudal blocks corresponding to Plates 42 to 47 and Plates 48 to 53, respectively, from The Mouse Brain in Stereotaxic Coordinates (Paxinos & Franklin 2008, 3rd Edition) (32). The BH blocks were transferred to RNAlater (Life Technologies, Inc., Grand Island, NE, USA) and stored overnight at 4 °C. The rostral and caudal parts of the Arcuate nucleus were dissected from slices using a dissecting microscope. Dissected tissue was stored at −80 °C. Total RNA was extracted from the combined nuclei (rostral and caudal arcuate) using Ambion RNAqueous-Micro Kits (Life Technologies, Inc.) according to the manufacturer’s protocol. Total RNA was also DNase I-treated, using the extraction kits, at 37 °C for 30 min to minimize any genomic DNA contamination. RNA quantity and quality were determined using a NanoDrop ND-2000 spectrophotometer (ThermoFisher, Inc., Waltham, MA, USA) and an Agilent 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies, Inc., Santa Clara, CA, USA).
6.3.5 Quantitative real-time PCR

cDNA was synthesized from 200 ng of total RNA using Superscript III reverse transcriptase (Life Technologies, Inc.), 4 μl 5x Buffer, 25 mM MgCl2, 10 mM dNTP (Clontech Laboratories, Inc., Mountain View, CA, USA), 100 ng random hexamer primers (Promega Corporation, Madison, WI, USA), 40 U/μl Rnasin (Promega) and 100 mM DTT in DEPC- treated water (Gene Mate, Bioexpress, Inc., Kaysville, UT, USA) in total volume of 20 μl. Reverse transcription was conducted using the following protocol: 5 min at 25 °C, 60 min at 50 °C, 15 min at 70 °C. The cDNA was diluted to 1:20 with Nuclease-free water (Gene Mate, Bioexpress) for a final cDNA concentration of 0.5 ng/μl and stored at −20 °C. BH test tissue RNA was used for positive and negative controls (no reverse transcriptase) and processed simultaneously with the experimental samples.

All primers were designed to span exon-exon junctions and synthesized by Life Technologies, Inc., using Clone Manager 5 software (Sci Ed Software, Cary, NC, USA). See Table 1 for a listing of all the primer sets used for quantitative real-time PCR (qPCR). For qPCR, 4 μl cDNA template (an equivalent of 2 ng total RNA) was amplified using either PowerSYBR Green master mix (Life Technologies) or Sso Advanced SYBR Green (BioRad, Inc., Hercules, CA, USA) on CFX-Connect Real-time PCR instrument (BioRad). Standard curves for each primer pair were prepared using serial dilutions of BH cDNA in triplicate to determine the efficiency (E=10(−1/m) − 1, m=slope) of each primer pair. All
efficiencies expressed as percent efficiency were approximately equal (one
doubling per cycle, 90–100%, Table 1). Therefore, the relative mRNA expression
data was analyzed using the ΔΔCT method (33,34). The amplification protocol
for all the genes was as follows: initial denaturing −95 °C for 10 min
(PowerSYBR) or 3 min (SsoAdvanced) followed by 40 cycles of amplification at
94 °C for 10 sec (denaturing), 60 °C for 45 sec (annealing), and completed with a
dissociation step for melting point analysis with 60 cycles of 95 °C for 10 sec, 65
°C to 95 °C (in increments of 0.5 °C) for 5 sec and 95 °C for 5 sec. The reference
genes used were Actb and/or Gapdh. Positive and negative controls were added
to each amplification run including a water blank. Quantification values were
generated only from samples showing a single product at the expected melting
point.

Final relative quantitation was done using the comparative CT method
(33,34) utilizing a calibrator of diluted (1:20) cDNA from the BH of an intact male.
The data were reported as relative mRNA expression. To determine the CT for
each transcript, the threshold was consistently set at the lowest point of the
exponential curve where the slope of the curve was the steepest for all plates.
The relative linear quantity of target molecules was calculated using the formula
$2^{-\Delta\Delta CT}$. All gene expression data were expressed as an $n$-fold difference
relative to the calibrator. The $n$-fold difference was averaged for each treatment
and analyzed statistically using a two-tailed Student’s $t$-test ($p \leq 0.05$).
6.3.6 Data analysis

All data was expressed as mean ± SEM. All the data from the bi-daily body weight measurements and CLAMS analysis were analyzed using a two-way ANOVA followed by a post-hoc Bonferroni-Dunn multiple comparison tests. All data from the food intake studies, body composition determination, quantitative real-time PCR experiments and other measurements were analyzed using a one-way ANOVA followed by a post-hoc Bonferroni-Dunn multiple comparison tests and/or Student’s t-test (unpaired). All data was analyzed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) and in all cases, effects were considered significant at \( p \leq 0.05 \).

6.4 Results

6.4.1 Serum E2 levels and uterine weights

Serum E2 levels were measured in all animals including intact females. Intact KIKO females had slightly higher serum E2 levels than intact WT females (WT: 3.3 ± 0.2 pg/ml (n=7) vs. KIKO: 4.7 ± 0.6 pg/ml (n=7); \( p<0.05 \)) respectively (Figure 2A). Intact ERKO females had significantly higher serum E2 levels (22.3 ± 4.2 pg/ml (n=7), \( p<0.01 \)) than both intact WT and KIKO females. In oil-treated, ovariectomized (ovx) WT, KIKO and ERKO females, the serum levels of E2 were not significantly different (3.9 ± 0.5 pg/ml (n=8); 8.5 ± 3.3 pg/ml (n=8); and 5.9 ± 1.3 pg/ml (n=8); respectively). In the E2-treated, ovx WT, KIKO and ERKO females, the serum levels of E2 were not significantly different (23.7 ± 3.5 pg/ml
(n=8); 26.5 ± 4.8 pg/ml (n=8) and 33.3 ± 7.5 pg/ml (n=8); respectively) but were significantly higher than in the oil-treated counterpart females (p<0.0001, p<0.01, p<0.01, respectively).

We measured uterine weights at the end of the experiment to confirm the presence of E2 in the WT ovx females (Figure 2B). In WT females, E2 replacement is known to increase uterine weights through an ERα-mediated mechanism (35). Intact WT females had significantly higher uterine weights/body weight than either intact KIKO or ERKO females (WT=2.61 ± 0.4 g/g; KIKO=1.7 ± 0.5 g/g; ERKO=0.6 ± 0.4 g/g; WT vs. KIKO: p<0.01; WT vs. ERKO: p<0.0001). In WT females, the oil-treated, ovx females had a uterine weight/body weight (g/g) that was significantly less than the E2-treated, ovx WT females (0.7 ± 0.3 g/g vs. 5.8 ± 0.5 g/g, p<0.0001). E2 also increased the uterine weight/body weight in ovx KIKO females, although the difference was not as great between oil- and E2-treated groups (0.9 ± 0.1 g/g vs. 1.5 ± 0.2 g/g, p<0.5). E2 replacement had no effect on ERKO uterine weights (0.5 ± 0.1 g/g vs. 0.5 ± 0.1 g/g). Furthermore, E2-treated WT females had significantly higher uterine weights than both E2-treated KIKO and ERKO females (p<0.0001 for both) and E2-treated KIKO females had significantly higher uterine weights than ERKO females (p<0.001).

6.4.2 Post-ovariectomy body weight gain and E2 attenuation
Ovariectomy in WT female mice results in significant body weight gain. Conversely, ovariectomy in ERKO females does not cause a significant increase in body weight (13). Prior to ovariectomy, there was no significant difference in body weights across or within the genotypes. WT females weighed 18.4 ± 0.3 g (oil) and 18.7 ± 0.4 g (E2); KIKO females weighed 17.7 ± 0.6 g (oil) and 17.9 ± 0.3 g (E2); and ERKO females weighed 18.9 ± 0.5 g (oil) and 19.3 ± 0.6 g (E2). In this study, oil-treated, ovx WT females gained more weight than both the KIKO and ERKO oil-treated, ovx females (Figure 3A). Significant differences appeared between the WT, KIKO and ERKO ovx females on day 15 and day 9, respectively (WT vs. KIKO: p<0.01, df=1, F=9.08; WT vs. ERKO: p<0.001, df=1, F=18.09). The weight gain in the ovx KIKO, as seen in Figure 3A, is intermediary between the ovx WT and ERKO females. Weight gain between the oil-treated, ovx KIKO and ERKO females was not significantly different (p=0.076) although the weight gain in the KIKO females was observably higher. This was due, in part, to a large amount of variation in the response to ovariectomy by ERKO females with three animals gaining weight and the remaining either not gaining weight or losing weight over the 27 days of the experiment.

Furthermore, post-ovariectomy body weight gain in WT was significantly greater than intact WT females (cumulative weight gain (day 27): intact WT: 1.4 ± 0.3 g vs. ovx WT: 3.8 ± 0.2 g; p<0.0001; Figure 3B). Intact KIKO females gained 1.4 ± 0.3 g over the 27 days while oil- treated, ovx females gain an average of 2.1 ± 0.3 g (p=0.11; Figure 3C). Intact ERKO females gained an average of 1.4 ±
0.9 g during the experiment while oil-treated, ovx ERKO females gained 0.5 ± 0.7 g (Figure 3D). However, ovx KIKO females did temporarily have greater cumulative weight gain at day 9 through day 13 compared to intact KIKO females (p<0.01) indicating a significant, initial post-ovariectomy weight gain that was not sustained like in the ovx WT females.

Only in ovx WT females did E2 replacement attenuate the post-ovariectomy body weight gain (Figure 3B; ANOVA: p<0.001, df=1, F=20.68). In KIKO females, E2 did not significantly reduce body weight gain although there was a clear separation in body weights between oil- and E2-treated KIKO females (Figure 3C). As previously reported, there is no effect of E2 on body weight gain in ovx ERKO females (Figure 3D). Similar to the response to ovariectomy, the response of the body weight in KIKO females to E2 replacement was intermediate to WT and ERKO females suggesting that ERE-independent signaling may be involved, but not sufficient for E2’s attenuation of post-ovariectomy body weight gain.

6.4.3 E2 effects on feeding behavior

During the 4 weeks of the experiment, food intake was measured every other day (QOD) for both intact and ovx females. The average 48 hr food intake was significantly higher in intact WT females (6.7 ± 0.3 g) than both intact KIKO (5.6 ± 0.2 g, p<0.01) and intact ERKO (5.7 ± 0.3 g, p<0.05) (Figure 4A). This pattern continued for both oil-treated and E2-treated ovx females. WT ovx
females, regardless of steroid treatment consumed more food than both the KIKO and ERKO ovx females (two-way ANOVA: p<0.0001, df=2, F=39.1).

Furthermore, oil-treated WT females consumed more food than their E2-treated counterparts (oil: 7.6 ± 0.2 g vs. E2: 6.9 ± 0.2 g, p<0.05) and their intact WT counterparts (p<0.05) (one-way ANOVA: p<0.05, df=2, F=4.8). Intact KIKO females ate significantly less food than either oil-treated or E2-treated KIKO females (oil: 6.2 ± 0.2 g; E2: 6.1 ± 0.1 g, p<0.05). There was no effect of E2 treatment on either KIKO or ERKO 48 hr food intake nor was there any difference between either intact ERKO food intake or oil-treated or E2-treated ERKO females.

During the 4 weeks of food intake measurements, we also observed a significant amount of food in the bottom of the cage. Therefore, we decided to measure the amount of food left in the cage every 48 hr and called it ‘food chewed’. The amount of food chewed correlated with the average 48 hr food intake in the intact females. Intact WT females chewed significantly more food than both the KIKO and ERKO intact females (WT: 2.7 ± 0.4 g vs. KIKO: 1.2 ± 0.3 g vs. ERKO: 1.1 ± 0.3 g, p<0.05) (Figure 4B). In the oil-treated, ovx females, the amount of food chewed was 1.3 ± 0.3 g for the WT females; 1.4 ± 0.4 g for the KIKO and 2.5 ± 0.5 g for the ERKO females (no significant effect of genotype). However, ovariectomy did decrease the amount of food chewed compared to intact females in the WT (p<0.05). In the E2-treated, ovx females, WT females (3.4 ± 0.6 g) chewed more food than KIKO females (1.1 ± 0.2 g;
p<0.01), but not ERKO females (2.0 ± 0.4 g). E2-treated WT females also chewed significantly more food than oil-treated WT females (p<0.01) and E2-treated ERKO females chewed more food than E2-treated KIKO (E2: p<0.05). Food chewing may be an indication of greater activity, anxiety/boredom or pica, non-nutritive food intake (36).

6.4.4 The effect of ovariectomy and E2 on body composition and metabolic chemistry

To determine the change in lean mass and fat accumulation over the course of the 4-week treatment, we measured body composition 5 days after ovariectomy and after 4 weeks using an EchoMRI Body Composition analyzer. The change in body fat was positive for both WT (22.3 ± 7.1%) and KIKO (13.3 ± 4.8%) females over the 4 weeks (Figure 5A). ERKO females did not have a significant change in body fat (−9.1 ± 13.7), and there were no significant differences between the genotypes. The change in body fat in intact WT and KIKO females correlates with the small increase in body weight over the 27 days of the experimental period. ERKO females did not increase in body fat significantly but had larger fat deposits at the start of the experiment than then WT and KIKO females (data not shown).

Amongst the oil-treated, ovx animals, the change in percent body fat was greatest in the WT compared to both the other oil-treated genotype groups and to the E2-treated WT females. Amongst the oil-treated females, the change in
percent body fat increased by 78.0 ± 10.2% in the WT, 49.2 ± 6.6% in the KIKO and 25.4 ± 12.3% in the ERKO (WT vs. KIKO: p<0.05; WT vs. ERKO: p<0.01). In the E2-treated WT females, the change in percent body fat was 49.3 ± 4.1% (WT oil vs. WT E2: p<0.01). The changes in percent body fat for KIKO (38.9 ± 7.9%) and ERKO (36.3 ± 8.1%) E2-treated females were not significantly different than the WT. There were no significant effects of steroid treatment or genotype on percent change in lean mass (Figure 5B) except for a significant difference between both oil-treated and E2-treated WT and their ERKO counterparts (WT oil vs. ERKO oil: p<0.01; WT E2 vs. ERKO E2: p<0.05).

We also measured non-fasting, whole blood triglyceride and glucose levels from collected trunk blood. We did not fast the animals prior to killing due to the effects of fasting on arcuate neuropeptide gene expression (37). E2 treatment increased the triglyceride levels in WT females (WT oil: 61.5 ± 3.0 mg/dl vs. WT E2: 74.3 ± 4.1 mg/dl, p<0.5). However, blood triglycerides were not significantly affected by genotype in the intact females or the oil-treated and E2-treated ovx females (Figure 5C). Blood glucose levels were highly variable and not significant different across the genotypes for intact or ovx females (Figure 5D).

6.4.5 The effect of ovariectomy and E2 on energy expenditure and locomotor activity

To measure changes in energy expenditure, we used a Comprehensive Lab Animal Monitoring System to measure oxygen consumption (V.O2), CO2
production (V.CO2), respiratory exchange ratio (RER), heat and activity (X and Z). Each female was analyzed for these parameters 5 days after ovariectomy for 48 hr and after 4 weeks of steroid treatment for another 48 hr. All data from the last 24 hr of the CLAMS run was used in the analysis and was further separated into day (7 am to 7 pm) and night (7 pm to 7 am). Comparisons were made between the pre-treatment and the post-treatment CLAMS results within genotype and across steroid treatment (intact, oil, E2).

In both day (data not shown) and night (Figure 6A), V.O2 was significantly lower in the animals post-treatment compared to pre-treatment for WT oil (p<0.001), WT E2 (p<0.01), KIKO oil (p<0.05) and KIKO E2 (p<0.05). There was no difference in V.O2 in either ERKO oil or ERKO E2 during the day or night between pre-treatment and post-treatment (Two-way ANOVA: day-night: p<0.0001, df= 1, F= 36.97). Furthermore, WT-oil, WT-E2, KIKO-oil and KIKO-E2 pre-treatment nighttime V.O2 was significantly higher than the ERKO-oil or ERKO-E2 pre-treatment nighttime V.O2 (WT oil: p<0.01; WT E2: p<0.01; KIKO oil: p<0.05; KIKO E2: p<0.05). These data indicate that the suppressive effects of ovariectomy on energy expenditure increase over time in both WT and KIKO females but not in ERKO females. These data also corroborates earlier research reporting that ERKO females have a lower energy expenditure compared to both WT and KIKO females (26).

After the 4 weeks of treatment, V.O2 is significantly lower during the daytime compared to the nighttime for all genotype-steroid treatments including
ERKO-oil and ERKO-E2 (Two-way ANOVA: day-night: p<0.0001, df= 1, F= 36.74; Figure 6B). The greatest difference was between WT-E2 day and WT-E2 night V.O2 (day: 3726 ± 130 ml/min/kg vs. night: 4657 ± 143 ml/min/kg, p<0.001). E2 only had a significant effect on V.O2 in the WT females during the nighttime hours (WT-oil: 4234 ± 168 ml/min/kg vs. WT-E2: 4657 ± 143 ml/min/kg, p<0.05). These data suggest that the attenuation of body weight gain in the WT females by E2 is due, in part, to a small but significant increase in nighttime V.O2.

Similar results were observed between the pre-treatment and post-treatment nighttime V.CO2 (Figure 6C). V.CO2 was significantly higher in the pre-treatment females compared to post-treatment females in WT-oil (p<0.001), WT-E2 (p<0.05), KIKO-oil (p<0.05), and KIKO-E2 (p<0.05) but not in the ERKO-oil or ERKO-E2 females (Two-way ANOVA: day-night: p<0.0001, df= 1, F= 30.85). V.CO2 in WT females prior to treatment was also significantly higher than in the ERKO females (p<0.05). Pre-treatment KIKO females were not significantly different from either WT or ERKO females. After 4 weeks of treatment, V.CO2 was significantly lower during the daytime compared to the nighttime for all genotype-steroid treatments (Two-way ANOVA: day-night: p<0.0001, df= 1, F= 77.71; Figure 6D). Although E2 had no significant effect on V.CO2 between genotypes either in the daytime or nighttime, the greatest difference between time periods was WT-E2 day and WT-E2 night V.O2 (day: 3501 ± 118 ml/min/kg vs. night: 4496 ± 151 ml/min/kg, p<0.001).
There was no effect of E2 on the respiratory exchange ratio (V.CO2/V.O2) or RER within the genotypes nor was there a significant effect of genotype on RER either during the daytime or nighttime (Figure 7A). However, there was a significant decrease in RER during the daytime compared to the nighttime between all genotype-treatments (Two-way ANOVA: day-night: \( p<0.0001 \), df = 1, \( F= 79.23 \)) with greatest difference between ERKO-E2 day and night (day: 0.87 0.01 vs. night: 0.99 0.02, \( p<0.0001 \)). Because RER is an estimation of the respiratory quotient, which indicates the type of fuel (fat or carbohydrates) being metabolized (38), we can infer that during the day the females are utilizing a mixture of fat and carbohydrates with more carbohydrates being utilized during the night, the primary feeding hours. We also measured body heat (CV x V.O2 or 3.815 + 1.232 x RER) using the CLAMS units (Figure 7B). As with RER, there was no significant effect of E2 on heat within genotypes during the daytime or nighttime but there was a significant decrease in heat between the daytime and nighttime between all genotype-treatments (Two-way ANOVA: day-night: \( p<0.0001 \), df = 1, \( F= 31.89 \)).

Locomotor activity was also measured using the CLAMS units. Dissimilar to V.O2 and V.CO2, there was no significant change between the nighttime pretreatment and post-treatment total X-plane activity, although there was a negative trend in the WT-oil females compared to the other treatments (Figure 7C). The decrease in nighttime WT-oil activity between pretreatment and post-treatment was not observed in the WT-E2 females. Indeed, the difference in the
X-plane beam breaks between the post-treatment WT-oil (347 ± 62 counts) and WT-E2 (535 ± 68 counts) females was the only significant effect of E2 on activity (p<0.05) (Figure 7C & 7D). There was also a significant decrease in locomotor activity between the daytime and nighttime between all genotype-treatments (Two-way ANOVA: day-night: p<0.0001, df= 1, F= 140; Figure 7D) with the greatest difference between the WT-E2 day activity (167 ± 19 counts) and the WT-E2 night activity (534 ± 68 counts), (p<0.0001). Similar results were observed for Z-plane activity (data not shown). These data indicate that the attenuation of body weight gain in the WT females by E2 is due, in part, to a small but significant increase in nighttime activity, which correlates to the increase in V.O2 (Figure 6B).

6.4.6 Neuropeptide genes regulated by E2 and across genotypes

To determine if there are any genotype or steroid effects on the expression of arcuate neuropeptide genes known to be involved in energy homeostasis, arcuate nuclei were dissected and processed for quantitative real-time PCR from all intact and ovx females (n are smaller than in the physiological experiments due to a loss of tissue samples during RNA extraction). We analyzed two anorexigenic genes, proopiomelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript (CART), and two orexigenic genes, neuropeptide Y (NPY) and agouti-related peptide (AgRP). In the arcuate, POMC
and NPY are co-expressed in the same neurons and NPY and AgRP are co-expressed.

In intact females, we observed no significant difference in gene expression between the genotypes (WT, ERKO, KIKO) for CART, NPY and AgRP (Figure 8A). For POMC, ERKO POMC expression was significantly lower than both WT and KIKO females (WT: 1.04 ± 0.14 (n=6); KIKO: 0.96 ± 0.11 (n=6); ERKO: 0.52 ± 0.17 (n=5); p<0.05 for both comparisons; genes were normalized to WT females for all genes). In the ovx females, POMC gene expression was significantly different within genotype between the oil-treated and E2-treated females in the WT (WT-oil: 1.09 ± 0.09 (n=8) vs. 1.52 ± 0.08 (n=8), p<0.01) (Figure 8B). In comparing gene expression across the genotypes when normalized to the WT-oil females, there was a significant effect of genotype-treatment (One-way ANOVA: p<0.0001, df=5, F=14.7). Indeed, the KIKO-E2, ERKO-oil and ERKO-E2 were all significantly lower than the WT-oil (KIKO-E2: p<0.05; ERKO-oil: p<0.05; ERKO-E2: p<0.001). KIKO-oil females were almost significantly less than the WT-oil (p=0.051).

Amongst the other three arcuate genes, CART expression in the WT females was the only pair to have significant change due to E2 treatment (Figure 8C). CART expression in WT- E2 (0.55 ± 0.1) was significantly lower than in WT-oil (1.08 ± 0.15; p<0.05). Across genotypes, there was a significant effect of genotype (One-way ANOVA: p<0.05, df=5, F=3.2) and both WT-oil and KIKO-oil (1.05 ± 0.19) was significantly higher than ERKO-E2 (0.43 ± 0.16; p<0.05). There
was no effect of E2 treatment within a genotype for either of the two orexigenic genes, NPY and AgRP (Figure 8D & 8E). However, in both genes there was a significant effect of genotype (One-way ANOVA: NPY - p<0.001, df=5, F=5.8; AgRP - p<0.05, df=5, F=3.4). In NPY, ERKO gene expression was higher than in the WT and KIKO females (WT vs. ERKO-oil (p<0.05) and ERKO-E2 (p<0.001); KIKO vs. ERKO-oil (p<0.05) and ERKO-E2 (p<0.01)). In AgRP, ERKO-E2 was significantly higher than WT-oil (p<0.001). These data collectively suggest that there are genotype differences in the gene expression of these neuropeptides that may be involved in the changes we measured in energy homeostasis.

6.5 Discussion

Previously, it has been shown that ovariectomy in wild-type female mice caused significant body weight gain, which is abrogated in ovx ERα KO (ERKO) female mice (13). Furthermore, intact, adult ERKO mice developed obesity and the knockin of ERα ERE- independent signaling in ERKO females (ERα KIKO) restored normal energy homeostasis in intact females (13, 26). We demonstrated here that ERE-independent signaling is not sufficient to either fully restore the positive weight gain observed in ovx WT females or fully reestablish the effects of E2 in attenuating post-ovariectomy body weight gain. In every measure of energy homeostasis conducted, the effects of E2 were only observed in WT females, suggesting that ERE-dependent signaling was necessary for effects of E2
replacement on feeding, oxygen consumption, activity, body fat accumulation and
gene expression of arcuate neuropeptides in ovx females.

Vehicle-treated, ovx WT females were shown to gain significantly more
weight than their E2-treated counterparts on regular and high-fat diets,
respectively (13,39–41). E2 replacement was associated with a suppression of
food intake (13,40) and an increase in oxygen consumption (41). Our data
supported these findings in WT females. E2 replacement in ovx WT females
increased oxygen consumption and activity and suppressed food intake
compared to oil-treated WT females. Together, these effects of E2 on energy
homeostasis attenuated the post-ovariectomy body weight gain in WT females.
Unexpectedly, none of these effects were observed in the E2-treated KIKO
females although there was an observable, but non-significant, attenuation of the
body weight gain. As in the WT females, ovariectomy did significantly suppress
oxygen consumption in KIKO females. However, E2 did not increase oxygen
consumption in the KIKO females suggesting that ERE-independent signaling
was not sufficient to restore E2’s augmentation of oxygen consumption. While
food intake did slightly increase due to ovariectomy in KIKO females, this
increase was significantly less than in the WT females regardless of treatment.
The smaller increase in the post-ovariectomy food intake in KIKO females is the
most plausible explanation for the lower amount of weight gain in the oil-treated,
ovx KIKO females. Therefore, the increase in body weight due to ovariectomy in
KIKO females is primarily through a decrease in metabolism, independent of activity, and through a small but significant increase in food intake.

Modulation of arcuate POMC and NPY neurons is known to have a role in E2’s suppression of food intake (42,43,16). For example, deletion of ERα in arcuate POMC neurons produced a hyperphagic response in intact female mice leading to an increase in body weight (16). In our study, an E2-induced increase in POMC gene expression was only observed in WT females, indicating that the suppressive effects E2 on food intake in this study was mediated, in part, by anorexigenic POMC neurons. Gene expression of the anorectic (POMC/CART) and orexigenic (NPY/AgRP) arcuate neuropeptides was significantly affected by genotype. ERKO females expressed more orexigenic peptides and less anorexigenic peptides than the WT counterparts. However, the differences in neuropeptide gene expression did not correlate with lower amount of food intake in ERKO females. This apparent discrepancy between gene expression and feeding behavior suggests a dysregulation of the intrinsic control of feeding behavior by arcuate neurons in ERKO females.

E2’s effects on metabolism are mediated by its action on neurons of the ventromedial nucleus of the hypothalamus (VMH). Deletion of ERα in the SF-1 neurons of the VMH (16) or silencing of ERα by RNAi administered in the VMH (4) produced a phenotype of decreased energy expenditure. Likewise, total knockout of ERα caused a decrease in energy expenditure compared to WT females, which is one of the primary causes of obesity in ERKO mice (12,26).
the current study, neither ovariectomy nor E2 replacement had an effect on energy expenditure in the ERKO females. Furthermore, intact WT and KIKO females had similar rates of oxygen consumption (26), as did WT and KIKO females within one week of ovariectomy. Long-term (12 weeks) ovariectomy in WT females caused a significant decrease in oxygen consumption leading to weight gain (44). Similar to WT females, long-term (5 week) ovariectomy significantly decreased energy expenditure in KIKO females. However, E2 attenuated post-ovariectomy body weight gain only in WT females, which was partially due to an increase in nighttime oxygen consumption. These data suggest that ERE-independent ERα signaling, possibly in the VMH, controls energy expenditure in intact females. This type of ERα signaling also has a role in the post-ovariectomy decrease in energy expenditure, but was not sufficient for E2’s augmentation of energy expenditure.

During the experiment, we also observed a difference in the amount of food chewed, but not fully consumed, between genotypes. In wild-type females, there was a significant decrease in the amount of food chewed after ovariectomy that was not seen in the other genotypes. E2 replacement in the WT females increased the amount of food chewed to similar levels observed in the intact. This behavior correlated with the higher levels of activity observed in the intact (data not shown) and E2-treated WT females. Another potential causes is compulsive or anxiety-associated feeding behaviors or “pica”, (36) some of which may be
augmented by E2 in mice (45), or increased anxiety due to the stress of single housing during the experiment (46).

The partial restoration of the post-ovariectomy body weight gain in KIKO females compared to WT females suggests a role for ERE-independent signaling. This role in energy homeostasis may function through the organizational effects of ERα signaling occurring during neurodevelopment. Thus, a potential cause behind the lack of body weight gain in ERKO females is the loss of ERα signaling during neurogenesis in the hypothalamus and other regions of the brain involved in homeostatic functions (47–49). While no such direct role of organizational ERα signaling in energy homeostasis has been demonstrated, the partial restoration of the response in body weight to ovariectomy in KIKO females indicates that a portion of these mechanisms are ERE-independent. This hypothesis is supported by the normal weight phenotype of the intact KIKO females (26). Therefore, we can hypothesize that ERα-mediated developmental programming and control of neural circuitry is necessary for the post-ovariectomy body weight gain. The development of a conditional ERα KO mice strain would be key in addressing this hypothesis.

Furthermore, it is unknown if the response to ovariectomy (body weight gain) is primarily mediated by central or peripheral organizational and activational mechanisms. A recently produced brain-specific ERKO strain exhibit higher body weight compared to wild-type littermates in both females and males (16). These mice exhibit higher food intake and visceral fat accumulation, lower heat
production and decreased locomotor activity indicating the central effects of E2 and ERα signaling are necessary for normal energy homeostasis. However, the response to ovariectomy has not been characterized in these mice. If there is a neurodevelopmental effect of ER signaling involved in the post-ovariectomy body weight gain, these mice should respond similar to the total ERKO females used in this study and others (13).

Peripheral expression of estrogen receptors includes organs and tissues involved in energy balance, metabolism and glucose homeostasis (50). Thus, the actions of ERα are involved in a range of metabolic processes in intact females, which are altered by ovariectomy (51,52). It is largely unknown if these peripheral effects of E2 and ERα are mediated by ERE-independent signaling. In liver-specific ERα knockout females, E2’s effects on insulin sensitivity and liver lipid deposition are impaired, without affecting E2’s suppression of adiposity on a high-fat diet (53). In intact KIKO females, glucose homeostasis and the response to high-fat diet are normalized compared to WT females (26), thus indicating that ERα ERE-independent signaling is involved in peripheral metabolic processes. Indeed, membrane-initiated ERα signaling is implicated in the regulation of gene expression and lipid synthesis in the liver through an AMPK-mediated pathway (54). Therefore, future experiments will examine the effects of high fat diets, liver function, glucose homeostasis and ovariectomy with or without E2 replacement in KIKO females (44,51,52).
One potential qualification to the current study is the genotype of the ERKO backbone to the ERα KIKO mice. The ERKO and KIKO females used in this study were genotypically different than those used in either Geary et al. (2001) or Park et al. (2010) studies, respectively. In the current study, Dr. Ken Korach recently produced the ERKO backbone by global deletion of exon 3 of the ERα gene (30) while the KIKO mice characterized in Park et al. (2010) were produced with the ERKO mouse produced by Dr. Pierre Chambon and colleagues (55). Furthermore, the ERKO mice originally described as not gaining significant weight post-ovariectomy in Geary et al. (2001) were a different ERKO strain produced by Dr. Ken Korach and colleagues (56). Potential differences between these three strains of ERα knockouts may account for the differences reported in the results between earlier studies (12,13), the Park et al., (2010) study and this current study.

In conclusion, ERα is the primary receptor in E2’s effects on energy homeostasis, although other ERs (Gq-mER, ERβ) have been recently implicated in mediating E2’s control of energy homeostasis either by suppressing feeding in ovx rodents (18–20) or by attenuating adiposity and maintaining normal glucose homeostasis (57,58). The multiple receptor-mediated mechanisms initiated by ERα are not fully understood yet previous studies (26,41,52) and the current study suggest that these mechanisms involve both ERE-dependent and -independent signaling pathways. Future studies must focus on the contributions of these two types of steroid signaling and their respective roles in both
organizational and activational effects of E2 on energy homeostasis. Such studies may provide multiple therapeutic targets for selective hormone replacement therapy with limited deleterious side effects.

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**Figures**

**Figure 1**

![Experimental protocol for ovariectomized and intact females.](image-url)
Figure 2

Figure 2. Serum 17β-estradiol levels and uterine weights from intact and ovariectomized females A. Serum E2 (pg/ml) were measured using a Mouse Calbiotech ELISA in WT (black bars), KIKO (dark gray bars) and ERKO (light gray bars) in intact females as well as oil-treated and E2-treated ovx females. Each intact genotype group had 7 females and each ovx group had 8 females. B. Uterine weights normalized to body weight (g/g) in the same animals. Data was
analyzed by a one-way ANOVA with *Bonferroni-Dunn* multiple comparison tests across and within genotypes (* p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001).

Figure 3
Figure 3. Post-ovariectomy body weight gain and the attenuation by E2A. Bi-daily (QOD) cumulative body weight gain in oil-treated, ovariectomized WT (black circles), KIKO (dark gray triangles) and ERKO (light gray squares). Each ovariectomized group had 8 females. Data was analyzed by a two-way ANOVA (genotype: p<0.01, df=2, F=9.84) with Bonferroni-Dunn multiple comparison tests (a = p<0.05; b = p<0.01; c = p<0.001; d = p<0.0001, compared to WT). B–D. Cumulative body weight gain in oil-treated, ovariectomized (black circles); E2-treated, ovariectomized (gray squares); and intact (dark gray triangles) females (B=WT, C=KIKO; D=ERKO). Ovariectomized groups had 8 females and the intact groups had 7 females each. Data was analyzed by a two-way ANOVA (WT: p<0.001, df=1, F=20.68; KIKO & ERKO: not significant (ns)) with Bonferroni-Dunn multiple comparison tests (a = p<0.05; b = p<0.01; c = p<0.001; d = p<0.0001, oil vs. E2 comparisons only).
Figure 4. Average 48 hr food intake and food chewed for both intact and ovariectomized females A. Average 48 hr food intake (g) over the course of the 4 weeks measured for each animal from each group. WT (black bars), KIKO (dark gray bars) and ERKO (light gray bars) in intact and oil-treated and E2-treated ovariectomized females. Data was analyzed by a two-way ANOVA (genotype: p<0.0001, df=2, F=39.1; steroid: p<0.01; df=2, F=5.25) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05; ** p<0.01). B. The average 48 hr amount of food chewed but not consumed by each animal within each group. Data was analyzed by a one-way ANOVA (Intact: p<0.01, df=2; F=6.28; E2: p<0.01, df=2, F=6.37; oil: ns) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05; ** p<0.01).
Figure 5. E2 replacement suppressed body fat accumulation and increases blood triglycerides levels

A. Average percent change in body fat/body weight during the 4 weeks averaged for each group. WT (black bars), KIKO (dark gray bars) and ERKO (light gray bars) in intact and oil-treated and E2-treated ovariectomized females. Data was analyzed by a one-way ANOVA (WT: p<0.001, df=2, F=13.6; KIKO: p<0.01, df=2, F=7.26; ERKO: p<0.05, df=2, F=4.26) with Bonferroni-Dunn multiple comparison tests across and within genotypes (** p<0.01, *** p<0.001).

B. The average percent change in lean mass/body weight averaged for each group. Data was analyzed by a one-way ANOVA (E2: p<0.05, df=2; F=3.65; oil: p<0.01, df=2, F=7.65; oil: ns) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05; ** p<0.01).

C. Blood triglycerides levels (mg/dl) averaged for each group. Data was analyzed by a one-way ANOVA with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05). D. Blood glucose levels (mg/dl) averaged for each group. Data was analyzed by a one-way ANOVA with Bonferroni-Dunn multiple comparison tests across and within genotypes.
Figure 6. Oxygen consumption (V.O2) and carbon dioxide production (V.CO2) decreases after ovariectomy, which is only attenuated by E2 in WT females

A. Average nighttime V.O2 (ml/min/kg) for each group before and after treatment. Unhatched bar = pre-treatment and hatched bars = post-treatment. Data was analyzed by a two-way ANOVA (genotype X steroid: p<0.001, df=5, F=4.09; pre-post: p<0.0001, df=1, F=36.97) with Bonferroni-Dunn multiple comparison tests across and within genotypes.

B. Average daytime and nighttime V.O2 (ml/min/kg) for each group post-treatment. Data was analyzed by a two-way ANOVA (day-night: p<0.0001, df=1; F=36.74; genotype X steroid: ns (p=0.06)) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05, ** p<0.01, *** p<0.001, compared to nighttime group).

C. Average nighttime V.CO2 (ml/min/kg) for each group before and after treatment. Unhatched bar = pre-treatment and hatched bars = post-treatment. Data was analyzed by a two-way ANOVA (genotype X steroid: ns; pre-post: p<0.0001, df=1, F=30.85) with Bonferroni-Dunn multiple comparison tests across and within genotypes.

D. Average daytime and nighttime V.CO2 (ml/min/kg) for each group post-treatment. Data was analyzed by a two-way ANOVA (day-night: p<0.0001, df=1; F=77.71; genotype X steroid: p<0.01, df=5, F=3.63) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, compared to nighttime group).

Figure 7
Figure 7. Respiratory Exchange Ratio (RER), heat production and activity
A. Average daytime and nighttime RER for each group post-treatment. Data was analyzed by a two-way ANOVA (day-night: p<0.0001, df=1; F=79.23; genotype X steroid: ns) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, compared to nighttime group). B. Average daytime and nighttime heat production for each group post-treatment. Data was analyzed by a two-way ANOVA (day-night: p<0.0001, df=1; F=31.89; genotype X steroid: p<0.0001, df=5; F=11.97) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05, ** p<0.01, compared to nighttime group). C. Average nighttime X-plane activity (counts) for each group before and after treatment. Unhatched bar = pre-treatment and hatched bars = post-treatment. Data was analyzed by a two-way ANOVA (genotype X steroid: p<0.0001, df=5, F=5.84; pre-post: ns) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05). D. Average daytime and nighttime X-plane activity for each group post-treatment. Data was analyzed by a two-way ANOVA (day-night: p<0.0001, df=1; F=140.2; genotype X steroid: p<0.0001, df=5, F=6.69) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, compared to nighttime group).

Figure 8
Figure 8. Genotype and steroid treatment affect relative expression of Arcuate neuropeptide genes. A. Average relative mRNA expression for POMC, CART, NPY and AgRP neuropeptides in the intact females. Data was analyzed by a one-way ANOVA (POMC: p<0.05, df=2; F=3.85) with Bonferroni-Dunn multiple comparison tests across and within genotypes (* p<0.05). B–E. Average relative mRNA expression in oil-treated and E2-treated, ovariectomized females for each neuropeptide. Data was normalized to WT-oil samples and analyzed by a one-way ANOVA (POMC: p<0.0001, df=5, F=14.7; CART: p<0.05, df=5, F=3.22; NPY: p<0.001, df=5, F=5.8; AgRP: p<0.05, df=5, F=3.44) with Bonferroni-Dunn multiple comparison tests across and within genotypes (a = p<0.05, b = p<0.01, c = p<0.001, d = p<0.0001).